



Original Research Article

Quantum of drug resistance among biofilm forming *Acinetobacter spp.* from intensive care units from a tertiary care hospital

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ABSTRACT

Keywords

Biofilm formation, *Acinetobacter spp.*, Drug-resistance

Biofilm formation on biotic and abiotic surfaces coupled with expression of multidrug resistance have helped *Acinetobacter spp.* emerge as successful hospital acquired pathogens. To study biofilm formation and drug-resistance in *Acinetobacter spp.* isolated from Endotracheal suction catheters from Intensive care unit patients. A total of seventy-five isolates of *Acinetobacter spp.* grown in pure culture from endotracheal suction catheters of patients admitted in intensive care units were included in the study. These isolates were subjected to anti-microbial sensitivity testing by modified Kirby Bauer disc diffusion method and biofilm formation by Microtitre plate method. : *Acinetobacter baumannii* was the main specie isolated. All isolates were biofilm producers. Quantum of drug resistance appeared proportionate to the strength of Biofilm forming capacity. *A.baumannii* has evolved has major public health problem. Biofilm formation and expression of high degree of drug resistance help them establish successfully. Since there is continuous shrinkage of available therapeutic options, measures to prevent inter and intra-hospital transmission of these deadly pathogens must be established in health care settings.

Introduction

Acinetobacter baumannii is one of the most important nosocomial pathogen to have drawn attention in the last couple of years owing to expression of multi-drug resistance. It is frequently isolated in pneumonia, urinary tract infections, bacteremia, meningitis, wound infections and above all from indwelling devices in the patients admitted especially in critical care areas. This can survive in hospital

environments despite unfavorable conditions such as desiccation, nutrient deficiencies and anti-microbial treatments. It is hypothesized that its ability to persist in these environments as well as its virulence is a result of its capacity to form biofilms. It can form biofilm on various abiotic surfaces such as polystyrene and glass as well as on biotic surfaces like epithelial cells. The adhesion and biofilm

forming characters of some clinical isolates seem to be related to the presence of multi-drug resistance. Of all the infections caused by this organism, the infections related to indwelling devices are most difficult to eradicate since organisms in biofilm are irreversibly attached to substratum or interface to each other embedded in a matrix of extracellular polymeric substances and activity of anti-microbial agents is greatly reduced under these conditions. The present study was undertaken to detect biofilm production and its association with quantum of drug resistance among the clinical isolates of *Acinetobacter baumannii*.

Materials and Methods

The study was conducted in the Department of Microbiology from December 2010 to November 2011. The study design was Prospective Observational. The Endotracheal (ET) suction catheters from patients between 18-75 yrs of age on assisted ventilation, which grew pure culture of *Acinetobacter spp* within 48 hours of sample inoculation on culture media, were included in the study. A total of seventy-five *Acinetobacter spp* were included and were tested for potential to form biofilm formation and antibiogram pattern.

The ET suction catheters were processed by Roll-Over Technique (Maki DG, *et al* 1977). The distal 5 cm of the catheter was transferred to the surface of a 90-mm 5% sheep BA (blood Agar) plate and rolled back and forth across the surface five times and incubated at 37°C overnight. Then, 2ml of Trypticase Soya Broth (TSB) was pushed through the lumen of the catheter and semi-quantitative culture was carried out (Cleri, D. J, *et al* 1980). The colonies morphologically resembling

Acinetobacter spp. were subjected to conventional biochemical tests. The isolates provisionally identified as *Acinetobacter spp* were further tested in Microscan WalkAway 40plus system for speciation and results were recorded after 48 hrs of incubation. Standard strains of organisms *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were tested for gram negative panels on weekly basis as a part of internal quality control for the microscan identification and antimicrobial sensitivity testing panels. The isolates were tested for antimicrobial susceptibility by Kirby-Bauer's disc diffusion method. Discs manufactured by Hi-Media (Mumbai); ciprofloxacin (5mcg), amoxicillin-clavulanic acid (10mcg), imipenem (10mcg), gentamycin (10mcg), amikacin (30mcg), piperacillin+tazobactam (100mcg/10mcg), ceftriaxone (30mcg), co-trimoxazole (1.25mcg/23.75mcg) were used for determination of anti-biogram. Isolates were subjected to ESBL determination by double disc synergy testing (DDST) using ceftazidime (30mcg) and ceftazidime-clavulanic acid (30mcg/10mcg) and detection of AmpC β -lactamases by using cefotaxime 30 μ g discs and cefotaxime-3 amino phenylboronic acid 30 μ g/300 μ g discs (Jacoby, G.A. *et al* 2009).

Semi-quantitative evaluation of biofilm formation was done by using Micro-titer plate method (Nural, C., *et al* 2008). Freshly sub-cultured isolates were taken and 3 isolated colonies were inoculated into the trypticase soya broth (TSB) with 0.25% glucose and incubated at 37°C for 18 -24 hrs. This inoculum was taken in 1:40 dilution (5 μ l of inoculum in 195 μ l of TSB with 0.25% glucose). 200 micro-liter of this was taken in the micro-titer plate and incubated at 37°C for 24 hrs. The

micro-titer plate was then washed with phosphate buffer saline and dried in inverted position. Then, it was stained with 1% crystal violet for 5 min. The wells were rinsed 5 times with phosphate buffer saline. Subsequently, 200 µl of ethanol-acetone (80:20, v/v) was added to solubilize 1% crystal violet. Optical density was measured at 595nanometer wavelength. Each isolate was tested in triplicate and the average value was taken. (Figure.1)The following values were assigned for biofilm formation OD₅₉₅<1, non-biofilm former; OD₅₉₅>1, weak; OD₅₉₅>2, medium; OD₅₉₅>3, strong. *Escherichia coli* ATCC 25922 was taken as negative control and *Pseudomonas aeruginosa* ATCC 27853 was taken as positive control.

Results and Discussion

Out of seventy-five *Acinetobacter spp.* isolates, seventy-one (94.67%) isolates were identified as *Acinetobacter baumannii* and four (5.33%) were identified as *Acinetobacter hemolyticus*. Biofilm formation of all the seventy five isolates was determined by using Microtitre plate method. All the isolates were biofilm producers. Strong biofilm formation was seen in twenty two (29.33%), medium in thirty one (41.33%) and remaining twenty two (29.33%) showed weak biofilm formation (Table-1). Anti-microbial susceptibility testing (AST) performed by Kirby Bauer disc diffusion testing showed 100% resistant to amoxycillin-clavulanic acid, ciprofloxacin, cotrimoxazole, gentamicin, amikacin, ceftriaxone. Piperacillin- tazobactam was resistant in 80% whereas imipenem was resistant in 69.33%. (Table.2) ESBL production could be demonstrated in eight (10.6%) out of seventy five isolates whereas sixty seven (89.33%) isolates gave in-determinant

result in the DDST. The isolates giving in-determinant results in DDST showed AmpC production in boronic acid double disc test. All the isolates which showed resistance to imipenem were subjected to disc potentiation test. Out of the fifty two imipenem resistant isolates, 9 (17.3%) isolates showed production of metallo-beta-lactamases.

Correlation between biofilm formation and drug resistance showed that all the strong biofilm formers were fully resistant to carbapenem group of drugs while for medium strength biofilm formers, the resistance to carbapenem was 90.32%. (Table.3).

Acinetobacter baumannii is ubiquitous, non-fermentative, gram-negative bacillus which plays a significant role in the colonization and infection of patients in hospitals especially intensive care facilities. Studies have shown that *A. baumannii* strains survive desiccation far better than any other *Acinetobacter* species and are usually more resistant to commonly used antimicrobial agents and have great potential to attach to various biotic and abiotic surfaces, and all these factors help *A.baumannii* establish itself as an impeccable nosocomial pathogen as compared to other *Acinetobacter spp* (Jawad, A., *et al* 1996. Musa, E.K., *et al* 1990).

Identification of the *Acinetobacter spp.* poses a difficult challenge. Of the few methods that have been used for identification of *Acinetobacter* species, DNA-DNA hybridization remains the reference method (Bouvet, P.J. and Grimont, P.A. 1986.). Species identification with manual system and semi-automated commercial identification systems like API 20NE, Vitek 2,

Table.1 Results for biofilm formation

TOTAL	BIOFILM FORMATION		
	WEAK	MODERATE	STRONG
75	22	31	22
PERCENTAGE	29.33%	41.34%	29.33%

Table.2 Results of AST by Kirby Bauer disc diffusion testing

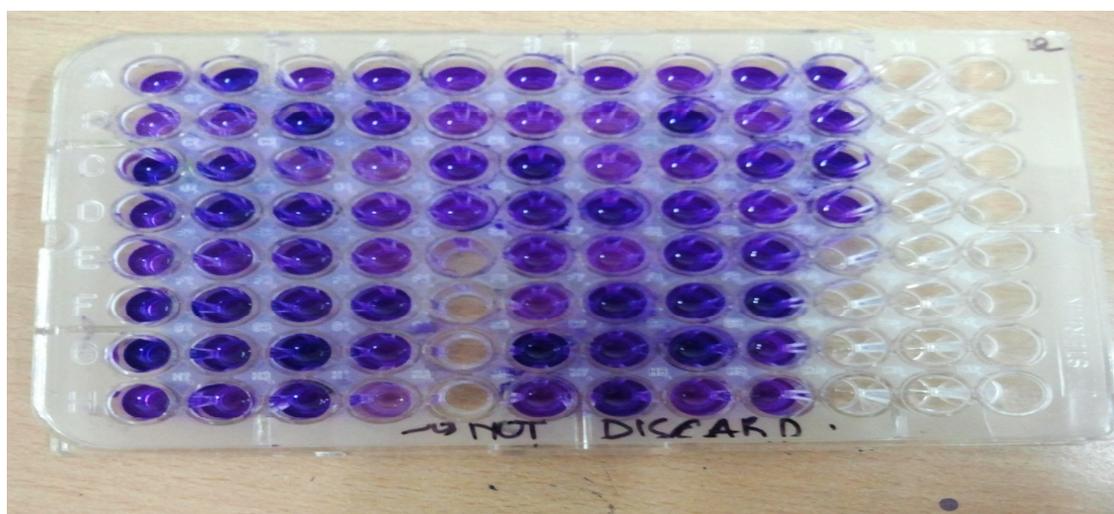
Antibiotics tested *	Ac	G	Ak	Co	Ci	Cf	Pt	Imp
Resistance	75	75	75	75	75	75	60	52
Resistance%	100	100	100	100	100	100	80	69.33

*Ac- Amoxicillin-clavulanic acid, G- Gentamicin, Ak- Amikacin, Co- Cotrimoxazole, Ci- Ceftriaxzone, Cf- ciprofloxacin, Pt- Piperacillin&Tazobactum, Imp- Imipenem

Table.3 Showing drug resistance when compared with biofilm formation

ACINETOBACTER SPP.	BIOFILM FORMATION		
	STRONG(22)	MEDIUM(31)	WEAK(22)
Carbapenem-resistant strains	22	28	2
Carbapenem-sensitive strains	-	3	20
MBL producers	9	-	-

Figure.1 Showing Micro-titer plate method for Biofilm Formation



Phoenix, and MicroScanWalkAway systems, are not completely satisfactory. This can be explained in part by their limited database content and because the substrates used for bacterial species identification have not been tailored specifically to meet the characterization requirements of non-fermenting organisms like; *Acinetobacter spp.*

A biofilm is a community of multiple bacterial cells associated with a surface, arranged in a tertiary structure in intimate contact with each other and encased in an extracellular matrix that consists of carbohydrates, nucleic acid and proteins and other macromolecules. The most common factors that can influence biofilm formation are; nutrient availability, bacterial appendages (pili & flagella), quorum sensing and macromolecular secretions. In present study, all the isolates were biofilm producers. This is consistent with finding of a study, which demonstrated the ability of *Acinetobacter spp.* to form biofilms isolated from catheter-related UTI, blood stream infections as well as a case of shunt related meningitis (Rodriguez-Bano, J., *et al* 2008).

In *Acinetobacter spp.*, resistance to all the major groups of antibiotics including tigecycline, colistin and polymixin has been reported (Navon, V.S, *et al* 2007). In present study, we found high level of drug resistance to all major groups of antibiotics limiting the therapeutic options for treatment. Bou *et al* (2000), in their investigation of a nosocomial outbreak found that all the isolates exhibited high level of resistance to semi-synthetic penicillins, ceftazidime, gentamicin, amikacin, netilmicin, cefepime including imipenem and meropenem (Bou, G., *et al* 2000).

Extended-spectrum beta-lactamases (ESBLs) have also been described for *A. baumannii*, but assessment of their true prevalence is hindered by difficulties with laboratory detection, especially in the presence of an AmpC. Presence of AmpC beta-lactamases interfere with demonstration of ESBL production with double disc synergy testing. This is consistent with the finding of our study which showed ESBL production in 10.6 % isolates while 89.33% showed indeterminate result. In the present study, 9 (17.3%) isolates showed MBL production. The only limitation to the study is that the modified hodge test was not performed; so complete presence of carbapenamases could not be detected. Authors suggest that modified hodge test should be preferred over disc potentiation test to detect complete spectrum of carbapenemase production. A very high level of carbapenem resistance seen in this study may be seen due to the presence of intrinsic type of beta lactamases i.e. AmpC-type cephalosporinase and an Oxacillinase. Presence of acquired beta-lactamases also i.e. MBLs (metallo-beta-lactamases) class B enzymes belonging to IMP-like, VIM-like, and SIM-1 and CHDLs (carbapenem hydrolyzing oxacillinase) class D enzymes has also been demonstrated. Resistance to carbapenems may also be explained by other mechanisms, such as porin loss or modification, presence of the CarO protein, AdeABC efflux system and by modification of penicillin-binding proteins (PBPs) (Fernandez-Cuenca, F , *et al* 2003).

Lee *et al* (2008) showed in their study that as a consequence of biofilm production, ability of *Acinetobacter spp.* to transfer genes horizontally might also enhance within these micro-communities and

facilitate the spread of antimicrobial resistance (Lee, H.W, *et al* 2008). In present study, 100% carbapenem resistance was seen in all strong biofilm forming isolates where as 90.32 % of the medium strength biofilm producers are carbapenem resistant. Twenty (90.9%) of the twenty two weak biofilm producing *Acinetobacter spp.* were carbapenem sensitive strains. Out of the fifty-two imipenem resistant isolates, 9 (17.3%) isolates showed MBL production and all these isolates were strong biofilm producers.

All the *Acinetobacter spp.* isolates were obtained from ETs were colonizing the endotracheal suction catheters and had potential to cause tracheobronchitis and ventilator associated pneumonia, which are nosocomial infections. *Acinetobacter spp.* has potential to form biofilm and spread resistance genes. They also have the largest antibiotic resistance island known so far (more than 40 resistance genes) and the genetic plasticity of *A. baumannii* enables it to benefit from a variety of resistance mechanisms to easily become more and more resistant when antibiotic pressure is maintained. With a few limited options for the treatment, imipenem and meropenem are among the drugs of choice to treat nosocomial infections due to multidrug-resistant *Acinetobacter baumannii*. However, their efficacy is being increasingly compromised by the emergence of carbapenem- hydrolyzing beta-lactamases of molecular Ambler class B (metalloenzymes) and D enzymes (oxacillinases). Combinations of polymyxin B, imipenem and rifampicin have been shown to be act synergistically in vitro. Tigecycline might also be useful, since tigecycline is known to be effective against some carbapenemase-producing

strains and might be a useful alternative to polymyxins. A combination of ampicillin and sulbactam has also been shown to be effective against multidrug resistant isolates for the treatment of life-threatening *Acinetobacter* infections. Strict infection control practices like use of medicated intravascular devices or changing peripheral lines before the organism is able to establish itself by forming biofilm formation, should be followed to prevent any outbreak due to this formidable pathogen.

A.baumannii is a very important pathogen that is gradually attracting more attention as a major public health problem. It is responsible for significant proportion of nosocomial infections among patients who are critically ill and receiving care in ICU facilities. With this situation together with the fact that biofilm phenotype of *A.baumannii* being highly refractile and recalcitrant to multiple drugs due to intrinsic resistance properties and those that can acquire resistant determinants with increasing propensity, makes this pathogen one of the most difficult challenge of the day.

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