Biofilm Production by Multi Drug Resistant Bacterial Pathogens Isolated From Patients in Intensive Care Units in Egyptian Hospitals

Elhabibi T1* and Ramzy S2
1Department of Microbiology and Immunology, Egyptian Russian University, Egypt
2Department of Microbiology and Immunology, Al-Azhar University, Egypt

Abstract
Antibiotic resistance among Multi drug resistant (MDR) Gram negative bacteria causing hospital acquired infections poses a great threat in ICU patients. The treatment of such infections has become increasingly problematic, due to their intrinsic and/or acquired resistance to variable classes of antibiotics. Moreover, the demonstrated ability of these bacteria to grow as biofilm is believed to have a major role in their ability to resist various antibiotics.

The aim of this study is to evaluate the role of the selected genes in biofilm formation in 3 significant MDR bacterial isolates (Acinetobacter baumannii and Pseudomonas aeruginosa and Stenotrophomonas maltophilia). In this study a total of 625 non replicated Gram negative non-fermenter bacterial isolates were isolated from different clinical specimens from intensive care units from hospitals in Egypt. These bacterial isolates were identified biochemically, API20E and genetically.

The antibiogram of all isolates was determined and revealed that all isolates were MDR and colistin was the most potent antibiotic against all A. baumannii and P. aeruginosa isolates. While trimethoprim/sulfamethoxazole combination was the most potent against all S. maltophilia isolates. Detection of biofilm formation of isolates was done by Tube method. While, Quantification of biofilm formation was done by the microtiter plate method using crystal violet (CV) assay. Screening for some selected genes responsible for biofilm formation was done by PCR as bap gene which is responsible for biofilm formation in A. baumannii, rhlI gene in P. aeruginosa strains and rmlA, spgM, rpfF genes in S. maltophilia. The results revealed the presence of these genes in both strong and weak biofilm producer isolates. These final results showed the significance of these genes in biofilm formation.

Keywords: Multi drug resistant; Bacterial pathogens; Intensive care

Introduction
Biofilm is considered as an accumulation of bacteria and their extracellular products forming a structured community on a surface [1]. Biofilms are complex bioactive structures composed of one or more bacterial species protected by a matrix of extracellular polysaccharides. Biofilm formation is initiated by the initial attachment of bacteria to a solid surface. The adherent cells form micro colony and accumulation of clusters of cells within bacterial polysaccharides and other types of matrices [2]. By time, the biofilm becomes thicker, denser and, when disrupted, leads to the dissemination of microbial cells [3]. Bacteria in biofilms become more resistant to antibiotics [4]. In the medical setting, biofilm-associated infections cause a significant problem that arises from the surface of different indwelling devices such as intravenous catheters, alloplastic materials, hydrocephalus shunts and others [2].

Multi drug resistant A. baumannii, P. aeruginosa and S. maltophilia species are nowadays widely spread. By nature, these microorganisms are more resistant than other Gram negative bacteria due to many reasons; the most significant reason is their outer membrane that is less permeable and their ability to form biofilm [5]. The ability of bacteria to form biofilms represents a prominent factor associated with A. baumannii, P. aeruginosa and S. maltophilia virulence. Strains of A. baumannii form biofilms in-vitro, and some of the molecular mechanisms associated with this phenotype have been extensively studied; the main gene associated with biofilm formation is bap (encoding the biofilm-associated protein [Bap]) [6].

One of the most common biofilm forming bacteria is P. aeruginosa [7]. P. aeruginosa has been extensively studied for genetic determinants that contribute to form biofilm. Recently, it was discovered that P. aeruginosa quorum-sensing (QS) signal molecules termed auto-

*Corresponding author: Elhabibi T, Department of Microbiology and Immunology, Egyptian Russian University, Egypt, Tel: +201004622554; Mobile: +201021411433; E-mail: tamer_elhabibi@yahoo.com

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Molecular detection of biofilm formation

Tube method (TM): A qualitative assessment of biofilm formation was evaluated as previously described by Christensen et al. [19]. 10 mm of trypoticase soya broth media with 10% glucose (TSB_B) was inoculated with loop-full of bacteria from overnight culture plates and incubated at 37°C for 24 h. Then the tubes were decanted and washed with phosphate buffer saline PBS (pH 7.4) and dried. Dried tubes stained with crystal violet (0.1%). Excess stain was rinsed off by gentle tapping and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for the formation of biofilm. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube.

Spectrophotometric assay method: The Spectrophotometric assay method described by Christensen et al. is the most commonly used and was considered as standard method for detection of biofilm production [20]. Previous reports have indicated the effect of culture media composition on biofilm formation; therefore we had determined biofilm formation in trypoticase soya broth media (TSB Difco), TSB with 1% glucose (TSB_B). Isolates obtained from fresh agar plates were inoculated in respective media and incubated at 37°C for 24 h in stationary condition and diluted 1:100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) were filled with 0.2 ml aliquots of the diluted cultures and bacteria free media was used as control. Then tissue culture plates were incubated at 37°C for 24 h. After incubation content of each well was removed by gentle tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.4) to remove free-floating planktonic bacteria. Biofilms formed by adherent sessile organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was removed by washing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader (model 680, Bio rad) at wavelength of 590 nm (OD590 nm). The microorganisms were classified into three groups according to the glyocalyx production and biofilm formation that was obtained by O.D. values. The experiment was performed in triplicate; and data was then averaged and plotted.

DNA extraction: DNA was extracted from the bacterial colonies using the QIAamp DNA mini kit (Qiagen Inc.) according to the manufacturer's recommendation. The DNA concentration and purity were determined by measuring the absorbance at 260 nm and by calculating the ratio of absorbance at 260 nm to that at 280 nm using a spectrophotometer (U.V-VIS.), U.V 2500 (Labomed. Inc.) [21]. DNA was used directly or stored at -20°C for future use.

Molecular detection of 16S ribosomal RNA gene for identification of A. baumannii strains by polymerase chain reaction (PCR) [22]: Amplification reaction mixtures (25 μl) containing 12.5 μl of GoTaq Green master mix 2X (Promega, USA), 2.5 μl (10 μM) for each forward (GCGAAGACTTCCTTGAGCAG) and reverse (GCGGAGTCATTCTTACGTCAG) gene primers (Kapa, USA), 5 μl of DNA template (100 μg) and 2.5 μl of PCR grade water (Promega). Amplification of the 16S rRNA was performed in a DNA thermal cycle, with the following cycling program: Initial denaturation at 95°C for 3 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

Molecular detection of 16S ribosomal DNA gene for identification of P. aeruginosa strains by polymerase chain reaction (PCR) [23]: Amplification reaction mixtures (25 μl) containing 12.5 μl of GoTaq Green master mix 2X (Promega, USA), 2.5 μl (10 μM) for each forward (GGCTGATGTTGTTGACGCT) and reverse (GCTGGTGTTTCTTCGCT) gene primers (Kapa, USA), 5 μl of DNA template (100 μg) and 2.5 μl of PCR grade water (Promega). Amplification of the 16S rDNA was performed in a DNA thermal cycle, with the following cycling program: Initial denaturation at 95°C for 2 min and 30 cycles of denaturation at 94°C for 20 s, annealing at 54°C for 20 s, extension at 72°C for 40 s and a final extension at 72°C for 1 min.

Molecular detection of 23S rRNA gene for identification of S. maltophilia strains by polymerase chain reaction (PCR): Amplification reaction mixtures (25 μl) containing 12.5 μl of GoTaq Green master mix 2X (Promega, USA), 2.5 μl (10 μM) for each forward (TACTCCTCCATGCCGTCGGAAGGTTGATG) and reverse (ACGCGATCTCCCGTGCG) gene primers (Kapa, USA), 5 μl of DNA template (100 μg) and 2.5 μl of PCR grade water (Promega). Amplification of the 23S rRNA was performed in a DNA thermal cycle, with the following cycling program: Initial denaturation at 94°C for 5 min, and 30 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 45 s and a final extension at 72°C for 10 min.

Molecular detection of bap gene which is responsible for biofilm formation in A. baumannii strains by PCR: Amplification reaction mixtures (25 μl) containing 12.5 μl of GoTaq Green master mix 2X (Promega, USA), 2.5 μl (10 μM) for each forward (GCTTGATGTTGCTGTCACCGG) and reverse (AGCGAGTCTACCTTCTG) gene primers (Kapa, USA), 5 μl of DNA template (100 μg) and 2.5 μl of PCR grade water (Promega). Amplification of the bap gene was performed in a DNA thermal cycle, with the following cycling program: Initial denaturation at 95°C for 3 min, and 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

Molecular detection of RhlI gene which is responsible for biofilm formation in P. aeruginosa strains by PCR [24]: Amplification reaction mixtures (25 μl) containing 12.5 μl of GoTaq Green master mix 2X (Promega, USA), 2.5 μl (10 μM) for each forward (GCGAGTCCTTACACGACGCG) and reverse (GCCTTTGACGTCGTGTTG) gene primers (Kapa, USA), 5 μl of DNA template (100 μg) and 2.5 μl of PCR grade water (Promega). Amplification of the RhlI gene was performed in a DNA thermal cycle, with the following cycling program: Initial denaturation at 94°C for 3 min, and 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

Molecular detection of rmlA, spgM, rpfF genes which is responsible for biofilm formation in S. maltophilia strains by PCR [14]: For each gene: Amplification reaction mixtures (25 μl) containing 12.5 μl of GoTaq Green master mix 2X (Promega, USA), 2.5 μl (10 μM)

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for each forward 5’-GCAAGGTCATCGACCTGG-3’ and 5’-TTGCGTGATAGACTCAGG-3’ for rmlA, 5’-GCTTCATCGAGGCG-TACTACCC-3’ and 5’-ATGCACGACACTCGTGCGC-3’ for spgM and, finally, 5’-CTGGTGACATCGTGGTG-3’ and 5’-TGATCCGCAT-CATTCCATGC-3’ for rpfF gene primers. Amplification of the rmlA, spgM, rpfF genes were performed in a DNA thermal cycler, with the following cycling program: Initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 45 s and a final extension at 72°C for 10 min.

The PCR was performed in a total volume of 25 µl reaction mixtures containing 150-200 ng of DNA as template, 0.5 µM of each primer and 1x of PCR master mix (Taq Master/High yield, Jena Bioscience) which provides 2.5 units per reaction of DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate, 1x PCR buffer (with 1.5 mM-MgCl). Amplification products were electrophoresed in 2% agarose gel in 0.5x TBE (Tris-borate-EDTA) at 70 Volts for 60 min and visualized under ultraviolet light. To be sure that the amplification products were of the expected size, a 1500 bp DNA ladder was run simultaneously as a marker. Presence of 750 bp, 618 bp, 278 bp, 1500 bp, 245 bp, 82 bp, 80 bp and 151 bp, respectively indicate positive results.

**Results**

Three hundred and seventy five, two hundred and fifty isolates of A. baumannii, P. aeruginosa and S. maltophilia respectively were identified and confirmed by different morphological, biochemical tests. All A. baumannii isolates showed positive results to catalase test and ability to grow on 44°C. All P. aeruginosa isolates reacted positively to motility, catalase and oxidase tests. All S. maltophilia isolates reacted positively to motility, catalase and Dnase tests. Bacterial isolates were confirmed by API 20E system showed 90.32% identification. All isolates of A. baumannii reacted positively to motility, catalase and Dnase tests. Bacterial isolates were confirmed by API 20E system showed 90.32% identification. All isolates of A. baumannii, P. aeruginosa and S. maltophilia respectively were confirmed by PCR assay using specific primers. Peaks for positive samples appeared at 750 bp, 618 bp and 278 bp, respectively as shown in Figures 1-3.

**Discussion**

**Detection of biofilm formation of biodherent isolates by PCR**

**Detection of bap gene in A. baumannii which is responsible for biofilm formation** [25]: Three hundred and seventy five isolates were tested for the presence of bap gene that is responsible for their abilities to produce biofilm using PCR. Three hundred and fifty nine isolates showed positive results and confirmed for the presence of the gene by showing a band on 1500 bp. As illustrated in Figure 4.

**Detection of RhlI gene in P. aeruginosa which is responsible for biofilm formation** [24]: Two hundred isolates were tested for the presence of RhlI gene that is responsible for their abilities to produce biofilm using PCR. All isolates showed positive results and confirmed for the presence of the gene by showing a band on 245 bp. As illustrated in Figure 5.

**Detection of rmlA, spgM, rpfF genes in S. maltophilia which are responsible for biofilm formation** [14]: Fifty isolates were tested for the presence of rmlA, spgM, rpfF genes that are responsible for their abilities to produce biofilm using PCR. 49, 50 and 50 Isolates showed positive results to the three genes respectively by showing a band on 82 bp, 80 bp and 151 bp, respectively as illustrated in Figures 6-8.

**Tube method**

A qualitative assessment of biofilm formation among six hundred and twenty five Gram negative microbial isolates was determined as described before by Christensen et al. [19] (Table 1).

**Tissue culture plate method (TCP)**

Assessment of biofilm production among six hundred and twenty five Gram negative microbial isolates was determined as described previously by Christensen et al. [20] (Table 2).

Biofilms play a significant role in bacterial colonization during infection, providing an opportunity for microbe to develop drug resistance.
Figure 2: PCR amplification with 16S rDNA gene primers for identification of *P. aeruginosa* strains. A 618 bp of 16S rDNA gene. Lane (L), DNA molecular size marker (500 bp ladder), Lanes (1-16) show positive result with positive bands at 618 bp.

Figure 3: PCR amplification with 23S rRNA gene primers for identification of *S. maltophilia* strains. A 278 bp of 23S rRNA gene. Lane (L), DNA molecular size marker (500 bp ladder), Lanes (1-18) show positive result with positive bands of 278 bp. Lane (19) positive control and Lane (20) negative control.

Figure 4: PCR amplification with bap gene primers. A 1500 bp of bap gene. Lane (L), DNA molecular size marker (500 bp ladder), Lanes (1-2), (6-7) and (9) show positive result with positive bands of 1500 bp. Lanes (3-5) and Lane (8) show negative result. Lane (10) positive control and Lane (11) negative control.
Figure 5: PCR amplification with RhlI gene primers.
A 245 bp of RhlI gene. Lane (L), DNA molecular size marker (500 bp ladder) and Lanes (1-15) show positive result with positive bands of 245 bp. Lane (16) positive control and Lane (17) negative control.

Figure 6: PCR amplification with rmlA gene primers.
A 82 bp of rmlA. Lane (L), DNA molecular size marker (500 bp ladder) and Lanes (1-9) show positive result with positive bands of 82 bp.

Figure 7: PCR amplification with spgM gene primers.
A 80 bp of spgM. Lane (L), DNA molecular size marker (500 bp ladder) and Lanes (1-13) show positive result with positive bands of 80 bp.
resistance [26]. Biofilms are estimated to be responsible for over 65% of nosocomial infections and 60% of all human bacterial infections [27,28]. The most commonly studied bacteria capable of forming biofilms are A. baumannii, P. aeruginosa and S. maltophilia. Biofilm formation is important in establishing infections on different host tissues as well as different medical devices in hospital acquired infections in Egyptian hospitals ICUs [7]. In these settings, the antibiotic resistance engendered by biofilms presents a serious challenge to the treatment of nosocomial infections caused by MDR bacteria [7].

Six hundred and twenty five Gram negative isolates (A. baumannii, P. aeruginosa and S. maltophilia) were isolated and tested for their abilities to form biofilm using tube method, tissue culture plate method and genetically. Twenty isolates (3.2%) were non/weakly adherent, 82 isolates (13.1%) were moderately adherent and 523 isolates (83.7%) was strongly adherent.

According to the results of the tube tests, 84% of the tested strains produced biofilm. The tube adherence assay is easy and simple although reading of the results may be somehow complicated. Furthermore, observers repeatedly have different interpretations about weak reactions [29]. However, other tests can give better interpretation as tissue culture plate method, where the bacterial culture is transferred to a well of a microtiter plate and read by ELISA plate reader, subsequently the approach was changing from a qualitative to a quantitative one. The quantitative microtiter-plate test predicts clinical applications more reliable than the tube testing [30]. In this study, no significant disagreement between the tube test results and microtiter-plate was observed. While in the study conducted by Abdi-Ali et al., considerably more strains were classified as weak adherent by the quantitative microtiter-plate method [31]. Factors that may affect the adherence of A. baumannii, P. aeruginosa and S. maltophilia including the hydrophobicity of the test tubes and shaking which raises the chances of bacterial interaction with the glass surface and uniform nutrients dispersion, which may be the main reasons for the difference in results between both studies.

Micro-organism Non/weakly adherent Strongly adherent Total

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Non/weakly adherent</th>
<th>Strongly adherent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO</td>
<td>%</td>
<td>NO</td>
</tr>
<tr>
<td>A. baumannii</td>
<td>75</td>
<td>20</td>
<td>300</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>20</td>
<td>10</td>
<td>180</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>5</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>16</td>
<td>525</td>
</tr>
</tbody>
</table>

Table 1: Table shows distribution of bioadherent isolates using tube method.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Non adherent</th>
<th>%</th>
<th>Moderately adherent</th>
<th>%</th>
<th>Strongly adherent</th>
<th>%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii</td>
<td>18</td>
<td>4.8</td>
<td>57</td>
<td>15.2</td>
<td>300</td>
<td>80</td>
<td>375</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>10</td>
<td>180</td>
<td>90</td>
<td>200</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>43</td>
<td>86</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>3.2</td>
<td>82</td>
<td>13.1</td>
<td>523</td>
<td>83.7</td>
<td>625</td>
</tr>
</tbody>
</table>

Table 2: Table shows distribution of bio adherent isolates using Tissue culture plate method (TCP).
and infection in the hospital setting [32,33]. Biofilms are difficult to get rid of as they are highly dense colonies of A. baumannii and exhibit high levels of resistance to antibiotics [34].

Our study revealed that 80% of A. baumannii isolates were strongly adherent, while 15.2% were moderately adherent and 4.8% were non-adherent. These results was higher than that presented by Abd-El-Ali et al. in Tehran, which presented that 28% of the isolates were strongly adherent, while 41% were moderately adherent and 25% were non-adherent [31]. This difference may come from the genetic diversity of MDR A. baumannii isolates between Egyptian and Iranian hospitals.

Detection of bap gene in A. baumannii which is responsible for biofilm formation showed 100% occurrence in all isolates which are moderately adherent and strongly adherent, these results nearly resembles that presented by Goh et al. which was 92% [25].

In our study, all isolates P. aeruginosa were biofilm producers by microtiter plate method using CV assay; 180 isolates (90%) were strong biofilm producers; 20 isolates (10%) were moderate biofilm producers and 4 isolates (8%) were weak biofilm producers. Similar results were obtained in another study in Egypt conducted by Abd El-Galil et al. as all isolates were biofilm producers by microtiter plate method using CV assay; 42 isolates (84%) were strong biofilm producers; 4 isolates (8%) were moderate biofilm producers and 4 isolates (8%) were weak biofilm producers [24]. Also, these results were confirmed by another study performed in Egyptian hospitals by Hisham et al., as all isolates were biofilm producing using CV assay; 16 isolates (80%) were strong biofilm producers; 2 isolates (10%) were moderate biofilm producers and 2 isolates (10%) were weak biofilm producers [35].

In the present study, RhlI gene was detected in all biofilm forming isolates suggesting that RhlI gene might be involved in biofilm production. This suggestion is confirmed by a previous study in Egypt by Abd El-Galil et al. who reported that 100% of P. aeruginosa isolates showed the presence of RhlI gene that proved that RhlI gene is responsible for biofilm formation [24]. Moreover, another study in Switzerland revealed that biofilm production was reduced by 70% in RhlI mutant strain of P. aeruginosa comparing to its parent strain indicating the role of RhlI gene in biofilm formation [36]. Also in another study in USA, they showed that if QS inhibitory compounds shut down RhlI expression, then all the other genes in the QS cascade would be shut down also, including those involved in biofilm formation that strengthens RhlI role in biofilm formation [37-39].

Conclusion

Our results revealed that rmlA, spgM, rpfP genes in S. maltophilia which are responsible for biofilm formation showed 100% occurrence in all isolates which are moderately adherent and strongly adherent, these results shows little difference than that presented by Pompilio et al. which showed an overall prevalence of 65.2, 88.8 and 61.8%, respectively [14]. This difference may come from the genetic diversity of MDR S. maltophilia isolates between Egyptian and overseas hospitals worldwide.

References


