Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
ISSN NO. 2320-5407

RESEARCH ARTICLE

Acinetobacter baumannii: Correlation between Biofilm Production and Multidrug Resistance

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Manuscript Info

Manuscript History

Received: 18 March 2015
Final Accepted: 22 April 2015
Published Online: May 2015

Key words:-
Acinetobacter baumannii, biofilm, multidrug resistance (MDR), intensive care unit (ICU), tube method, microtiter plate.

Abstract

Background: Recently, *Acinetobacter baumannii* has received much interest owing to its increasing enrollment in a number of serious infections in healthcare settings.
Aim: To assess *in-vitro* biofilm formation and examine the correlation between biofilm production and antibiotic resistance among clinical isolates of *A. baumannii*.
Materials and methods: A prospective cohort study was conducted over a period of 12 months starting from January 2014 to December 2014. A total of 56 *A. baumannii* isolates from different clinical specimens were recovered from patients admitted to the intensive care unit (ICU) of Emergency Hospital, Mansoura University, Mansoura, Egypt. Antimicrobial susceptibility testing was evaluated by the Kirby-Bauer's disc diffusion method. Biofilm formation was studied by both tube method and microtiter plate assay.
Results: *A. baumannii* isolates were resistant to most of the tested antibiotics except polymyxin B. Of these isolates, 62.5% were able to produce biofilms as shown by both tube method and microtiter plate assay. The highest tendency of biofilm production was shown by *A. baumannii* isolated from endotracheal aspirates (51.4%). About 77.1% of the biofilm-forming isolates were multidrug-resistant (MDR).
Conclusion: The present study revealed a high predilection among *A. baumannii* isolates to form biofilm, together with a significant association between biofilm production and MDR.

Introduction:-

Acinetobacter baumannii is strictly aerobic, catalase-positive, oxidase-negative, non-motile, non-fermenting, Gram negative coccobacillus that is widely distributed in clinical settings [1]. This opportunistic bacterium is associated with a wide range of infections in hospitalized patients, including; bacteraemia [2], pneumonia [3], meningitis [4], urinary tract infections [5] and wound infections [6].

Acinetobacter baumannii isolates are often resistant to high concentrations of antimicrobial drugs because of both intrinsic and acquired mechanisms, such as increased production of multidrug resistance (MDR) efflux pump proteins [7]. Besides, bacterial cells often exist as a biofilm, which is a structured community of bacterial cells enclosed in a self-produced polymeric matrix adherent to an inert or living surface [8]. *In-vitro* studies have demonstrated that biofilms can survive antibiotic concentrations of up to 1.000 times the minimum inhibitory

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concentration (MIC) for a planktonic culture, and *in-vivo*, bacteria that survive antibiotic exposure in a biofilm state can cause recurrence of infection upon cessation of antibiotic treatment [9].

Several investigators have yielded contradictory results as regard to the association between biofilm formation and MDR. For example, Gurung et al. (2013) studied 60 *A. baumannii* isolates and announced a positive relationship between biofilm formation and antibiotic resistance [10]. On the other hand, other co-workers did not find an association between biofilm production and MDR in *A. baumanii* isolates [11]. Therefore, this work was undertaken to study the *in-vitro* biofilm formation and verify the relevance between biofilm production and MDR in *A. baumannii* isolates.

Materials and methods:-

This prospective cohort study was conducted over a period of 12 months starting from January 2014 to December 2014.

Bacterial isolates. Clinical samples collected from patients admitted to the intensive care unit (ICU) of Emergency Hospital, Mansoura University, Mansoura, Egypt were referred to the microbiology laboratory at the Microbiology Diagnostics and Infection Control Unit (MDICU), Faculty of Medicine, Mansoura University for evaluation. All media used in this study were purchased from Oxoid (Basingstoke, UK) and prepared according to the manufacturer's instructions. *A. baumannii* isolates were identified based on their colony morphology, Gram staining characters and results of standard biochemical reactions [12].

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined by the Kirby-Bauer's disc diffusion method. Interpretation of the results was done according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [13]. Briefly, bacterial inocula were prepared by suspending the freshly grown bacteria in normal sterile saline adjusted to a 0.5 McFarland standard. The test strains were applied to the surface of Muller-Hinton agar (MHA) plates. Antibiotics tested (Oxoid, UK) included; amoxicillin (AML; 25 µg), amoxicillin/clavulanic acid (AMC; 20/10 µg), piperacillin/tazobactam (TZP; 100/10 µg), ceftazidime (CAZ; 30 µg), ceftriaxone (CRO; 30 µg), cefotaxime (CTX; 30 µg), cefepime (FEP; 30 µg), cefoperazone/sulbactam (SCF; 75/30 µg), aztreonam (ATM; 30 µg), imipenem (IPM; 10 µg), meropenem (MEM; 10 µg), amikacin (AK; 30 µg), gentamicin (CN; 10 µg), doxycycline (DO; 30 µg), polymyxin B (PB; 300 units), ciprofloxacin (CIP; 10 µg), ofloxacin (OFX; 5 µg) and trimethoprim/sulfamethoxazole (SXT; 1.25/23.75 µg). *Escherichia coli* (*E. coli*) ATCC 25922 was used for quality control.

Screening for ESBL-producing A. baumannii strains. This was done as part of the routine susceptibility testing according to the criteria set by the CLSI [13]. Two discs, ceftazidime (30 µg) and cefotaxime (30 µg), were used. Strains showing zone of inhibition of ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime were selected for conformational tests of ESBLs.

Phenotypic confirmation for production of ESBLs. Production of ESBLs by *A. baumannii* isolates was confirmed by the double-disc synergy test (DDST) [14]. Amoxicillin-clavulanic acid (20/10 µg) disks were placed in the center of MHA plates. The antibiotic discs of cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg) and aztreonam (30 µg) were placed with center to center distance of 30 mm to the centrally placed amoxicillin-clavulanic acid disc. The plates were incubated at 37◦C overnight. Increase of more than 5 mm in zone diameter in the presence of clavulanic acid was interpreted as positive result for ESBLs.

Assessment of biofilm formation. Both qualitative and quantitative tests were used to detect the biofilm-forming ability of *A. baumannii* isolates. The qualitative test was done by the tube method as described previously by Christensen and his co-workers [15]. According to this method, a loopful of the test strain was inoculated in 10 mL of trypticase soy broth [(TSB); Oxoid, UK] with 1% glucose in the test tubes. The tubes were incubated at 37◦C for 24 hours, then, the content of each tube was decanted, washed with phosphate buffered saline [(PBS); pH 7.3, Sigma-Aldrich, USA] and dried. The tubes were then stained with 0.1% crystal violet (Sigma-Aldrich, USA) for 15 minutes. Excess stain was washed with distilled water. The tubes were dried in an inverted position. The scoring for tube method was done according to the results of the control strains (positive control; *Staphylococcus epidermidis* ATCC 35984). Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as: I) weak/None, II) Moderate and III) High/strong. All tests were carried out in triplicates and the results were averaged.

-Also a quantitative test described by Christensen et al. (1985) [16] was performed. In brief, the test strains isolated from fresh agar plates were inoculated into 10 mL of TSB with 1% glucose. Inoculated broth media were incubated at 37◦C for 24 hours. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom microtiter plates (Sigma-Aldrich, USA) were filled with 200 µL of the diluted cultures. The positive control strains were treated in the same way and added to separate wells of microtiter plates, while negative controls contained only TSB. The plates were covered with a lid and incubated at 37 ◦C for 24 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µL of PBS (pH 7.3) four times to remove free floating bacteria, dried in an inverted position and stained with 0.1% crystal violet for 15 minutes. Excess stain was removed with distilled water and the plates were kept for drying. The optical density at 570 nm (OD 570) was determined using microplate reader. According to the absorbance values, the adherence capability of each isolate was classified into the following four categories: none (−), weak (+), moderate (++) and strong (+++) adherent cells. The cut-off absorbance value (ODc) was considered as three standard deviations above the mean OD of the negative control. Each assay was performed in triplicates and the average OD was considered.

Table 1. Adherence classification based on microtiter plate method (Stepanovic et al., 2007) [17]

Mean OD value	Adherence	Biofilm formation
OD ≤ ODc (< 0.09)	None	None
ODc < OD ≤ 2 ODc (0.09 - 0.18)	Weak	Weak
2 ODc < OD ≤ 4 ODc (0.18-0.36)	Moderate	Moderate
4 ODc < OD (0.36)	Strong	High

Legend. Optical density cut-off value (ODc) = average OD of negative control+ 3x standard deviation (SD) of negative control.

Statistical analyses:

All statistical analyses were performed using IBM-SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Qualitative data were described in the form of number and percentage. The statistical association of significance was assessed with the Chi-square (X^2) test. A *p*- value of <0.05 was considered to be statistically significant.

Results:-

Fifty six clinical isolates of *A. baumannii* were detected during the study period. Of these isolates; 20 were recovered from endotracheal aspirates (35.8%), 12 (21.4%) from sputum, 9 (16%) from blood, 5 (8.9%) from urine, 5 (8.9%) from wound swabs, 2 (3.6%) from bronchoalveolar lavage (BAL), 2 (3.6%) from pus and 1 (1.8%) from CSF. *A. baumannii* strains represented 28.7% of the total isolates.

Table 2. Antibiotic testing result of *Acinetobacter baumannii* isolates by disk diffusion method

Antibiotic	Resistant strains	
	Total isolates (56)	Percent (100%)
Amoxicillin	53	94.6
Amoxicillin/clavulanic acid	51	91
Cefotaxime	44	78.6
Ceftriaxone	43	76.8
Ceftazidime	42	75
Aztreonam	40	71.4
Doxycycline	38	67.9
Trimethoprim/sulfamethoxazole	36	64.3
Cefepime	35	62.5
Ciprofloxacin	34	60.7
Ofloxacin	34	60.7
Cefoperazone/sulbactam	33	58.9
Gentamicin	33	58.9
Amikacin	32	57.1
Imipenem	31	55.4
Meropenem	31	55.4
Piperacillin/tazobactam	29	51.8
Polymyxin B	2	3.6

Antimicrobial susceptibility testing of *A. baumannii* isolates by disk diffusion method revealed that resistance to amoxicillin and amoxicillin/clavulanic acid was the most common where 94.6% and 91% of the isolates were resistant, respectively. On the other hand, there was a high degree of susceptibility to polymyxin B where only 3.6% of the isolates (n=2) exhibited resistance to this antibiotic. Furthermore, 62.5% (n=35) of the examined *A. baumannii* isolates were MDR [table 2]. Out of the 56 tested *A. baumannii* isolates, 55.4% of the isolates (n=31) were found to be phenotypically ESBL-producers.

The qualitative tube method for screening for biofilm production explored that 35 *A. baumannii* isolates (62.5%) were positive for biofilm formation. On the other hand, the quantitative microtiter plate assay was strongly positive in 35 isolates (62.5%) while the remaining isolates were either weakly-adherent (n=7) or biofilm non-producers (n=14) [figure 2]. The weakly-adherent isolates were considered negative for biofilm production. The highest propensity of biofilm production was shown by *A. baumannii* recovered from endotracheal aspirates (51.4%) followed by sputum (28.6%), urine samples (14.3%) and bronchoalveolar lavage (5.7%) [figure 1].

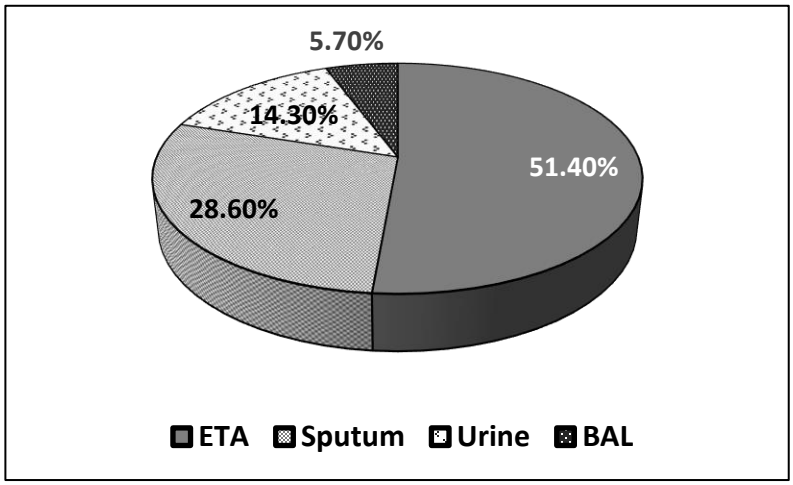

Fig. 1. Distribution of biofilm-producing *Acinetobacter baumannii* isolates according to different kinds of clinical specimens

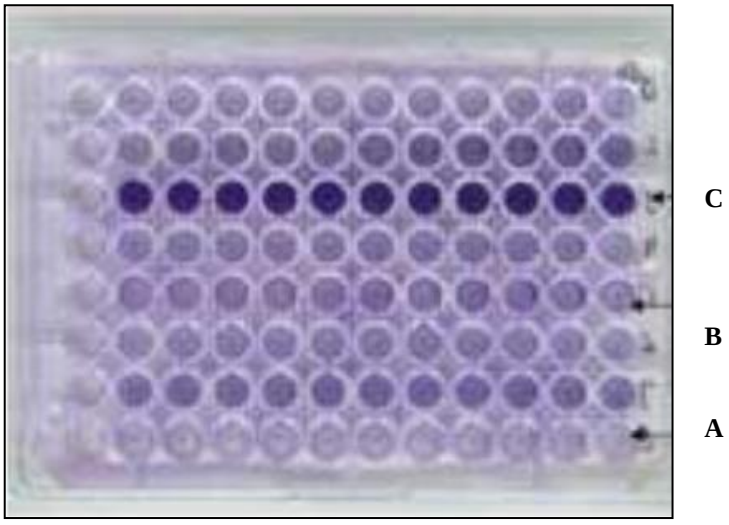

Fig. 2. Microtiter plate assay for detection of biofilm formation among *Acinetobacter baumannii* isolates
A: No biofilm (OD_{570} < 0.09)
B: Weak biofilm (OD_{570}: 0.09 - 0.18)
C: High biofilm (OD_{570}: > 0.36)

Table 3. Comparison of antibiotic resistance profile among biofilm-positive and biofilm-negative *Acinetobacter baumannii* isolates

Antibiotic	Biofilm positive-resistant isolates (%)	Biofilm negative-resistant isolates (%)	No. of resistant isolates
AML (25 µg)	35 (66%)	18 (34%)	53
AMC (20/10 µg)	35 (68.6%)	16 (31.4%)	51
CTX (30 µg)	35 (79.5%)	9 (20.5%)	44
CRO (30 µg)	35 (81.4%)	8 (18.6%)	43
CAZ (30 µg)	35 (83.3%)	7 (16.7%)	42
ATM (30 µg)	35 (87.5%)	5 (12.5%)	40
DO (30 µg)	31 (81.6%)	7 (18.4%)	38
SXT (1.25/23.75 µg)	25 (69.4%)	11 (30.6%)	36
FEP (30 µg)	25 (71.4%)	10 (28.6%)	35
CIP (10 µg)	20 (58.8%)	14 (41.2%)	34
OFX (5 µg)	21 (61.8%)	13 (38.2%)	34
SCF (75/30 µg)	25 (75.8%)	8 (24.2%)	33
CN (10 µg)	21 (63.6%)	12 (36.4%)	33
AK (30 µg)	21 (65.6%)	11 (34.4%)	32
IPM (10 µg)	24 (77.4%)	7 (22.6%)	31
MEM (10 µg)	23 (74.2%)	8 (25.8%)	31
TZP (100/10 µg)	20 (69%)	9 (31%)	29

Legend: AML; amoxicillin, AMC; amoxicillin/clavulanic acid, CTX; cefotaxime, CRO; ceftriaxone, CAZ; ceftazidime, ATM; aztreonam, DO; doxycycline, SXT; trimethoprim/sulfamethoxazole, FEP; cefepime, CIP; ciprofloxacin, OFX; ofloxacin, SCF; cefoperazone/sulbactam, CN; gentamicin, AK; amikacin, IPM; imipenem, MEM; meropenem, TZP; piperacillin/tazobactam.

Concerning antibiotic resistance pattern among both biofilm positive and negative *A. baumannii* isolates, a higher antibiotic resistance profile was observed among biofilm-producing isolates compared to biofilm non-producers [table 3] with a statistically significant difference (p <0.05). In addition, it was noticed that 77.1% of biofilm-producing *A. baumannii* isolates (27/35) were considered to be MDR compared to 38% of biofilm non-producing strains with a statistically significant difference (p <0.05). Noteworthy, DDST for detection of ESBLs was positive in 57.1% (n=20) of biofilm-producing *A. baumannii* isolates.

Discussion:-

During the past few decades, *A. baumannii* has emerged as an important opportunistic pathogen in hospitalized patients, particularly in ICUs [18], and this is probably related to the increasingly invasive diagnostic and therapeutic procedures used in these clinical settings [19]. Due to their widespread resistance to antibiotics, management of such infections can be very difficult contributing to prolonged stay in ICUs and increased patients' mortality [20]. Accordingly, this research was accomplished to check biofilm formation by *A. baumannii* isolates retrieved from ICU, Emergency Hospital, Mansoura University and to detect a potential association between establishment of biofilm and MDR using phenotypic methods.

In the current work, most of *A. baumannii* isolates were recovered from endotracheal aspirates (35.8%), followed by sputum (21.4%), blood (16%) and urine (8.9%). Concomitant with the results of this study, Mostofi et al. reported that amongst the investigated *A. baumannii* isolates, 30% originated from endotracheal aspirates, 12% from wound swabs and 8% from urinary tract infections [21]. Likewise, Dheepa et al. from a study performed in India, realized isolation rates of about 24% from endotracheal aspirates and 16 % from sputum samples [22]. On the contrary, Hsueh and co-workers detected a high isolation rate of *A. baumannii* from sputum samples accounting for 71.4% [23].

Different definitions of MDR *A. baumannii* have been adopted in the biomedical literature. However, the most widely accepted one is resistance to more than three classes of antibiotics [24]. In this study, the prevalence of MDR isolates was 62.5% (n=35). This finding runs parallel to those reported by Dent et al. (2010) [25] and De Francesco et al. (2013) [26] where MDR *A. baumannii* isolates comprised 72% and 54%, respectively. In contrast, higher prevalence was reported by Begum et al. (2013) where 100% of the *A. baumannii* isolates collected from a tertiary

care hospital in Islamabad, Pakistan were found to be MDR [27]. On the opposite side, Soroush et al. (2010) indicated that only 40.6% of their *A. baumannii* isolates were MDR [28]. In the present study, a remarkably high susceptibility rate of *A. baumannii* isolates to polymyxin B was observed where only 3.6% of the isolates were resistant to such antibiotic. In agreement to this finding, Mak et al. (2009) claimed that polymyxin B was the most effective drug in controlling this pathogen [29]. Moreover, a study in New Delhi, India recorded 96.4% sensitivity to polymyxin B among *A. baumannii* isolates [30]. Another Indian study showed 100% sensitivity to polymyxin B [31]. Similarly, only 1.6% of the *A. baumannii* isolates exhibited resistance to polymyxin B in a study based in Korea [32].

Phenotypic identification of ESBL-producing isolates has been achieved using the DDST. From a total of 56 isolates, 55.4% (n=31) were identified to produce ESBLs. Similar result was mentioned by Mohajeri et al. (2014) where 61.9% of their *A. baumannii* isolates were confirmed to be ESBL-producers [33]. Nevertheless, lower prevalence rate was recognized by Safari et al. (2015) as only from a total of 100 samples, 7% *A. baumannii* isolates were identified to produce ESBL enzymes [34].

Biofilm formation is thought to be an important pathogenic feature in the establishment and spread of *A. baumannii* infections [35]. Both qualitative tube method and quantitative microtiter plate assay have been employed in this study for detection of biofilm-producing *A. baumannii* isolates. Subsequently, both methods concluded that 35 isolates (62.5%) were strong biofilm producers, indicating a significant agreement between the results of both methods. Nonetheless, seven weakly-adherent isolates were additionally detected by the quantitative microtiter plate assay asserting its sensitiveness, being the gold-standard method for biofilm screening [36]. However, as adherence alone may not complete the cycle of biofilm formation and there might be additional mechanisms that could explain adherence, the weakly-adherent isolates were classified as biofilm negative strains [37]. Similar occurrence of 63% and 62% biofilm formers have also been reported by Rodriguez et al. [38] and Rao et al. [39], respectively. However, a study done in India by tube method reported 50% positivity for biofilm formation in *A. baumannii* isolates [10].

It is worth mentioning that the majority of biofilm-forming isolates were recovered from endotracheal aspirates (51.4%), followed by sputum (28.6%) and urine samples (14.3%). These results are in accordance with those of Donlan (2001) who reported a strong relationship between biofilm-producing bacteria and urinary catheters [40]. Besides, Mansour and co-worker (2012) mentioned that the majority of biofilm-producing *A. baumannii* were from tracheal aspirates and urine specimens (94.4%) [11].

In this study, the association between biofilm-forming ability and individual drug resistance of *A. baumannii* was analyzed. The resistance rates of most of the tested antibiotics were found to be greater in biofilm-forming isolates compared to the biofilm non-forming group. This result is in concordance with the finding of Hassan et al. (2011) [41]. Similarly, Nahar et al. (2013) has reported 100% resistance to amoxicillin, ceftriaxone, ceftazidime and aztreonam in biofilm-forming *Acinetobacter* species. Resistance to gentamicin, amikacin, ciprofloxacin and imipenem was also higher among biofilm-forming *Acinetobacter* isolates in the same study [42]. Abdi-Ali et al. (2014) confirmed that 92% of their biofilm-forming *A. baumannii* isolates were resistant and 6.6% were susceptible to ciprofloxacin, although the results for imipenem were 68% and 24%, respectively. They attributed the high level of resistance to ciprofloxacin to its considerable use in their hospitals [43]. It was interesting to note that 57.1% of the biofilm-producing *A. baumannii* isolates were also ESBL-producers. On the other hand, Gurung et al. (2013) stated higher prevalence, as 73% of their biofilm-forming *Acinetobacter* isolates were ESBL-producers [10].

A variety of reasons for the increased antimicrobial resistance of microorganisms in biofilms have been postulated. Although bacteria in biofilms are surrounded by an extracellular matrix that might physically impede the diffusion of antimicrobial agents, this does not sound to be a dominating mechanism of biofilm-associated antimicrobial resistance. Nutrient and oxygen depletion within the biofilm can cause some bacteria to enter a stationary state, in which they are less susceptible to growth-dependent antimicrobial killing. A subpopulation of bacteria may differentiate into a phenotypically resistant state. Lastly, some organisms in biofilms have been shown to express biofilm-specific antimicrobial resistance genes that are not necessary for biofilm formation [44].

Conclusion:-

Globally, the results of this study displayed that most of the clinical isolates of *A. baumannii* have the potential to produce biofilms. Besides, a positive correlation was disclosed between biofilm formation and MDR in *A. baumannii*. However, farther research should be done in the future in this area, to provide better understanding of biofilm processes in *A. baumannii* in order to combat this real threat, thereby, improving patient management.

Funding:
No funding sources.
Conflicts of interest:
None.

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