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Effect of amikacin over biofilm formation of carbapenam resistant *Pseudomonas aeruginosa* on urinary catheters.

KEYWORDS: MBL Pseudomonas aeruginosa, catheter tips, biofilm, amikacin.

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ABSTRACT *Pseudomonas aeruginosa is a clinically troublesome Gram negative pathogen. Pseudomonas aeruginosa generally demonstrates resistance to various antimicrobial agents. Biofilm formation occurs in these indewelling devices leading to morbidity and mortality among the hospitalized patients. Any antibiotic or enzymes should be capable of inhibiting the biofilm formation, ready to detach the biofilm formed or capable of increasing the susceptibility of these biofilm producing isolates. In our study, in vitro effect of amikacin over the biofilm produced by the carbapenem resistant isolates was carried out and the impact produced by the drug over the biofilm producer was analysed. The multidrug resistant strains of Pseudomonas aeruginosa, resistant to carbapenem drugs were included in the study. Three different strains 3, 9 and 21(strain number) of MBL positive strains of P.aeruginosa were taken for the study of biofilm forming characteristics on urinary catheter. Once the biofilm formation of the MBL producing P.aeruginosa is confirmed by the quantitative viable count method, the isolates are subjected to the biofilm prevention by amikacin. Significant decrease in biofilm formation as indicated by the CFU was seen on 5 days and 10 days of observation. Thus amikacin-agarose coated catheter tips significantly reduced the biofilm formation.*

Pseudomonas aeruginosa is a clinically troublesome Gram negative pathogen that causes a wide range of opportunistic infections and nosocomial outbreaks. *Pseudomonas aeruginosa* generally demonstrates resistance to various antimicrobial agents associated with enzyme production, alteration in outer membrane permeability or due to effective efflux systems limiting the effect of drugs over the strains.[1] Carbapenams are potent agents against the multidrug resistant strains of *Pseudomonas aeruginosa* and the β lactamases produced by these organisms render them ineffective.[2] These carbapenem hydrolysing enzymes were identified as metallo β lactamases belonging to the Amber Class B classification.[3,4]

A significant medical advancement particularly in treatment of critically ill patients is the use of indwelling devices. Biofilm formation occurs in these in dewelling devices leading to morbidity and mortality among the hospitalized patients. The incidence of bacterial infections among the patients with the devices like urinary, endotracheal, intravenous and implants leads to the biofilm formation. [5]

The attachment to solid surface initiates the biofilm formation, stimulating the microbial aggregation and proliferation, forming microcolonies, excreting an exopolysaccharide 'slime", consolidates the attachment to the surfaces forming the microaggregates latter getting differentiated into the biofilms. [6] The biofilm differentiation depends upon the concentration gradient dependent signals being generated by the quorum sensing molecules which control and alter expression of a large number of genes. [7]

Microbial biofilms also present serious challenges to the immune system because expression of bacterial antigens within the encasing polysaccharide matrix is suppressed and the colonies are highly resistant to phagocytosis by polymorphonuclear cells.[8]

The biofilm forming property of the isolates makes them a persistent state of infection, difficult to eradicate thereby increasing the morbidity as they are resistant to antibiotics and host defence mechanism. [9] Any antibiotic or enzymes should be capable of inhibiting the biofilm formation, ready to detach the biofilm formed or capable of increasing the susceptibility of these biofilm producing isolates. In our study, in vitro effect of amikacin over the biofilm produced by the carbapenem resistant isolates was carried out and the impact produced by the drug over the biofilm producer was analysed.

Material and methods:
Isolates of *Pseudomonas aeruginosa* from various clinical specimens were taken for the study. They were subjected to the susceptibility testing against antipseudomonal antibiotics by disc diffusion test as per the Clinical and laboratory standards institute (CLSI) guidelines. [10]

The multidrug resistant strains, resistant to carbapenem drugs were included in the study. The metallo β lactamase production was confirmed by the ceftazidime, ceftazidime + EDTA MIC (Radianz biotechnologies) (Franco MRG et al., 2010). [11]

These isolates were positive for bla_{VIM} gene subtypes by PCR amplification, the primers used were *bla*-$_{VIM}$ VIM1A-5'-TCT ACA TGA CCG CGT CTG TC-3' and VIM1B- 5'-TGT GCT TTG ACA ACG TTC GC-3' (Giakkoupi.P,et al., 2003) [12] and then subjected for the in vitro test for the biofilm formation.

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Biofilm formation:
Three different strains 3, 9 and 21(strain number) of MBL positive strains of *P.aeruginosa* were taken for the study of biofilm forming characteristics on urinary catheter. The biofilm formation of the isolates was studied in different time intervals. They were tested in plain broth without catheter tips (control) and with catheter tips.

Biofilm prevention:
Once the biofilm formation of the MBL producing *P.aeruginosa* is confirmed by the quantitative viable count method,[13] the isolates are subjected to the biofilm prevention by amikacin. Amikacin (Ai) 200mg concentration in presence of agarose was coated on outer surface of 1cm catheter tip with sterile precaution. Broth without tips were used as controls, tips without Ai but containing agarose – 0.5% semisolid, tips with Ai agarose were used. The viable counts were analysed both for control and test at 0 day, 5th day and 10 days. The nonadherent cells were removed by washing with sterile saline and the adherent cells were removed from the surface with sterile blade.

Result:
The *bla* $_{VIM}$ gene positive metallo β lactamase producers were subjected to the biofilm production(Table 1). The better biofilm producers, showing increased viable count on the catheter were selected for biofilm inhibition / prevention by amikacin. The MBL producers were susceptible to amikacin when compared with other antibiotics. The biofilm growth was measured by colony forming units (CFU) on the catheter surfaces coated with fixed concentration of amikacin. The biological assay was confirmed using confocal microscopy. (JASMN LABORATORY)

The viable count of SJ 3, CFU was $\log_{10}$ 5.9, 8.3 and 10.0 on 0 day, 5 days and 10 days respectively in broth without catheter (Table 2). The viable count, CFU was $\log_{10}$ 5.14, 8.0 and 10.84 on 0 day, 5 days and 10 days respectively on catheter surface. There was an increase in the CFU formation as the time progressed. The increase was more on catheter surface.

The viable count for strain SJ 9, CFU was $\log_{10}$ 5.9, 8.3 and 10.0 on 0 day, 5 days and 10 days respectively in broth without catheter. The viable count, CFU was $\log_{10}$ 5.95, 8.0 and 10.9 on 0 day, 5 days and 10 days respectively on catheter surface (Figure 1). CFU for the strain SJ 21 was done in similar way. There was an increase in the CFU formation as the time progressed. The increase was slightly more on catheter surface. Of the three strains, strain 9 showed CFU formation and there was an increase in the CFU formation as the time progressed and the increase was slightly more on catheter surface which was higher than that of the others.

Based on the preliminary performance of the strain SJ 9 in forming the biofilm slightly better than others (Figure 1), it was selected for further work. The results of biofilm formation in broth and amikacin-agarose coated catherter tips is given in table 3.Significant decrease in biofilm formation as indicated by the CFU was seen on 5 days and 10 days of observation. There was a decrease in 1.5 $\log_{10}$ difference seen on the fifth day. On 10th day there was 3.3 $\log_{10}$ difference in CFU was found (Figure 2). Thus amikacin-agarose coated catheter tips significantly reduced the biofilm formation.

Discussion:
P.aeruginosa colonizes urinary catheters, forms biofilm, responsible for persistent and recurrent nosocomial catheter associated urinary tract infections, which possibly increases the morbidity and mortality among the immunocompromised patient. Quorum sensing in *P.aeruginosa* plays a key role in biofilm formation. [14] According to Hoiby et al., [15] the effect of antibiotics on bacteria biofilm growing was very less and not similar to our study. Among the antimicrobials, amikacin seems to be very effective in case of infection by Gram negative bacilli and can be used as effective monotherapy against the pandrug resistant *P.aeruginosa*. [16]

Our study is similar to the in vitro study done by Jamal M et al., 2013[17] in the prevention of biofilm formation of multidrug resistant strain. Ai-agarose is effective in the prevention of biofilm formation of the carbapenem resistant *Pseudomonas aeruginosa*. It is similar to the work of Chauhan et al., 2012 by the use of antibiotic lock therapy that constitutes an adjuvant therapy for catheter related infection. [18]

Our study was parallel to the gentamicin antibiotic lock solution associated with complete eradication of gram positive and gram negative bacterial biofilm developed in totally implantable venous access ports.

The in vitro study over the bacterial growth measurement based on colony forming units showing the significant inhibition of amikacin agarose when compared with controls $p<0.001$ were in concordance with study of Zelichenko .G *et al.*, 2013. [19]

The $bla_{VIM\ 3, VIM\ 9, VIM\ 21}$ when analysed for the biofilm production all were capable of producing them and $bla_{VIM\ 9}$ was found to be showing enhanced biofilm production. This strain was isolated from dialysis fluid. The inhibition of biofilm formation was effective showing more than 2 log 10 difference which was statistically significant $P < 0.001$.

Conclusion:
Bacterial biofilms are one of the predominant causes of chronic infections and infection associated with catheter related devices and may be due to the enhanced tolerance to antimicrobials, bactericidal and immunological challenge. Through many studies are related to the bacteria but the attention over the biofilm producers –the carbapenem resistant isolates are less documented. Carbapenem resistant *Pseudomonas aeruginosa* biofilm exhibits marked susceptibility to amikacin agarose.

Prevention of biofilm formation by carbapenem resistant *Pseudomonas aeruginosa* in this study is more promising and future detailed studies over these isolates can be recommended as a novel pathway where the clinicians can use them as one of the ways of prevention of the catheter associated urinary tract infections.

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Table 1:
***bla*$_{VIM}$ positive strain used for biofilm screening:**

Strain no	MBL production	*bla*$_{VIM}$ gene sequencing	Biofilm formation
3	Positive	Positive	Positive
9	Positive	Positive	Positive
21	Positive	Positive	Positive

Table 2:
The results of biofilm formation with strain 3 bla$_{VIM}$ positive *P.aeruginosa*.

Biofilm formation	0 day	5 day	10 day
Stain no 3	5.146128	8	10.8451
Control	5.90309	8.30103	10

Table 3:
Biofilm formation of strain 9 in broth and amikacin –agarose coated catheter tips.

Sample	Time	Detail	Mean (log 10)	SD	Comparison	P value	Significance
1	0 day	broth	6.95	0.229	1vs 4	>0.5	Not significant
4		tip	6.77	0.15			
2	5 days	broth	9.07	0.035	2vs5	<0.001	Significant
5		tip	8.2	0.05			
3	10 days	broth	12.02	0.015	3vs6	<0.001	Significant
6		tip	8.73	0.25			

Figure 1:
Biofilm formation of MBL producing *bla* VIM positive *P.aeruginosa* on catheter surface

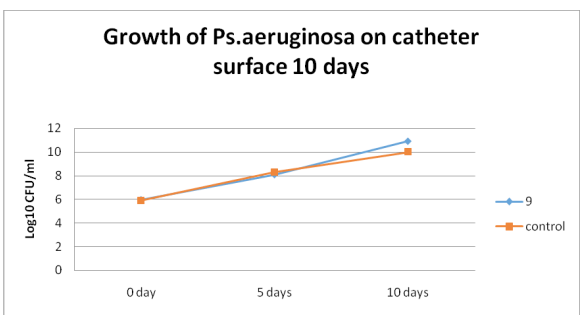
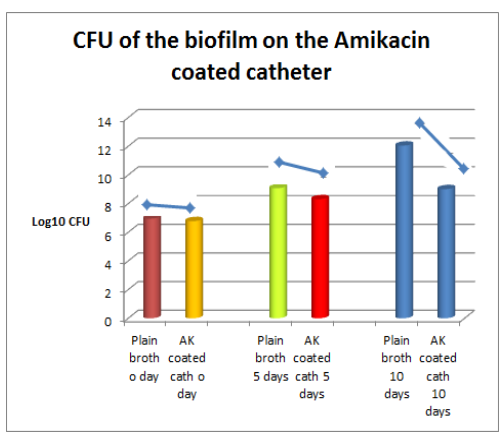


Figure 2:
Effect of amikacin coated catheter over the biofilm formation on the *bla*$_{VIM}$ positive *P.aeruginosa*.



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