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THE ASSOCIATION OF BIOFILM FORMATION WITH ANTIMICROBIAL RESISTANCE IN ACINETOBACTER BAUMANII COMPLEX ISOLATED FROM HOSPITAL ADMITTED PATIENTS

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ABSTRACT

Background: *Acinetobacter baumanii* is one of the important emerging opportunistic pathogens that lead to serious nosocomial infections. Its major virulence properties, such as the ability to adhere to surfaces, the capacity to form microbial communities, and resistance to antimicrobial agents, make it difficult to control and eliminate. Therefore, this study was conducted to detect the association of biofilm formation with antimicrobial resistance in Acinetobacter baumanii complex (ABC) isolated from hospital admitted patients. **Methods**: One hundred clinical isolates of ABC derived from sputum, endotracheal tube secretions, blood, urine, and pus were obtained from patients admitted to Chitwan Medical College and Teaching Hospital. The isolates were identified by standard microbiological procedures. Biofilm formation was detected by the tissue culture plate method. Antimicrobial susceptibility was tested using modified Kirby-Bauer disk diffusion method as per CLSI guidelines. Multidrug resistance, extensive drug resistance and pandrug-resistance were determined and compared them among biofilm producers and non-producers. **Results:** ABC was remarkably associated with biofilm formation, i.e. 80% of the isolates were biofilm producers. Interestingly, all ABC isolated from endotracheal tube secretions were biofilm producers. The biofilm producing ABC showed a significantly higher (P<0.05) incidence of antimicrobial resistance compared to the biofilm non-producers for 17 antimicrobial agents tested. Of a total of 80 biofilm positive isolates, 71.25% were multidrug resistant, 3.75% were extensively drug resistant and 2.5% were pandrug resistant isolates. Multidrug resistance in biofilm producing isolates was found to be significantly (p<0.05) higher than in biofilm non-producing isolates. **Conclusion:** ABC isolates that have a high propensity to form a biofilm have a significant association with multiple drug resistance, aiding their ability to promote colonization and leading to persistent nosocomial infections. Therefore, early detection of the presence of a biofilm could aid the selection and delivery of an effective antimicrobial treatment strategy.

KEYWORDS: A. baumanii complex, biofilm, antimicrobial resistance.

INTRODUCTION

Acinetobacter baumannii complex (ABC), once neglected in the clinical setting has now emerged as the pathogen that accounts for almost 80 % of all reported *Acinetobacter* infections.[1] They are responsible for various serious infections such as ventilator-associated pneumonia, bacteremia, meningitis, peritonitis, urinary tract infections, and wound infections.[2] Infections due to these pathogens are even more vulnerable when associated with medical devices, e.g., vascular catheters, cerebrospinal fluid shunts, foley catheters etc.[3] This is due to the ability of ABC to adhere and form biofilms (extracellular polymeric substances, EPS) on biotic and abiotic surfaces (inanimate objects). The action of antimicrobials may be decreased due to the excessive production of EPS and persistent metabolically inactive cells in the biofilm. Therefore, resistance rate has been increased, limiting few therapeutic options.[4,5]

In recent years, multidrug-resistant (MDR) *A. baumannii* are increasingly held responsible for nosocomial infections. Unfortunately, MDR *A. baumannii* clones are spreading into new geographic areas with increasing

number of strains acquiring many resistance genes.[4] Furthermore, newer extended-spectrum-beta-lactamases and different carbapenemases are emerging fast, leading to pan-resistant strains of *A. baumannii*. In addition, this bacterium has shown a remarkable tendency to develop resistance to virtually every antibiotic class.[6] Thus, this study was conducted to detect the prevalence of biofilm in ABC isolated from hospitalized patients and its association with antimicrobial resistance.

MATERIALS AND METHODS

The study was carried out in patients admitted to Chitwan Medical College and Teaching Hospital (CMCTH), Bharatpur, Nepal. The specimens were collected from patients admitted to different wards including intensive care unit (ICU) and emergency department and processed by standard microbiological procedures. The isolates were identified by Gram stain, cultural characteristics and biochemical characteristics including the capacity of strains to grow at 41°C and 44°C.[7] A total of 100 isolates of ABC obtained from sputum, endotracheal tube secretions, blood, urine and pus were included in this study.

Biofilm detection

Biofilm formation was detected by tissue culture plate method (TCP). This assay proposed by Christensen et al is generally considered to be the gold-standard technique for biofilm detection.[8] A colony of bacteria was grown overnight at 37 °C in 2 mL of trypticase soy broth (TSB). The bacterial culture was then diluted 1:100 with sterile fresh medium. Each well of a 96-well flat-bottomed polystyrene tissue culture plate (3 wells for each strain) was filled with 200 µL of the diluted culture. The biofilm producing reference strain of A. baumannii ATCC 19606 was used as positive control. Sterile broth was used as negative control. The plates were covered with a lid and incubated aerobically for 24 hours at 37 °C. After incubation, the content of each well was removed and the wells were carefully washed, three times, with 0.2 mL of phosphate buffer saline (pH 7.2) in order to remove free floating bacteria. Adherent bacteria were fixed with 99% methanol for 10-15 min. The plates were decanted and allowed to dry. Then, plates were stained for 10min with 0.1% crystal violet (CV). Excess stain was rinsed off by washing with tap water. Optical density (OD) of stained adherent biofilm was measured by using a micro-ELISA reader (Human) at wavelength 570 nm. The experiment was performed in triplicate. The interpretation of biofilm production was done as per the criteria of Stepanovic et al[9] and categorized as weak, moderate and strong biofilm producers.

Antimicrobial susceptibility test

The antimicrobial susceptibility tests of the isolates against various antimicrobials were performed using Muller Hinton agar (MHA) by the standard disk diffusion technique (modified Kirby-Bauer method) and interpreted as per the CLSI guidelines.[10] The following antimicrobial agents were tested: amikacin (30 µg), ampicillin/sulbactum (10/10µg), aztreonam (30µg), cefepime (30µg), cefotaxime (30 µg), ceftazidime(30µg), ciprofloxacin (5µg), cotrimoxazole (25µg), doxycycline (30µg), gentamicin (10µg), imipenem(10µg), levofloxacin (5µg), meropenem (10 µg), minocycline (30µg), netillin (30µg), piperacillin/tazobactam (100/10 µg), tetracycline (30µg), ticarcillin clavulanic acid (75/10 µg), (Hi-media Laboratories India).

Identification of multidrug- resistant (MDR), extensively drug- resistant (XDR) and pandrug-resistant (PDR) isolates

MDR, XDR and PDR isolates were identified according to the combined guidelines of the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC).[11] The isolates resistant to at least one agent in three or more antimicrobial categories were regarded as multidrug resistant (MDR). Extensively drug resistance was considered as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) whereas as pandrug resistance as non-susceptibility to all agents in all antimicrobial categories (i.e. no agents tested as effective for that organism).

Statistical Analysis

SPSS software (SPSS Inc no.17) was used for data analysis. Chi-square ($\chi 2$) test was used for analysis of categorical data. A P-value of < 0.05 was considered statistically significant.

RESULTS

Detection of biofilm by TCP method

Among 100 clinical isolates obtained from hospital admitted patients, biofilm formation was observed in 80 isolates (80%). 10% isolates produced biofilm strongly, 40 % isolates did moderately and 30 % isolates did weakly (table 1).

Table 1: Detection of biofilm by Tissue culture plate method.

	Biofilm	S N (%)	M N (%)	W N (%)	Total N (%)
TCP	Positive	10 (10)	40 (40)	30 (30)	80 (80%)
	Negative	N/A	N/A	N/A	20 (20%)
Abbreviations; S; strong, M; moderate, W; weak, N/A; Not applicable					

Distribution of biofilm forming isolates on various specimens

Among total isolates, all the isolates collected from ET were biofilm producers whereas 85.7%, 78.6%, 71.4% and 66.7% from sputum, blood, pus and urine respectively were biofilm producers (figure 1).

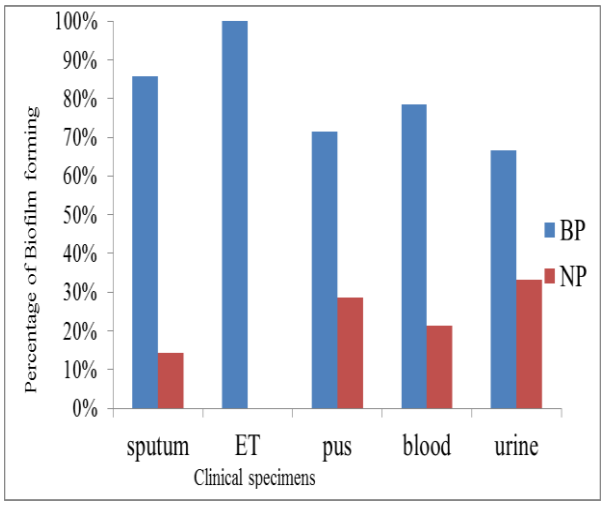

Figure 1. Distribution of biofilm forming isolates based on various specimens.

Distribution of biofilm forming isolates on hospital wards

Of all the isolates obtained from different wards, 20/20 (100%) isolates from ICU, 18/22 (81.8%) isolates from non-ICU (wards other than ICU) and 42/58 (72.4%) isolates from emergency department were biofilm producers (figure 2).

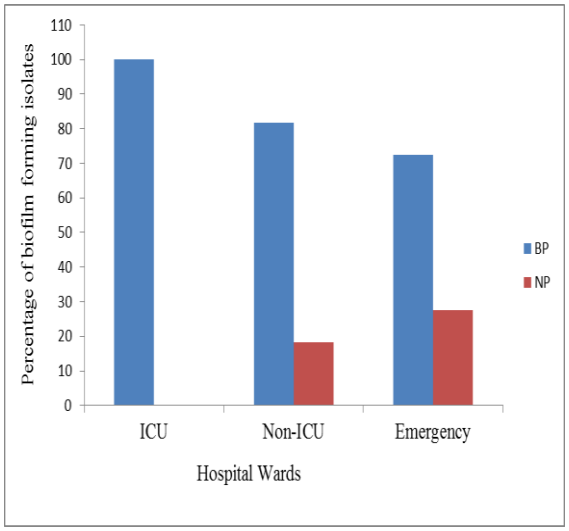

Figure 2. Distribution of biofilm forming isolates based on hospital wards.

Antimicrobial resistant patterns

The biofilm producing ABC showed significantly higher incidence of resistance to the following antimicrobials compared to the biofilm non-producers: amikacin, ampicillin/sulbactum, aztreonam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, cotrimoxazol, gentamicin, levofloxacin, meropenem, minocycline, netillin, piperacillin/tazobactam, ticarcillin clavulanic acid and tobramycin ($p<0.05$). However, resistance pattern was higher but not significant for imipenem, doxycycline, polymyxin B and tetracycline (table 2).

Table 2: Antimicrobial resistance patterns in biofilm producers and non-producers.

Antimicrobials	**BP(N=80)**	**BN(N=20)**	***P* value**
Aminoglycosides			
Amikacin (30µ g)	34(42.5)	0(0)	0.000*
Gentamicin (10µ g)	46(57.5)	6(30)	0.280
Netillin (30µ g)	46(57.5)	0(0)	0.000*
Tobramicin (10µ g)	42(52.5)	2(10)	0.001*
Antipseudomonal penicillins with β lactamase inhibitor			
Piperacillin/tazobactam (100/10µ g)	58(72.5)	4(20)	0.000*
Ticarcillin clavulanic acid (75/10µ g)	46(57.5)	4(20)	0.003*
Carbapenem			
Meropenem (10µ g)	46(57.5)	6(30)	0.028*
Imipenem (10µ g)	32(40)	4(20)	0.960
Extended spectrum cephalosporins			
Cefotaxime (30µ g)	74(92.5)	8(40)	0.000*
Ceftazidime (30µ g)	74(92.5)	8(40)	0.000*
Cefepime (30µ g)	74(92.5)	10(50)	0.000*
Fluoroquinilones			
Ciprofloxacin (5µ g)	58(72.5)	8(40)	0.006*
Levofloxacin (5µ g)	46(57.5)	0(0)	0.000*
Folate pathway inhibitor			
Cotrimoxazole (25µ g)	52(65)	8(40)	0.041*
Monobactum			
Aztreonam (30µ g)	72(90)	6(30)	0.000*
Penicillin with β lactamase inhibitor			

Ampicillin/sulbactum (10/10µ g)	26(32.5)	0(0)	0.003*
Polymyxin B (10µ g)	4(5)	0(0)	0.307
Tetracyclines			
Tetracycline (30µ g)	40(50)	6(30)	0.108
Doxycycline (30µ g)	14(17.5)	2(10)	0.413
Minocycline (30µ g)	34(42.5)	2(10)	0.007*
Abbreviation; * statistically significant			

MDR, XDR and PDR isolates

Of a total of 80 biofilm positive isolates, 57 (71.25%) were MDR, 3 (3.75%) were XDR and 2 (2.5%) were PDR. Multidrug resistance was found to be significantly (p<0.05) higher in biofilm producing isolates than in biofilm non-producing isolates (table 3).

Table 3: MDR, XDR, PDR isolates in biofilm producers and non-producers.

	MDR	XDR	PDR
Biofilm positive (n=80)	57 (71.25%)	3 (3.75%)	2 (2.5%)
Biofilm negative (n=20)	8(40%)	0 (0)	0 (0)

DISCUSSION

ABC plays a significant role in colonization and infection of patients admitted to hospitals. They have been implicated in a variety of nosocomial infections, particularly ventilator associated pneumonia in patients confined to intensive care units (ICU).[3,4] They may form biofilm even in the harsh environment, resist the antimicrobial therapy and survive for the extended period of time.

In the present study, the rate of biofilm formation by ABC (80%) was found to be higher as compared to that observed by Dheepa et al (60%)[12] and Babapour et al (66.66%)[13]. Acinetobacter infections have been associated closely with surgery or the use of artificial devices. Nahar et al[14] observed that 100% Acinetobacter species isolated from central venous catheter blood, 66.7% from endotracheal tube and 84.2% from tracheal aspirates formed biofilm. Interestingly, 100% of the isolates from ET in the current study formed the biofilm. The biofilm formation rate in the isolates obtained from other specimens [(sputum (85.7%), blood (78.6%), pus (71.4%) and urine (66.7%)] is almost similar to that observed by Cevahir et al.[15]

A. baumanii is one of the common opportunistic pathogens colonizing patients particularly in ICUs, where multiple manipulations following surgery, as well as the endotracheal tubes and intravascular, ventricular or urinary catheters are frequently used.[15] In our study, 100% isolates from ICU, 81.8% from non-ICU and 72.4% from emergency department produced biofilm, the observation is slightly higher than the report of Nahar et al[14] who noticed that 87.5% of Acinetobacter isolates from ICU and 55.0% isolates from non ICU were biofilm producers.

More than 60% of hospital-acquired infections worldwide are due to bacteria forming biofilms on medical devices.[16] The temporary implantation of a vascular catheter, a urinary catheter or an endotracheal tube can become a site for *A. baumannii* adhesion causing infection.[17] Further, Feldman et al [18] also showed that interior of the ETT was colonized by bacteria within a biofilm. This fact becomes clear by the ability of *A. baumanii* to survive in the inanimate objects even after exposure to dry conditions.

Based on adherence property, our study showed 10% strong, 40% moderate, and 30% weak biofilm producers. Our result is in accordance with the finding of Dheepa et al.[12] who also reported higher number of moderate biofilm producers. However, Abdi-Ali et al[19] noted 18% strong, 10% moderate and 41% weak biofilm producers. The variation of adherence property may be due to the difference in the number of clinical isolates from different sources and also the factors such as surface area, type of surface (rough/ smooth), porosity, charge on the surface and surface hydrophobicity.[19] In the current study, intrinsic ability of the clinical isolate to form biofilms on the culture plate but not on the indwelling medical devices or tissues was assessed.

In the present study, significantly higher incidence of antimicrobial resistance was observed in biofilm producing ABC compared to biofilm non-producers (p<0.05). Resistance in biofilm producers was >90% to cephalosporins and aztreonam, >40% to carbapenem, and aminoglycosides, and 72.2% to pipracillin/tazobactum and ciprofloxacin, 65% to cotrimoxazole, and 57.5% to ticarcillin/clavulanic acid. Only biofilm producers were resistant to amikacin, ampicillin/sulbactum, levofloxacin, netillin, and polymyxin B. Although carbapenem has been the antibiotic of choice for treatment of infections caused by this organism, resistance to this antimicrobial will be alarming because only few therapeutic options may remain. In the present study, 40% of the isolates were resistant to imipenem, this finding being similar to the observation of Houang et al who reported 43% resistance to imipenem.[20] Another study showed that biofilm producing Acinetobacter isolates exhibited 100% resistance to imipenem, 82%, to amikacin 88% to cephotaxime, 70% to ciprofloxacin and 38% to aztreonam.[21] Our results were also in accordance with the data reported by Dheepa et al[12] who observed resistance to ceftazidime as 95%, cefepime 95%, aztreonam 85%, ciprofloxacin 85%, amikacin 80%,

gentamicin 70%, imipenem 65%, pipercillin+tazobactum 40% and netilmicin 20% in biofilm producing *A. baumanii*. Mak et al claimed that polymyxin B is the most effective drug in controlling this bacterium.[22] However, we observed 5% resistance against this antimicrobial which indicates the limitation of the therapeutic options for the infections associated with ABC.

In this study, multidrug resistance in the biofilm forming isolates (71.25%) is significantly higher ($p<0.05$) than the multidrug resistance in biofilm non producers. A previous literature has also reported high (90%) multidrug resistance in the biofilm producers.[13] Moreover, in the present study, only biofilm producers were XDR (3.25%) and PDR (2.25%) isolates. ABC might acquire resistance to multiple drugs within biofilm communities. The high colonizing capacity of ABC combined with its resistance to multiple drugs, will contribute to the organism's survival and further dissemination in the hospital setting. Thus, the functions of the biofilm formed by ABC encompass its ability to resist antimicrobial therapies as well as to protect from external stresses such as dehydration and limited nutrient availability.[5, 20]

CONCLUSION

The results showed that most clinical isolates of ABC isolated from hospitalized patients have the ability to produce biofilms. This could potentially promote colonization of antibiotic-resistant bacteria in hospital environments. The persistence of nosocomial infection leads to increase the morbidity and mortality. Thus, a continuous monitoring of biofilm and antibiotic susceptibility in clinical isolates of ABC in every hospital seems necessary. Further, early detection of biofilm can aid to formulate effective antimicrobial treatment strategy and infection control.

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CONFLICT OF INTEREST

None

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