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Original Article

Antimicrobial resistance pattern of biofilm forming *Pseudomonas aeruginosa* isolated from patients with nasogastric and endotracheal tubes

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ABSTRACT

Biofilms are communities of microorganisms attached to a surface. It has become clear that biofilm-grown cells express properties distinct from planktonic cells, one of which is an increased resistance to antimicrobial agents. A total of 79 non replicate gram negative bioadherent isolates from 113 patients in intensive care units with nasogastric and endotracheal tubes were collected, identified, then tested for their abilities to form biofilm using tube method, tissue culture plate method and genetically. Antimicrobial susceptibility testing and time-kill assay were done to figure out the role of biofilm formation in antimicrobial resistance. Gram negative isolates were *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae* and *Citrobacter koseri*. The microorganisms were classified into three groups (strongly adherent, moderately adherent and non adherent) according to the biofilm formation that was obtained by optical density (O.D.) values. The antibacterial susceptibility testing revealed that more than 70% of the bioadherent isolates were multi-drug resistant (MDR) with resistance to more than 4 antimicrobials. So it has been observed that the resistance of bacteria in biofilms to antibiotics is increased compared with what is normally seen with planktonic cells.

Key words: resistance pattern, antimicrobial, biofilm, nasogastric tube, endotracheal tube

INTRODUCTION

Biofilm is an accumulation of microorganisms and their extracellular products forming a structured community on a surface, or defined as surface attached microbial populations of either single or multiple species [1]. The first observations of biofilm were obtained through scanning electron microscopy which showed primary attachment of monolayer bacterial consortia, embedded in an amorphous mucous structure on the surfaces of medical devices. This phenotype was initially referred to as slime formation. In retrospect, in most cases, the 'slime' was very likely polysaccharide intercellular adhesin (PIA), and so PIA and 'slime' are considered to be the same. Today, this special mode of thick extracellular matrix (maturation phase) is generally termed biofilm formation [2]. Antimicrobial agents effective against planktonic bacteria frequently fail to eradicate bacterial biofilms. The problem is that choosing of antibiotics is based on bacterial cultures derived from planktonic bacteria which differ in behavior and in phenotypic form from bacteria in biofilm. The failure of antimicrobial agents to treat biofilms has been associated with a variety of mechanisms:1-agents often fail to penetrate the full depth of the biofilm (extrinsic resistance), 2- organisms in the biofilm grow more slowly; therefore, they are more resistant to antimicrobial agents that require active growth, 3-antimicrobial binding proteins are poorly expressed in these bacteria, 4-bacteria within a biofilm activate many genes that alter the cell envelope, the molecular targets, and the susceptibility to antimicrobial agents (intrinsic resistance), 5-bacteria in a biofilm can survive in the presence of antimicrobial agents at a concentration 1,000–1,500 times higher than the concentration needed to kill planktonic cells of the same species [3].

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Aminoglycosides and beta-lactam antibiotics were showed to be able to prevent the formation of "young" biofilms, while fluoroquinolones are effective in case of both "young" and "older" biofilms because of their good penetrative qualities [4].

MATERIALS AND METHODS

Seventy nine non replicated gram negative bioadherent isolates from 113 patients in medical intensive care unit (MICU) and surgical intensive care unit (SICU) with nasogastric and endotracheal tubes were collected from Al-Demerdash Hospital, Cairo, Egypt in the period from September 2011 to June 2012, identified morphologically, microscopically, and biochemically, then tested for their abilities to form biofilm using tube method, tissue culture plate method and genetically.

a- ***Tube method (TM):*** A qualitative assessment of biofilm formation was determined [5]. Ten mls of **t**rypticase soya broth with 10% glucose **(T**$SB_{glu)}$ was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with phosphate buffer saline PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation.

b- ***Spectrophotometric Assay method:*** The Spectrophotometric Assay method [6] is considered as standard test for detection of biofilm formation. Previous reports have indicated the influence of media composition on biofilm production; therefore we had evaluated biofilm production in tryticase soy broth (TSB Difco), TSB with 1% glucose (TSB_{glu}). Isolates from fresh agar plates were inoculated in respective media and incubated for18-24 hour at 37°C in stationary condition and diluted 1:100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, Kolkata, India) were filled with 0.2 ml aliquots of the diluted cultures and bacterial free media was used as control. The tissue culture plates were incubated for 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Optical densities (O.D.) of stained adherent bacteria were determined with a micro ELISA auto reader (Elx 808, Biotek, USA) at wavelength of 590 nm (OD590 nm). The microorganisms were classified into three groups according to biofilm formation that was obtained by O.D. values as shown in table 1. Experiment was performed in triplicate; the data was then averaged and calculated.

Effect of biofilm formation on antimicrobial resistance pattern: The antibacterial susceptibility testing of the gram negative bioadherent isolates was done using the following antimicrobial discs: aztereonam (30 µg), cefoxitin (30 g), chloramphenicol (30 µg), imipenem (10 µg), levofloxacin (5 µg), nalidixic acid (30 µg), piperacillin (100 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg) (Oxoid, UK).

Time kill assay:

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ciprofloxacin, amikacin and ceftriaxone: Antimicrobial suscebtibility was determined by standard broth microdilution method as described by the clinical laboratory standards institute [7]. Serial dilutions of antimicrobial agent were prepared in 96 well microtiter plates. The range tested was 0.125 µg/ml to 128 µg/ml. the bacterial suspensions were standardized to yield a final inoculum size of 1×10^6 to 1×10^7 CFU/well. The inoculum size and purity of all isolates was confirmed by plate counts. The MIC was defined as the lowest concentration that inhibit growth after 18 to 24 hours of incubation at 37°C. All wells showing no visible growth were subcultured on Muller-Hinton agar plates. The MBC was defined as the lowest concentration that decreased the viable cells by 99.9% as compared with the control after 18-24 hours of incubation at 37°C.

Time kill curves: Viable counts of strongly adherent isolates were determined at various time intervals in the presence of MBC and 2MBC of ciprofloxacin, amikacin and ceftriaxone. In suspension: the organisms were cultured in broth for 16-18 hours, diluted, and standardized to contain 10^7-10^8 CFU/ml using McFarland standards. The antimicrobial agent in minimum bactericidal concentration and 2 MBC were added to the bacterial suspension and incubated with

shaking at 37°C. Samples were diluted in saline containing 0.1% tween 80 and plated at 0, 2, 4, 6, 8, 12 and 24 hr of incubation. Viable counts at these time periods in presence of antimicrobial agent were compared to those of controls. In biofilm: the bacterial suspensions were cultured for 16-18 hr, standardized to 10^5-10^6 CFU/ml. aliquots (0.1 ml) of the diluted cultures were added to the wells of sterile 96 wells polystyrene flat bottom tissue culture plates. The plats were incubated for 24 hrs at 37°C to form biofilms. Aliquots (0.1 ml) of fresh broth containing the antimicrobial agent were added to each of the preformed biofilms in experimental rows. The contents of the wells were mixed vigorously for 30 seconds to resuspend the biofilms, diluted with saline containing 0.1% tween 80, vortexed for 1 min and plated at 0, 2, 4, 6, 8, 12 and 24 hr of incubation. The viable counts were compared to those of resuspended biofilms in the wells without antimicrobial agent.

Statistical analysis: Data were presented as mean ± SD. Two way Analysis of variance (ANOVA) was used for testing the significance using GraphPad Prism®. $p<0.05$ was taken as a cut off value for significance.

Molecular detection of algD genes responsiple for biofilm formation in Pseudomonas aeruginosa strains by PCR: PCR amplifications were performed in a final volume of 50 µl by containing about 3 µl of the genomic DNA as the template, 2 µl of 400 nmol/L gene specific primers each, 25 µl of 2x AmpliTaq gold PCR master mix and the volume was completed with 18 µl deionized water, two primers were used, VIC Forward 5'-TTCCCTCGCAGAGAAAACATC-3' and VIC Reverse 5'-CCTGGTTGATCAGGTCGATCT-3' [8]. Cycling conditions for *algD* gene were as follows: Initial denaturation at 94°C for 5min followed by 30 cycles of denaturation at 94°C for 1min, annealing at 60°C for 1min, extension at 72°C for 1min. final extension at 72°C for 7min, then hold at 4°C for 10min Amplified products were controlled by 1.3% agarose gel electrophoresis in 0.5 x TBE buffer (Fig. 5).

RESULTS

Distribution of gram negative bioadherent isolates form nasogastric and endotracheal tubes: Gram negative isolates were *Pseudomonas aeruginosa, Klebsiella pneumoniae*., *Escherichia coli, Enterobacter cloacae* and *Citrobacter koseri* with the following percentages respectively (60.7%, 14%, 11.4%, 11.4% and 2.5%). The microorganisms were classified into three groups according to the glycocalyx production and biofilm formation that was obtained by O.D. values (Fig. 1).

Effect of biofilm formation on antimicrobial resistance pattern: The antibacterial susceptibility testing of the gram negative bioadherent isolates against different antimicrobial discs revealed that 29.1% of the isolates were resistant to more than 6 drugs while 45.6% were resistant to 4 to 6 drugs and 25.3% were resistant to 2 to 3 drugs which emphasize the role of biofilm in antimicrobial resistance.

Time kill assay: Viable counts of strongly adherent isolates were determined at 0, 2, 4, 6, 8, 12 and 24 hrs in suspension and biofilm in the presence of MBC and 2MBC of ciprofloxacin, amikacin and ceftriaxone**.** Viable counts at these time periods in presence of antimicrobial agents were compared to those of controls (Fig. 2-4).

DISCUSSION

In our study, 29.1% of the bioadherent isolates were resistant to more than 6 drugs, where 45.6% were resistant to 4 to 6 drugs and 25.3% were resistant to 2 to 3 drugs. So that in comparing antimicrobial resistance to the ability of biofilm formation in the individual strains, we observed that strains capable of forming biofilms were more frequently observed to be an MDR phenotype. However, as previous studies have shown that biofilm formation is higher in MDR strains [9-11] and can promote antimicrobial resistance by selecting for highly resistant strains following treatment with sub-inhibitory antimicrobial concentrations [12,13], the ability of a strain to develop biofilms may have an important, yet not fully understood role in the development of multidrug resistance.

CONCLUSION

The results of the time kill assay revealed that the sensitivity of the biofilm forming organisms toward antimicrobials is greatly affected by the growth condition of the organism, by paying close attention to the growth phase of planktonic cells and biofilm cells, it is obvious that the contribution of a slow growth rate to biofilm cell survival against antibiotics. Gilbert and colleagues examined growth-rate-related effects under controlled growth conditions for planktonic cultures and biofilms of *P. aeruginosa*, *E. coli* and *S. epidermidis*. They made the general observation that the sensitivities of both the planktonic and biofilm cells to either tobramycin or ciprofloxacin increased with increasing growth rate, thus supporting the suggestion that the slow growth rate

of biofilm cells protects the cells from antimicrobial action. For *P. aeruginosa* at slow growth rates, both the planktonic and intact biofilm cells were equally resistant to ciprofloxacin. However, as the growth rate was increased, the planktonic cells became more susceptible to ciprofloxacin than the biofilm cells. This result supports the idea that some other property of the biofilm, and not just growing slowly, was important for the observed recalcitrance of biofilms to antimicrobial treatment [14].

Table 1: Classification of bacterial adherence by TCP method at wavelength of 590 nm

Mean OD* values	Adherence	Biofilm formation
<0.120	Non	Non / weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

*optical density

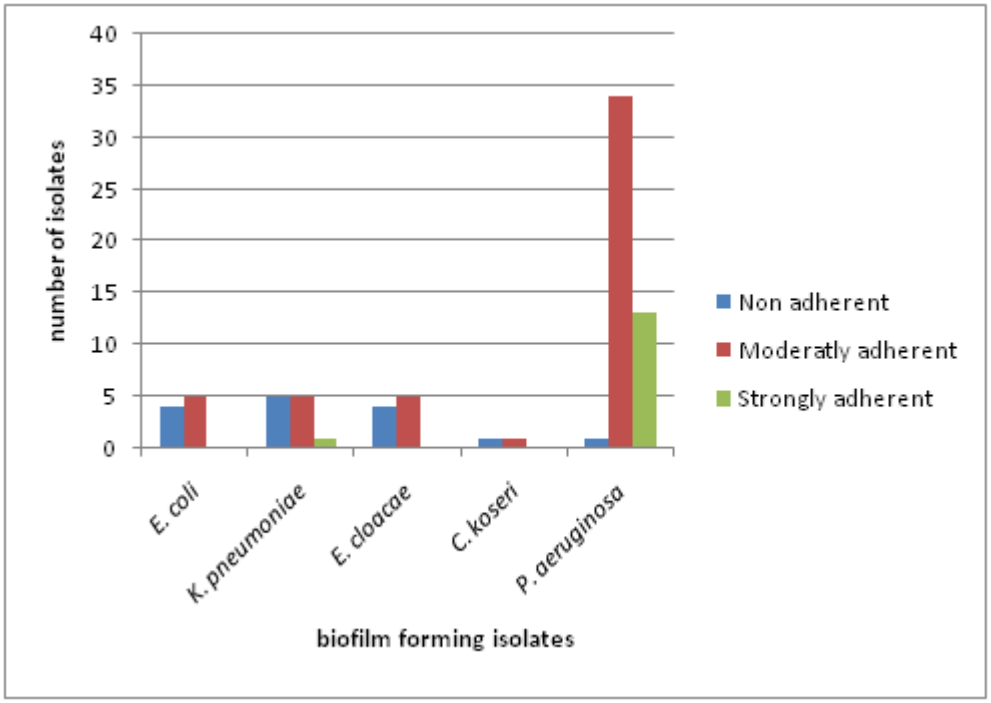

Figure 1: Distribution of biofilm forming gram negative isolates.

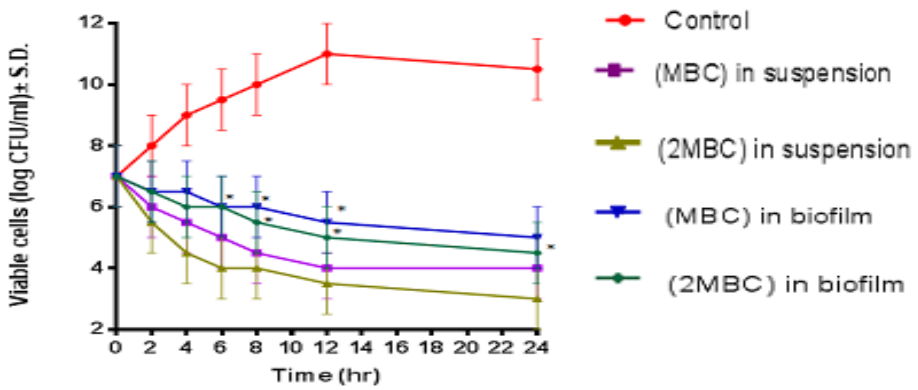

Figure 2: Control growth curves and time–kill plots of the effect of ciprofloxacin (CPX) on strongly adherent *P. aeruginosa* isolates as planktonic cells and biofilm. Points represent means and error bars. ***:** significantly different (P <0.05) from corresponding time point of treated suspension group.

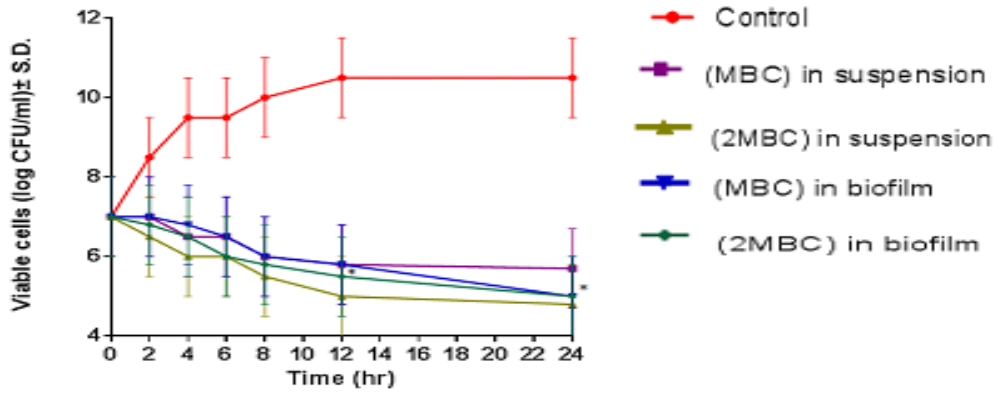

Figure 3: Control growth curves and time–kill plots of the effect of amikacin (AMK) on strongly adherent *P. aeruginosa* isolates as planktonic cells and biofilm. Points represent means and error bars. ***:** significantly different (P <0.05) from corresponding time point of treated suspension group.

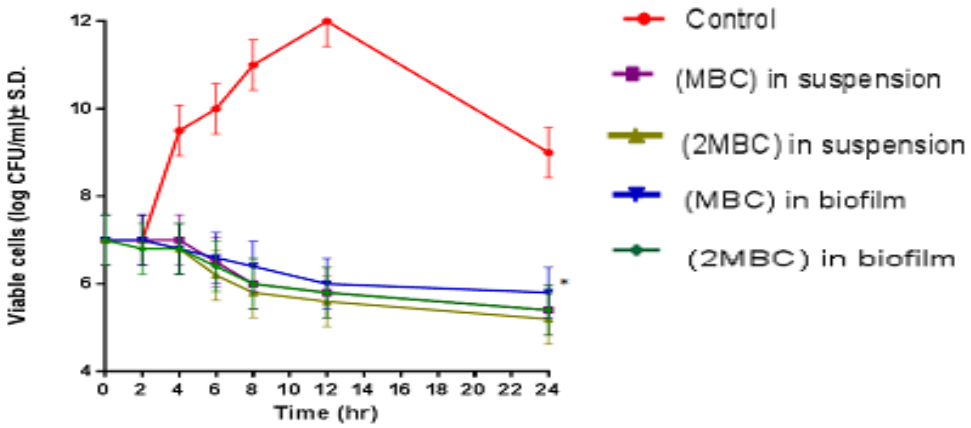

Figure 4: Control growth curves and time–kill plots of the effect of ceftriaxone (CTX) on strongly adherent *P. aeruginosa* isolates as planktonic cells and biofilm. Points represent means and error bars. ***:** significantly different (P <0.05) from corresponding time point of treated suspension group.

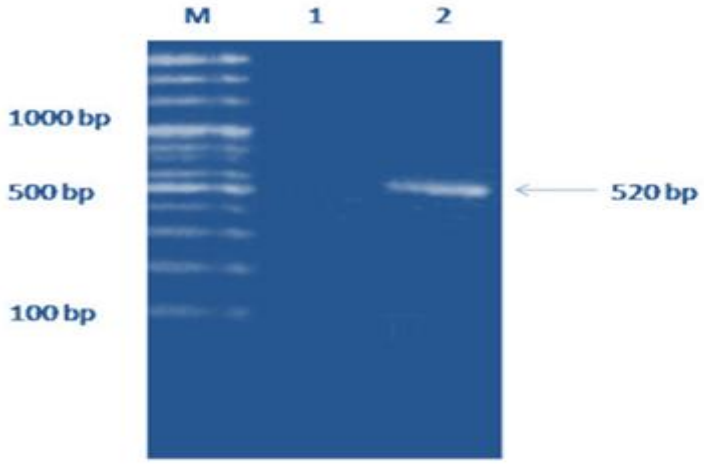

Figure 5: PCR amplification with *VIC* primers. A 520-bp of *algD* gene. Lane M, 100bp DNA size marker, lane 1 negative control, lane 2 *P. aeruginosa* isolate producing *algD* gene.

REFERENCES

1. Tenke P et al. Update on biofilm infections in the urinary tract. World J Urol 2012; 30:51-7.
2. Hall-Stoodley L et al. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004; 2:95–108.
3. Tenke P. The role of biofilm infection in urology. World J Urol 2006; 24:13-20.
4. Reid G. Oral fluoroquinolone therapy results in drug adsorption on urethral stents and prevention of biofilm formation. Int J Antimicrob Agents 2001; 17:317-9.
5. Christensen GD et al. Adherence of slime–producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 1982; 37:318–26.
6. Christensen GD et al. Adherence of coagulase negative Staphylococi to plastic tissue cultures: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985; 22:996–1006.
7. Clinical Laboratory Standard Institute. Zone diameter Interpretive Standards and corresponding minimal inhibitory concentration interpretive break point. 2012 Supplement M44-S1. Clinical and Laboratory Standard Institute, Wayne, PA.
8. Ferreira LV et al. PCR identification of *Pseudornonas aeruginosa* and direct detection in clinical samples from cystic fibrosis patients. J Med Microbiol 1999; 48:357-61.
9. Kwon AS et al. Higher biofilm formation in multidrug-resistant clinical isolates of *Staphylococcus aureus*. Int J Antimicrob Agents 2008; 32:68–72.
10. Rao RS et al. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii*. Indian J Med Microbiol 2008; 26:333–7.
11. Reiter KC et al. High biofilm production by invasive multiresistant staphylococci. APMIS 2011; 119:776–81.
12. Rachid S et al. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesion expression in biofilm-forming *Staphylococcus epidermidis*. Antimicrob Agents Chemother 2000; 44:3357–63.
13. Wang Q et al. Enhancement of biofilm formation by subinhibitory concentrations of macrolides in icaADBC positive and negative clinical isolates of *Staphylococcus epidermidis*. Antimicrob Agents Chemother 2010; 54:2707–11.
14. Evans DJ. Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. J Antimicrob Chemother 1991; 27:177–84.