CORRELATION BETWEEN BIOFILM PRODUCTION AND MULTIPLE DRUG RESISTANCE IN IMIPENEM RESISTANT CLINICAL ISOLATES OF ACINETOBACTER BAUMANNII

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Abstract

Purpose: To study the qualitative and quantitative methods for the investigation of biofilm formation and to examine the correlation between biofilm and antibiotic resistance among the clinical isolates of Acinetobacter baumannii. We also verified the association between biofilm and presence of extended spectrum β-lactamases, particularly, bla\(_{\text{PER-1}}\).

Methods: A total of 55 isolates were subjected to susceptibility testing by disc diffusion method for 13 clinically relevant antibiotics. Screening for biofilm production was done by both qualitative and quantitative methods through tube and microtitre plate assay respectively. The presence of bla\(_{\text{PER-1}}\) was checked by PCR. Results: A. baumannii isolates showed very high resistance (>75%) to imipenem, cephrotaxime, amikacin and ciprofloxacin. Only cefoperazone, netilin and norfloxacin were found to be effective agents. Results of microtitre and tube methods were concordant with 34 isolates (62%) showing biofilm formation. Resistance to four antibiotics such as amikacin (82% vs. 17.6%, \(P<0.001\)), cephrotaxime (88% vs. 11%, \(P<0.001\)), ciprofloxacin (70% vs. 29%, \(P=0.005\)) and aztreonam (38% vs. 11%, \(P=0.039\)) was comparatively higher among biofilm producers than non-biofilm producers. Microtitre assay additionally detected 14 weakly adherent isolates. Only 11 isolates had \(\text{bla}_{\text{PER-1}}\) gene and among these two were strong biofilm producers, while remaining were weakly adherent isolates. Conclusion: Microtitre plate method was found to be a more sensitive method for biofilm detection. This study demonstrates a high propensity among the clinical isolates of A. baumannii to form biofilm and a significant association of biofilms with multiple drug resistance. Presence of \(\text{bla}_{\text{PER-1}}\) appears to be more critical for cell adherence than for biofilm formation.

Key words: A. baumannii; biofilm; \(\text{bla}_{\text{PER-1}}\); MDR

Acinetobacter baumannii has emerged as an important and problematic human pathogen as it is the causative agent of several types of infections including pneumonia, meningitis, sepsicaemia, and urinary tract infections. It ranked second after Pseudomonas aeruginosa among the nosocomial, aerobic, non-fermentative, gram negative bacilli pathogens.\(^1\) Furthermore, this organism frequently causes infections associated with medical devices, e.g., vascular catheters, cerebrospinal fluid shunts, Foley catheters etc.\(^2\)-\(^4\) A. baumannii has emerged recently as a major cause of hospital acquired infections because of the extent of its antimicrobial resistance and its propensity to cause large, often multi-facility nosocomial outbreaks.\(^2\)-\(^4\) Mortality in patients suffering from A. baumannii infections can be as high as 75%.\(^2\) Infections due to A. baumannii often prove difficult to treat due to high level of resistance to multiple antibiotics as a result of both intrinsic and acquired mechanisms.\(^5\)-\(^7\) The potential ability of A. baumannii to form biofilms might also explain its outstanding antibiotic resistance, survival properties and increased virulence.\(^2\)-\(^7\) Indeed one of the recent studies showed a positive correlation between the biofilm formation and presence of extended spectrum of β-lactamase (ESBL) \(\text{bla}_{\text{PER-1}}\) among the A. baumannii isolates.\(^8\) A contrary observation was also noticed in yet another study.\(^9\)

Biofilm formation is a well-known pathogenic mechanism in device related infections in hospitals.\(^10\) Moreover, the environmental survival of some nosocomial pathogens may be facilitated by biofilm formation on abiotic surfaces. Little is known concerning the biofilm formation and its mechanisms in A. baumannii.\(^7\)-\(^11\)-\(^13\)

Therefore, the present study was undertaken on clinical isolates of A. baumannii to determine the frequency of biofilm formation by different methods and correlate biofilm formation with development of multiple antibiotic resistance and ESBL production. Furthermore, we also verified existence of any association between biofilm formation and presence of ESBL particularly, \(\text{bla}_{\text{PER-1}}\).

Materials and Methods

Bacterial isolates

A total of 55 isolates of A. baumannii in the study were obtained from Pondicherry Institute of Medical Sciences Hospital (PIMS), Puducherry, India, from various clinical
specimens like endotracheal aspirates, cerebrospinal fluid, wound swabs, urine and blood culture specimens from patients admitted in ICUs and acute medical care units during January to April 2007. All the isolates were characterised to the species level using phenotypic tests as described elsewhere.\(^{[14,15]}\) Antimicrobial susceptibility testing was performed for 13 different therapeutically relevant antibiotics by Kirby Bauer disk diffusion method according to norms of Clinical Laboratory Standards Institute (CSLI).\(^{[16]}\) Antibiotics tested included amikacin (10 μg), aztreonam (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), cefepine (30 μg) cefoperazone (30 μg), cefazolin (5 μg), imipenem (10 μg), netilin (30 μg), norfloxacain (30 μg) and ofloxacin (30 μg) which were obtained from Hi-Media, Mumbai.

**Biofilm production**

Biofilm formation was checked only for 51 isolates out of 55 isolates. Biofilm production was estimated qualitatively for all the isolates by tube method as described previously by Christensen et al.\(^{[17]}\) Biofilm production was determined quantitatively using modified microtitre plate method described elsewhere.\(^{[18]}\) *A. baumannii* ATCC 19606 strain was taken as the positive control and a non-biofilm forming bacteria (*Escherichia coli* strain) as a negative control.

All tests were carried out in triplicates and the results were averaged. In the present study, only strongly adherent ones were taken as biofilm positive while weakly adherent ones were taken as negative for biofilm production.

**PCR for bla\(_{\text{PER-1}}\)**

All isolates of *A. baumannii* were tested for the presence of *bla*\(_{\text{PER-1}}\) gene by PCR. Detection of *bla*\(_{\text{PER-1}}\) gene was performed using primers that were described in previous investigation.\(^{[9]}\) The PCR reaction mixture (10μL) contained 1μL of reaction buffer (containing 1.5 mM of MgCl\(_2\)), 50 μM of each deoxyribonucleoside triphosphate, 50 pmol of each PER-1 primer, 0.5U of Taq polymerase (Bangalore Genel) and 30 ng of DNA template. PCR amplifications were performed using thermocycler (Eppendorf, Germany) with following cycling conditions: (i) initial denaturation step of 5 minutes at 94°C, (ii) 30 cycles of PCR, with each cycle consisting of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, and (iii) a final extension step of 5 minutes at 72°C. PCR end products were analysed on 1.2% agarose gel, stained with ethidium bromide. Statistical analysis was performed wherein categorical variables were compared using the Chi-square test (or Fisher’s exact test, if required). Differences were significant if the *p* value associated with the test was < 0.05.

**Results**

Qualitative tube method of biofilm screening showed 34 isolates (62%) positive for biofilm production. Most of the isolates showed thick blue ring at the liquid-air interface. Some of the isolates showing positive results in tube method are shown in figure 1. In the quantitative assay for the biofilm production, the isolates were classified as strongly biofilm producing (strongly adherent), weakly adherent isolates and non-biofilm producers (non-adherent). Quantitative microtitre assay for biofilm formation was strongly positive in 34 isolates (62%) while the remaining isolates were either weakly adherent (14) or non-biofilm producers (3). Fourteen weakly adherent isolates were considered as negative or non-biofilm producers. Both the methods for biofilm detection thus showed similar results. Isolates screened for biofilm formation by microtitre plate assay method are shown in figure 2.

The overall percentage of resistance observed among all the *A. baumannii* isolates including biofilm producers and biofilm non-producers for 13 antibiotics tested, is given in table 1. *A. baumannii* isolates showed 100% resistance to imipenem, 89% resistance to cefotaxime, 80% to amikacin.

![Figure 1](image1.png)

**Figure 1:** Biofilm formation on glass surfaces at liquid-air interface under static growth conditions, Tube 1 - Positive control *A. baumannii* ATCC 19606; Tube 2 to 6 - *Acinetobacter* isolates positive for biofilm; Tube 7 - Negative control (non-biofilm producer - *Escherichia coli* strain)

![Figure 2](image2.png)

**Figure 2:** Quantitative assay for biofilm formation on microtitre plate, Rows A-E - Clinical isolates of *A. baumannii* isolates tested for biofilm, Row F - Negative Control, Row G - Positive control *A. baumannii* ATCC 19606

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and 73% to ciprofloxacin. Cefoperazone and norfloxacin were found to be more effective against most of the isolates. Almost all the multiple antibiotic resistant isolates tested positive for biofilm formation.

The multi-drug resistance patterns of the biofilm producing *A. baumannii* isolates is shown in Table 2. All the biofilm forming isolates showed maximum resistance to imipenem (100%), followed by cephapirnin (88%), amikacin (82%) and ciprofloxacin (70%). Both biofilm producers and non-producers were completely resistant to imipenem. However, resistance to other three antibiotics such as amikacin (82% vs. 17.6%, *P* < 0.001), cephapirnin (88% vs 11%, *P*<0.001), and ciprofloxacin (70% vs 29%, *P*=0.005) was comparatively higher among biofilm producers than non-biofilm producers (Table1). Resistance among biofilm producers to aztreonam was also high (38% vs 11%, *P*=0.039) when compared with non-biofilm producers. Least resistance (5%) was noticed only for cefoperazone.

Majority of clinical isolates of *A. baumannii*, which were isolated from wound infection (44%) showed strong positive results for the biofilm formation. Thirty percent of biofilm producers were from nosocomial pneumonia patients where endotracheal aspirate was the clinical specimen. The third highest number of biofilm producers (12%) were isolated from urinaty tract infection. In all remaining sites, the isolation of biofilm producers was less than 6%.

The maximum multiple resistance among the isolates was noticed for antimicrobial agents such as imipenem, cephapirnin, amikacin, cefazolin and ciprofloxacin. Isolates predominantly showed five unique drug combination profiles (Table 2). Highest number of isolates (14) showed the profile Ce, Cf, Cz, I, followed by Ak, Ce, Cz, Of, I which had 12 isolates. The resistograms of 13 isolates obtained from different clinical specimens displayed definite patterns (Table 3). Isolates collected from patients’ endotracheal aspirate showed three distinct profiles of resistance namely 1A, 1B, 1C in which a group of nine isolates were resistant to three sets of the same antibiotics. Similarly the isolates from wound infection showed two sets (1C, 2) of unique resistance profiles and two of the isolates from urine showed one unique profile.

Presence of *bla*<sub>PER-1</sub> gene was confirmed by detection of DNA fragment of the size 900bp. Eleven isolates showed the presence of *bla*<sub>PER-1</sub> gene, four from respiratory infection, five from wound infection and one each from traumatic meningitis and urinary tract infection (Fig. 3). The amplicon size was ~875bp in three of the isolates. Among the 11 *bla*<sub>PER-1</sub> Positive isolates, only two showed strong adherence while the remaining nine were weakly adherent. All the 11 *bla*<sub>PER-1</sub> Positive isolates were multi-drug resistant and were also resistant to all the β-lactam antibiotics tested. Two biofilm positive isolates (strain ID No. 14 and 28) that were *bla*<sub>PER-1</sub> Positive were found to be multi-drug resistant having resistant patterns Ak, Ce, Cf, Ci, Cpm, Cz, I, Nt, Of and Ak, Ce, Cf, I respectively.

**Discussion**

*A. baumannii* infections present a global medical challenge. They are opportunistic pathogens and are

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**Table 1:** Antibiotic susceptibility results (percentage) of the *A. baumannii* isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Biofilm positive isolates (n=34)</th>
<th>Biofilm negative isolates (n=17)</th>
<th>Resistance of all isolates (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>82.3</td>
<td>17.6</td>
<td>80</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>38.2</td>
<td>61.8</td>
<td>29</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>32.3</td>
<td>67.7</td>
<td>36.3</td>
</tr>
<tr>
<td>Cephotaxime</td>
<td>88.2</td>
<td>11.7</td>
<td>89</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>70.5</td>
<td>29.4</td>
<td>72.7</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>35.2</td>
<td>64.8</td>
<td>41.8</td>
</tr>
<tr>
<td>Cefepime</td>
<td>26.4</td>
<td>73.6</td>
<td>30.9</td>
</tr>
<tr>
<td>Cefaperazone</td>
<td>5.0</td>
<td>95.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>64.7</td>
<td>35.3</td>
<td>63.6</td>
</tr>
<tr>
<td>Imipenem</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>20.5</td>
<td>79.5</td>
<td>27.2</td>
</tr>
<tr>
<td>Oxacin</td>
<td>11.7</td>
<td>88.3</td>
<td>9.0</td>
</tr>
<tr>
<td>Ofloxacine</td>
<td>52.9</td>
<td>47.1</td>
<td>56.3</td>
</tr>
</tbody>
</table>

*Ak- amikacin, Ao- aztreonam, Cf- ciprofloxacin, Cz- cefazolin, I- imipenem, Nx-netillin, Nx-norfloxacin, Of- ofloxacine*

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**Table 2:** Multiple drug resistant patterns of biofilm producers

<table>
<thead>
<tr>
<th>Multiple Drug combinations</th>
<th>Number of isolates showing resistance</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ak, Ao, Ce, Cf, Cz, I, Of</td>
<td>5</td>
<td>14.7</td>
</tr>
<tr>
<td>Ak, Ao, Ce, Cz, I</td>
<td>10</td>
<td>29.4</td>
</tr>
<tr>
<td>Ce, Cf, Cz, I</td>
<td>14</td>
<td>41.1</td>
</tr>
<tr>
<td>Ak, Ce, Cz, I, Of</td>
<td>12</td>
<td>35.2</td>
</tr>
<tr>
<td>Ao, Ce, Cf, I</td>
<td>7</td>
<td>20.5</td>
</tr>
</tbody>
</table>

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**Table 3:** Unique resistogram patterns of biofilm producers with respect to their site of isolation

<table>
<thead>
<tr>
<th>Profile no.</th>
<th>Site of isolation</th>
<th>No. of isolates</th>
<th>Resistogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 A</td>
<td>ETA</td>
<td>5</td>
<td>Ak, Ao, Ce, Cf, Cz, I, Of</td>
</tr>
<tr>
<td>1 B</td>
<td>ETA</td>
<td>2</td>
<td>Ak, Ce, Cf, I, Of</td>
</tr>
<tr>
<td>1 C</td>
<td>ETA, Wound</td>
<td>2</td>
<td>Ak, Ao, Ce, Cz, I, Of</td>
</tr>
<tr>
<td>2</td>
<td>Wound</td>
<td>2</td>
<td>Ak, Ce, Cf, Cz, I, Nt, Of</td>
</tr>
<tr>
<td>3</td>
<td>Urine</td>
<td>2</td>
<td>Ca, I, Nx</td>
</tr>
</tbody>
</table>

*ETEA- Endotracheal aspirate, Ak- amikacin, Ao- aztreonam, Cf- ciprofloxacin, Cz- cefazolin, I- imipenem, Nx-netillin, Nx-norfloxacin, Of- ofloxacine*
particularly successful at colonizing and persisting in the hospital environment. They are able to resist desiccation and survive on inanimate surfaces for years.\[^{2-4,6}\] Interest in this organism has been growing rapidly because of the emergence of multi-drug-resistant strains, some of which are pan-resistant to antimicrobial agents.\[^{2,6,19}\] It is also among the most common causes of device-related nosocomial infection that results when the organism is able to resist physical and chemical disinfection, often by forming a biofilm.

Biofilm formation is thought to be a key pathogenic feature, especially in relation to intravascular line infections and ventilator associated pneumonia. Generally, two properties are often associated with biofilm producing bacteria, namely, the increased synthesis of exopolysaccharide (EPS) and the development of antibiotic resistance.\[^{20}\] One can assume that increased production of EPS in \textit{A. baumannii} is likely to create a protective environment leading to difficulty in antibiotic penetration leading to development of resistance. In addition, there appears to be some differences in the cellular physiology of cells within the biofilm that also results in increased drug resistance.\[^{21}\] Thus infections due to bacteria that form biofilms are a tenacious clinical problem. Only, few reports have described the ability of clinical isolates of \textit{A. baumannii} to attach and form biofilms on glass surfaces.\[^{7,8,12}\]

In our study, among the 34 biofilm positive isolates, the resistograms of 13 isolates isolated from different clinical sources like endotracheal aspirate, wound, urine, CSF and blood, displayed distinct patterns (antibiotypes 1A, 1B, 1C, 2, 3). Such antibiotypes imply that the same isolate is being isolated from different patients over a period of time and it also emphasises on persistence and dissemination of \textit{A. baumannii} in hospital environment. Here, gaining the ability to form biofilm could be a good strategy to enhance the survival and persistence under stressed conditions, e.g. during host invasion or following antibiotic treatment. As a consequence of biofilm development it is said that the ability of \textit{Acinetobacter} to transfer genes horizontally might also enhance within these micro communities facilitating the spread of antibiotic resistance.\[^{8}\] Thus, \textit{A. baumannii} isolates capable of forming biofilm might be selected under antibiotic pressure, or conversely, \textit{A. baumannii} might acquire resistance to multiple drugs within biofilm communities. In either case, it appears that the high

Biofilm forming isolates were also less frequently resistant to imipenem and ciprofloxacin in that study\[^{9}\] while our results showed positive association in biofilm positivity and multiple drug resistance particularly to certain antibiotics. Compared to non-biofilm producers our study also detected significantly higher resistance to cefotaxime, amikacin, ciprofloxacin and aztreonam among biofilm producers. Non-biofilm producers showed increased resistance only for netillexin and ofloxacin.

Interestingly, it was observed that 44% of isolates (15) among the biofilm positive isolates were isolated from wound infections. These isolates were strongly positive for biofilm. Additionally, 30% of biofilm producers were from pneumonia patients. This indicates that in wound and respiratory infections biofilm may contribute to antibiotic resistance. Appropriate antibiotic selection for the treatment of such biofilm associated infections is extremely important. Carbenpens have been the antibiotics of choice for treatment of infections caused by this organism, but resistance to carbenpens is becoming common, and very few therapeutic options remain. In our study, all the isolates were imipenem resistant which is the most common antibiotic prescribed in this hospital. In addition, high percentage of resistance was also witnessed to cefotaxime, amikacin and ciprofloxacin. The potential ability of \textit{A. baumannii} to form biofilms could explain this outstanding antibiotic resistance.\[^{17}\] In concurrence with the other studies\[^{9,22}\] we noted that 62% of our isolates that were strong biofilm producers, also showed nearly complete resistance to all the antibiotics tested. Nevertheless, a few of the strong biofilm forming isolates were sensitive to some of the antibacterial agents namely ceftoperazone, norfloxacin and netillexin. This could be due to the ability of some of these antibiotics to penetrate the biofilms, thereby inhibiting the bacterial growth.

In our study, among the 34 biofilm positive isolates, the resistograms of 13 isolates isolated from different clinical sources like endotracheal aspirate, wound, urine, CSF and blood, displayed distinct patterns (antibiotypes 1A, 1B, 1C, 2, 3). Such antibiotypes imply that the same isolate is being isolated from different patients over a period of time and it also emphasises on persistence and dissemination of \textit{A. baumannii} in hospital environment. Here, gaining the ability to form biofilm could be a good strategy to enhance the survival and persistence under stressed conditions, e.g. during host invasion or following antibiotic treatment. As a consequence of biofilm development it is said that the ability of \textit{Acinetobacter} to transfer genes horizontally might also enhance within these micro communities facilitating the spread of antibiotic resistance.\[^{8}\] Thus, \textit{A. baumannii} isolates capable of forming biofilm might be selected under antibiotic pressure, or conversely, \textit{A. baumannii} might acquire resistance to multiple drugs within biofilm communities. In either case, it appears that the high
colonising capacity of A. baumannii, combined with its resistance to multiple drugs, contributes to the organism’s survival and further dissemination in the hospital setting. By acquiring various kinds of resistance mechanisms, biofilm phenotype of A. baumannii has developed into one of the most difficult hospital pathogens to control and treat. Nevertheless, not much is known about the mechanism that triggers the development of biofilm in A. baumannii.

Lee et al.,[8] have previously reported that there is a strong relationship between biofilm formation and the production of PER-1 β-lactamases. However in our study, only two strong biofilm (strongly adherent) forming isolates were positive for PER-1 β-lactamases. Interestingly, rest of the PER-1 β-lactamase positive isolates (n=9) were weakly adherent or produced negligible biofilm. This observation is in concurrence with the study of Sechi et al.[13] wherein no correlation existed between PER-1 β-lactamase positive isolates and biofilm producers. Nonetheless, these bla

PER-1 positive isolates were strongly associated with cell adhesion.[13] From the above observations, we believe that presence of bla

PER-1 is more critical for cell adhesion than for biofilm formation.

Overall, the present study demonstrated a high propensity among the clinical isolates of A. baumannii to form biofilm and there was a significant association of biofilms with multiple drug resistance. bla

PER-1 presence is likely to facilitate cell adherence. Biofilm production in A. baumannii might promote increased colonization and persistence leading to higher rates of device related infections. A greater understanding of the nature of biofilms and intercellular communications within the biofilm and also their role in serious infections of A. baumannii will help in development of new and more effective treatment for Acinetobacter infections.

References


