

The Correlation Between Biofilm Formation and Drug Resistance in Nosocomial Isolates of *Acinetobacter baumannii*

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Background: *Acinetobacter baumannii* has become a major cause of hospital-acquired infections due to its resistance to common antibacterial agents. Biofilm formation is a well-known pathogenic mechanism involved in *A. baumannii* infections.

Objectives: The aim of this study was to determine the association between biofilm formation and antibiotic resistance, production of AmpC and Extended-Spectrum β -lactamases (ESBL) in clinical isolates of *A. baumannii* collected from two hospitals of Tehran.

Materials and Methods: Sixty isolates of *A. baumannii* were employed of which, 30 were burn and 30 were non-burn isolates. Biofilm formation was measured by the microtiter plate assay. The production of AmpC was detected by the AmpC disc test with cloxacillin, and ESBL production was determined using the double disc synergy test.

Results: Biofilm production occurred in 61.7% of the isolates among which, non-burn isolates (59.5%) produced more biofilm compared to the burn strains (40.5%). Multidrug resistance was observed in both biofilm positive and negative strains. However, the non-burn isolates were significantly more resistant to meropenem and tobramycin regardless of their potential to form biofilm. Interestingly, biofilm-producing non-burn isolates were significantly more resistant to amikacin, gentamicin, tobramycin and meropenem. Production of AmpC was also significantly higher in biofilm-producing non-burn isolates. Conversely, ESBL production was significantly higher in burn isolates. There was an association between biofilm formation and AmpC but not ESBL-production among non-burn isolates.

Conclusions: The potential to form biofilm correlated with antibiotic resistance and AmpC production in non-burn burn isolates of *A. baumannii*. On the other hand, the burn strains produced significantly higher amounts of ESBL yet biofilm production was unrelated to antibiotic resistance or ESBL-production.

Keywords: *Acinetobacter baumannii*; Burn; Biofilms; Antibiotic Resistance

1. Background

Members of the genus *Acinetobacter* are aerobic, non-fermentative Gram-negative bacilli that can easily be obtained from soil, water, food and sewage (1). The most important member of the genus, *Acinetobacter baumannii*, has emerged as one of the most troublesome pathogens for health care institutions, globally. Outbreaks of *A. baumannii* nosocomial infections including urinary tract, secondary meningitis, burn infections and nosocomial pneumonia, have made the organism the leading cause of mortality in hospitalized patients (1-3). The remarkable ability of the organism to accumulate diverse resistance mechanisms has led to the emergence of multidrug resistant isolates over the past 15 years (4). Resistance to most commercially available antibiotics including extended-spectrum penicillins, cephalosporins and carbapenems is mostly due to the production of a number of β -lactamases including AmpC and Extended-Spectrum β -lactamases (ESBL), which limit the therapeutic options for treatment of these infections (5). Another factor that contributes to the establishment and spread of infection is the ability of the organism to form biofilm on medical

devices and biological surfaces such as epithelial cells (6-8). The ability of *A. baumannii* to form biofilms is multifactorial and diverse depending upon the surface with which the cells are interacting (7, 8). Bacteria which grow in biofilms are often resistant to numerous antibacterial agents and products of the immune system and are extremely difficult to eradicate (2, 9, 10). Hence, it is important to establish correlations between biofilm formation and drug resistance in clinical isolates of *A. baumannii*.

2. Objectives

The aim of this study was to pursue the correlation between biofilm formation and antibiotic resistance, ESBL and AmpC production in burn and non-burn nosocomial isolates of *A. baumannii*.

3. Materials and Methods

3.1. Bacterial Isolates

Sixty clinical isolates of *A. baumannii* were employed of

which, 30 were from burn infections collected from Shahid Motahari Hospital and 30 were non-burn isolates obtained from Imam Hossein Hospital of Tehran, between October 2011 to April 2012. The burn isolates were mostly from wounds (n = 25) followed by blood (n = 3) and urine (n = 2). The majority of the non-burn isolates were collected from the intensive care unit (n = 19) and were mostly from sputum (n = 17) followed by wound specimens (n = 4), catheters (n = 3), blood (n = 3), cerebral spinal fluid (n = 2) and trachea (n = 1). Bacterial identification was confirmed by conventional biochemical methods and the isolates were stored at -20°C in brain heart infusion broth (Oxoid, UK) containing 10% dimethyl sulfoxide (v/v). Biofilm positive and negative *Staphylococcus epidermidis*, RP62A and RP62NA, strains were used as controls (11).

3.2. Antibacterial Susceptibility

Antibacterial susceptibility was determined by disc diffusion as recommended by the Clinical and Laboratory Standards Institute (CLSI) using the following antibiotic discs (Mast, UK): aztreonam (30 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), cefepime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), piperacillin (100 µg) and piperacillin/tazobactam (100/10 µg) (12).

3.3. Extended-Spectrum β-Lactamases Production

The Combined Disc Test (CDT) was used as recommended by the CLSI (12). Briefly, the turbidity of an overnight culture of the test isolate was adjusted to 0.5 McFarland's standard prior to inoculation of Muller Hinton Agar (MHA, Liofilchem, Italy) plates containing 200 mg of cloxacillin/L to inhibit AmpC β-lactamases. Ceftazidime (30 µg) and ceftazidime/clavulanic acid (30/10 µg) discs were placed 30 mm apart on the bacterial lawn and the plates were incubated at 37°C overnight. An increase of ≥ 5 mm of the inhibition zone around the ceftazidime/clavulanic acid disc compared with ceftazidime alone was interpreted as positive for ESBL production.

3.4. AmpC Production

Production of AmpC was screened by the AmpC disc test (13). Briefly, a blank disc moistened with sterile saline was inoculated with a few colonies of the test strain and placed next to a 30 µg cefoxitin disc on the surface of MHA plate, previously inoculated with an overnight culture of *E. coli* ATCC 25922. After overnight incubation at 37°C, flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disc containing the test strain was interpreted as positive for AmpC β-lactamase production (13).

3.5. Biofilm Assay

Biofilm formation was determined in vitro by the microtiter plate assay (Mtp) as previously reported (11).

Briefly, a 1:200 dilution of overnight grown bacterial cultures in trypticase soy broth (TSB, Liofilchem, Italy) was prepared and aliquots (200 µL) of each culture were inoculated to four wells of 96-well flat-bottomed polystyrene plates for each test organism. Following incubation at 37°C for 22-24 hours, the wells were washed twice with 200 µL of PBS, dried at room temperature and stained with 0.1% safranin solution in water for 15 minutes. The plates were then washed in distilled water, dried and the optical density of the biofilms was measured at 492 nm using an ELISA reader (Stat Fax 2100, Awareness Tech Inc., USA). Optical densities below 0.12 were considered as biofilm negative and OD > 0.12 were reported as biofilm positive (11). Each test was repeated on three different days and the results were reported as the mean of the obtained values.

3.6. Statistical Analyses

Non-parametric analyses were performed using the two-tailed Mann-Whitney U test using the SPSS software (version 19) for comparison of antibiotic resistance profiles, biofilm formation, AmpC and ESBL production between burn and non-burn groups.

4. Results

All isolates were resistant to cefepime, ceftazidime, cefotaxime, piperacillin, piperacillin-tazobactam, ciprofloxacin and imipenem. For the rest of the antibiotics, comparison between the burn and non-burn isolates is shown in Figure 1. As observed, resistance to amikacin, aztreonam and gentamicin were similar between burn and non-burn isolates (96.7 vs. 90%, 96.7 vs. 100% and 53.3 vs. 56.7%, respectively). On the other hand, resistance to tobramycin (60 vs. 10%) and meropenem (93.3 vs. 43.3%) was significantly higher in non-burn isolates compared to the burn strains ($P < 0.05$). Biofilm production occurred in 37 isolates (61.7%) among which, 22 (59.5%) were non-burn and 15 (40.5%) were burn isolates indicating that non-burn strains significantly produced more biofilm compared to the burn strains ($P < 0.05$). Figure 2 shows that biofilm-producing non-burn isolates were significantly more resistant to amikacin, meropenem and tobramycin compared to the biofilm negative strains within the same group ($P < 0.05$). Gentamicin resistance was also higher in the biofilm-producing non-burn strains than the non-producers but the difference was not significant (30% vs. 20%). On the other hand, the rate of resistance to all four antibiotics was similar among the burn strains regardless of their potential to form biofilm. There was no association between antibiotic resistance and biofilm formation among the burn isolates (Figure 2). Comparison of AmpC and ESBL production between burn and non-burn isolates is shown in Figure 3. Overall, regardless of the potential to form biofilm, AmpC production was significantly higher in non-burn isolates compared to the burn group (80% vs. 10%, $P = 0.00$). On the other hand, ESBL

production was significantly higher in the burn isolates (83.4% vs. 43.3%, $P < 0.01$). Finally, despite the low number of the isolates, co-production of AmpC and ESBL was much higher among the non-burn isolates compared to the burn samples (33.0% vs. 3.3%, $P < 0.05$) (Figure 3). Correlation between biofilm formation and production of AmpC (not ESBL) was only observed among the non-burn isolates.

5. Discussion

Biofilm formation is thought to be an important pathogenic feature in establishment and spread of *A. baumannii* infections (6). We found that the majority of our *A. baumannii* isolates, especially the non-burn strains, were capable of biofilm production. Multidrug resistance has been shown to correlate with the ability to form biofilms on abiotic and biological surfaces by *A. baumannii* (7, 14). Rao et al. (15) reported that a multidrug resistant isolate of *A. baumannii* was capable of forming significant amounts of biofilm, and biofilm production correlated with the accumulation of certain outer membrane proteins. In addition, antibiotic resistance mechanisms such as production of chromosomally encoded β -lactamases, efflux pumps and mutations in antibiotic target molecules also contribute to the survival of bacteria in biofilms (16). Biofilm-forming *A. baumannii* have been reported to show high levels of resistance ($> 75\%$) to imipenem, ciprofloxacin, cefotaxime, amikacin and aztreonam in India (15, 17). In an Iranian study, 92% and 68% of the isolates from urinary catheter isolates were resistant to ciprofloxacin and imipenem, respectively (18). Our results showed 100% resistance to both imipenem and ciprofloxacin. We found high levels of antibiotic resistance in both biofilm positive and negative test isolates and hence, were not able to observe an association between biofilm formation and resistance to most of the test antibiotics. However, despite the high levels of antibiotic resistance, there was a significant association between biofilm production and resistance to amikacin, tobramycin and meropenem in non-burn isolates. Nucleo et al. (19) showed that sub-minimum inhibitory concentrations of imipenem induced biofilm formation in a clinical isolate of *A. baumannii* in vitro. We found significantly higher susceptibility to meropenem compared to imipenem in both biofilm positive and negative test isolates. It has been shown that carbapenem-associated outer membrane protein channels possess an imipenem (but not meropenem) binding site depending on their primary structure (20). Hence, carbapenem susceptibility of clinical specimens should not be based on testing with imipenem only (21). The influence of β -lactamase production on biofilm formation in *A. baumannii* has also been demonstrated. In fact, a strong relationship was found between production of PER-1 β -lactamase and biofilm formation in clinical isolates of *A. baumannii* (14, 22). Similarly, a correlation between PER-1 β -lactamase and biofilm formation was observed in *Pseudomonas aeruginosa* (23).

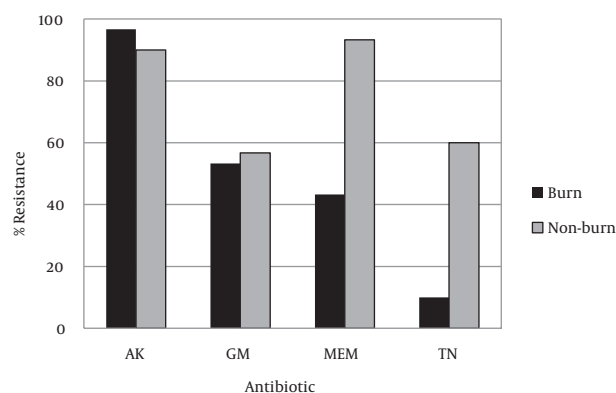


Figure 1. Comparison of Resistance Rates to Amikacin (AK), Gentamicin (GM), Meropenem (MEM), Aztreonam (ATM) and Tobramycin (TN) Between Burn and Non-Burn Isolates of *Acinetobacter baumannii*

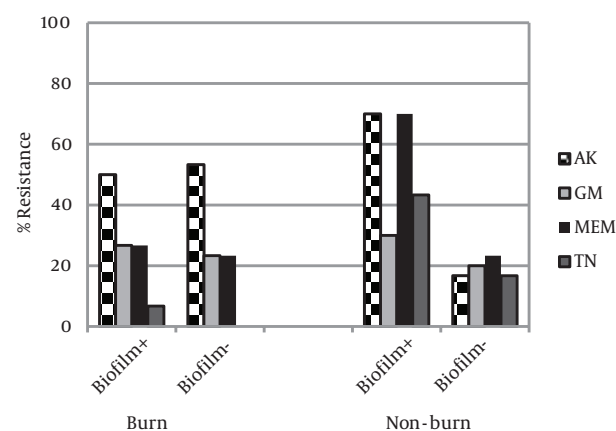


Figure 2. Distribution of Resistance Patterns to Amikacin (AK), Gentamicin (GM), Meropenem (MEM) and Tobramycin (TN) Among Biofilm Positive and Negative Burn and Non-Burn Isolates of *Acinetobacter baumannii*

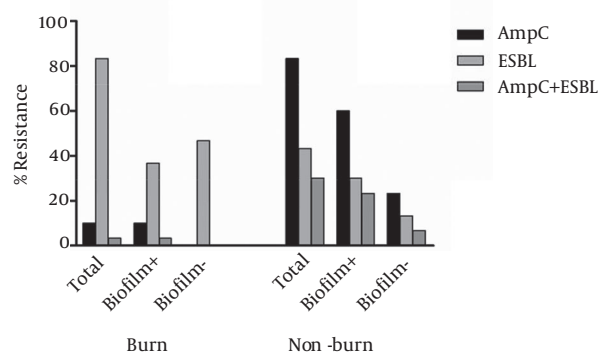


Figure 3. Correlation Between AmpC and ESBL Production and Biofilm Formation Among Burn and Non-Burn Isolates of *Acinetobacter baumannii*

In our study, AmpC production was significantly higher among the non-burn isolates regardless of their potential to form biofilms. Conversely, ESBL production was significantly higher in the burn group. Since the majority of our non-burn isolates (63%) came from ICU patients, we can assume that the antibiotic resistant strains may have had a chance to spread among patients by various means such as contact between patients and health care personnel during hospitalization. We did not find an association between AmpC and ESBL production and the potential to form biofilm among the burn isolates. However, a correlation was observed between biofilm formation and production of AmpC among the non-burn strains. Revdiwala et al. (24) showed that biofilm formation correlated with ESBL production in a number of Gram-negative clinical isolates including *A. baumannii*. Considering the differences found between the isolates from the two hospitals, further studies comparing the DNA fingerprints of the two groups are needed to determine whether the isolates from different health centers share common origins. The present study showed a higher rate of antibiotic resistance and AmpC production among the biofilm forming, non-burn isolates of *A. baumannii*. On the other hand, the burn strains produced significantly higher rates of ESBL but biofilm production was unrelated to antibiotic resistance or ESBL production.

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