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Aminoglycoside antibiotics for biofilm mediated infections: Correlation between concentration of selected antibiotics and biofilm persistence

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ABSTRACT

Bacteria in a biofilm are highly resistant to the antimicrobials and are responsible for the chronicity and persistence of infection. The MIC and minimum biofilm inhibitory concentrations (MBIC) of aminoglycoside, quinolone and cephalosporin antibiotics were tested against reference strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The aminoglycoside antibiotics amikacin and gentamicin were effective against Staphylococcus biofilm formation. In certain cases they even showed higher activity towards the biofilm bacteria than to the planktonic cells. The effect of antibiotic concentration on biofilm persistence was studied. Ciprofloxacin was found to induce biofilm persistence in all the tested bacteria. Cephalosporin was found to promote the biofilm persistence inone of the *S. aureus* strains while this was not showing any significant change with respect to other organisms. Aminoglycosides were not favoring biofilm persistence in any of the bacteria and they inhibited biofilm formation in one of the *S. aureus* strain.

INTRODUCTION

The major challenge in antimicrobial chemotherapy is the development of microbial resistance. Many of the existing antibiotics are microbial products which may take part in microbial competition within environmental niches. Development of antimicrobial resistance against these microbial products by other bacterial species may be an adaptive mechanism for their own survival [1]. Biofilm formation is one of the reasons for the development of antimicrobial resistance and biofilms comes intothe picture, when the clinical antimicrobial therapy fails in spite of having sensitivity in the laboratory tests.

Biofilms are bacterial communities which differ greatly from their planktonic counterparts and the biofilm forming bacteria may offer upto 1000 times more resistance to antibiotics than their free planktonic cells [2-4]. Normally when a patient is reported to the hospital with an infection, the infectious agent would have already adhered and might have started biofilm formation. So, rather than testing antibiotic susceptibility or the effect of antibiotic concentration on biofilm formation,the effect of antibiotic concentration on preformed biofilm will be ideal. Several researchers have determined the minimum biofilm eradication concentrations of various antibiotics against several bacterial biofilms. The concentration of antibiotic needed for the inhibition of biofilm bacteria was found to be pretty high compared to their minimum inhibitory concentrations[5-6]. Before using high concentrations of antibiotic for the treatment of biofilm mediated infections, it is important to study their effects on preformed biofilms. The effects of concentrations of various antibiotics on preformed biofilms of different bacteria were examined in this study and the concentration dependent biofilm persistence was analyzed using statistical methods. This will give an idea about the prognosis of a disease when we use different antibiotic concentrations for antimicrobial chemotherapy.

MATERIALS AND METHODS

All the test strains chosen for the study are control strains for antibiotic susceptibility testing. The organisms used in the study were procured from the Microbial Type Culture Collection(MTCC) and Gene Bank, Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), Pune, India. The details of bacterial strains used, along with their equivalent ATCC numbers are as follows: *Staphylococcus aureus* subsp. au MTCC 96(ATCC 9144) and MTCC 1430 (ATCC 12600), *Escherichia coli* MTCC 739 (ATCC 10536) *and Pseudomonas aeruginosa NCIM 5029 (ATCC 27853).*

Qualitative and quantitative determination of biofilm formation

The phenotypic evaluations of biofilm producing ability of the bacteria were tested qualitatively by congo red agar method [7]. The interpretation was done according to the reference scale for biofilm formation [8]. The biofilm formation was done using 96 well microtiter plates [9] and was quantified by modified microtiter plate assay [10]. Optical density was measured at 590nm using a microtitre plate reader (ELx800).

Minimum inhibitory concentration (MIC)

MICs were determined according to the standard CLSI guidelines [11]. Amikacin, Gentamicin, Ciprofloxacin and Cefotaxim were tested against all the bacterial strains. Each test was done in triplicate and positive and negative controls were kept.

Minimum biofilm inhibitory concentration (MBIC)

MBICs were determined by an antibiotic susceptibility assay [12] using 96 well microtiter plates. The first well antibiotic concentration was 500 µg/ ml.

The effect of antibiotics on pre-formed biofilms

This was tested by the assay earlier described with some modifications [13]. Eighteen to twenty hours old biofilms were grown by the method given above. After incubation the planktonic cells were removed, washed and fresh TSB with serial twofold dilutions of the antibiotic (antibiotic stock solution with 20mg ml^{-1} concentration) was added to the wells and kept for 18 to 20 h. of incubation at 37°C. Biofilm quantification was done by crystal violet staining. Each antibiotic was tested in triplicates for every bacterial strain. Biofilm persistence after antibiotic treatment was determined using the following formula [14].

$$\text{Percentage of biofilm persistence} = \left(\frac{(A_{590}x - A_{590}\ \text{negative control})}{(A_{590}\ \text{positive control} - A_{590}\ \text{negative control})}\right) 100$$

Where, x corresponds to the antibiotic used.

The percentage of biofilm persistence was plotted as scatterplots of X and many Y. Logarithmically transformed antibiotic concentration (common logarithm [in] antibiotic concentration) was plotted onthe X axis. The reason for transforming the antibiotic concentration was to obtain a more normal distribution.

Statistical analysis

In order to quantify the strength of linear relationship between the two quantitative variables, antibiotic concentration and percentage of biofilm persistence, the correlation coefficient was calculated. The two variables were taken as x and y; and the data were taken in the form of n pairs (i.e. $[x_1, y_1]$, $[x_2, y_2]$, $[x_3, y_3]$..... $[x_n, y_n]$), then the correlation coefficient was calculated by the following equation:

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 \sum_{i=1}^{n}(y_i - \bar{y})^2}}$$

Where $\bar{x}$ is the mean of the x values and $\bar{y}$ is the mean of the y values. The value of Pearson correlation coefficient r, close to +1 indicates a strong positive linear relationship, a value close to -1 indicates a strong negative linear relationship and a value close to 0 indicates no linear relationship. The strength of the relationship can be obtained from the 95% confidence interval. The hypothesis test of correlation was done. The null hypothesis is that the population correlation coefficient equals zero. The statistical package SPSS 16 was used for the analysis.

RESULTS

Estimation of biofilm formation

Biofilm production of different microorganism in Congo red agar is given in Table I. The quantitative determination of biofilm formation was done using crystal violet staining and the biofilm production of each strain was classified in Table II. Biofilm formations on the sides of the wells are shown in Fig. 1. The congo red agar method revealed all the bacterial strains to be biofilm producing except *P. aeruginosa* which formed very red colonies in congo red agar indicative of non-biofilm producer. The modified microtiterplate method gave similar results for *Staphylococcus aureus*. According to crystal violet assay *P. aeruginosaand E. coli* strainswerefound to be capable of strong adherence and high biofilm production. Two aminoglycosides, one cephalosporin and a fluoroquinolone were tested against two Gram positive and two Gram negative strains. MIC values of the antibiotics are given in Table III.

MIC and MBIC

The MICs obtained fell within the suggested ranges for MIC [15]. In MBIC assay the antibiotic concentration which can inhibit the biofilm formation was found out. The MBIC values together with MBIC/MIC ratio are given in Table IV. A marked difference noticed between the MIC and MBIC Values. Amikacin and Cefotaxim were found to be comparatively effective for *P. aeruginosa* strain. In all other cases the MBIC to MIC ratio was quite high for all the antibiotics.

Table 1. Biofilm production of microorganisms in CRA

Microorganism	24hrs	48hrs	Results
S.aureus MTCC 1430	Very black	Very black	Producer
S.aureus MTCC 96	Very black	Very black	Producer
E.coli MTCC 739	Almost black	Almost black	Weak producer
P.aeruginosa NCIM 5029	Very red	Very red	Non producer

Table 2. Biofilm production of different strains, their adherence capability and the biofilm classification

Microorganisms	Biofilm formation OD-TSB (24hr, A590)	Biofilm adherence	Biofilm classification
S. aureus MTCC 96	0.96	Strong	High
S. aureus MTCC 1430	0.75	Strong	High
E. coli MTCC 739	0.72	Strong	High
P. aeruginosa NCIM 5029	0.9	Strong	High

Table 3. MIC values of the antibiotics

Antimicrobial agent	MIC ($mgml^{-1}$)			
	S. aureus MTCC 96	*S. aureus* MTCC 1430	*E.coli* MTCC 739	*P.aeruginosa* NCIM 5029
Amikacin	0.11	0.080	0.20	0.20
Gentamicin	.004	0.01	0.01	0.01
Ciprofloxacin	0.0004	0.001	0.001	0.001
Cefotaxim	0.01	0.005	0.005	0.01

Table 4. MBIC values and MBIC/MIC ratio

Antimicrobial agent	*S.aureus* MTCC 96		*S.aureus* MTCC 1430		*E.coli*MTCC 739		*P.aeruginosa*NCIM 5029	
	MBIC	Ratio	MBIC	Ratio	MBIC	Ratio	MBIC	Ratio
Amikacin	5	510.20	5	128.21	20	1025.64	1.25	19.05
Gentamicin	5	2083.33	5	510.20	2.5	128.21	0.63	47.36
Ciprofloxacin	1.25	3906.25	0.16	195.25	0.63	195.31	0.16	195.25
Cefotaxim	1.25	125	0.63	126	1.25	250	0.16	16

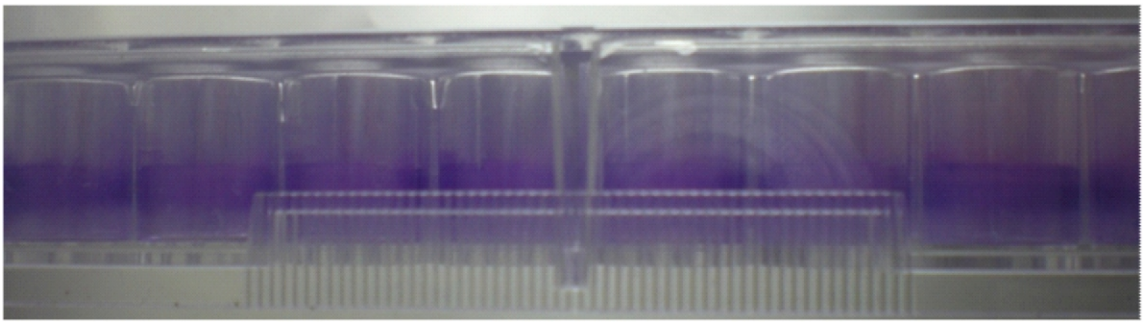

Fig 1. Biofilm production on the side of the microtiter wells upon crystal violet staining

Table 5. Correlation between antibiotic concentration and biofilm persistence

Antibiotic	Micro organism	Correlation coefficient(r)	95% confidence interval	Two tailed p value	Significance
Amikacin	*S.aureus* MTCC 96	-0.01	-0.58 to 0.57	0.9737	ns[d]
Gentamicin		-0.09	-0.63 to 0.51	0.7869	ns[d]
Ciprofloxacin		0.94	0.79 to 0.98	< 0.0001	es[c]
Cefotaxim		-0.23	-0.71 to 0.40	0.4805	ns[d]
Amikacin	*S.aureus* MTCC 1430	-0.60	-0.87 to -0.04	0.0393	s[a]
Gentamicin		-0.65	-0.89 to -0.12	0.0226	s[a]
Ciprofloxacin		0.75	0.32 to 0.93	0.0046	vs[b]
Cefotaxim		0.94	0.78 to 0.98	< 0.0001	es[c]
Amikacin	*P.aeruginosa*MTCC 5029	-0.13	-0.66 to 0.48	0.6823	ns[d]
Gentamicin		-0.15	-0.67 to 0.47	0.6460	ns[d]
Ciprofloxacin		0.62	0.08 to 0.88	0.0303	s[a]
Cefotaxim		-0.26	-0.73 to 0.37	0.4120	ns[d]
Amikacin	*E.coli*MTCC 739	-0.05	-0.61 to 0.54	0.8837	ns[d]
Gentamicin		0.26	-0.37 to 0.73	0.4152	ns[d]
Ciprofloxacin		0.67	0.16 to 0.90	0.0171	s[a]
Cefotaxim		-0.44	-0.81 to 0.18	0.1506	ns[d]

[a]s: significant, [b]vs: very significant, [c]es: extremely significant, [d]ns: not significant

Effect of antibiotics on pre-formed biofilms

The correlation between antibiotic concentration and biofilm persistence, the confidence interval and their significance are given in table V. The Pearson correlation coefficient and the p value gave sufficient evidence to suggest the linear relationship between various antibiotics concentration and percentage of biofilm persistence of various microorganisms. Amikacin and gentamicin concentrations have got a negative linear relationship with percentage of *S. aureus* MTCC 1430 biofilm persistence. The ciprofloxacin concentration had a positive linear relationship with the percentage of biofilm persistence of *S. aureus* MTCC 96, *S. aureus* MTCC 1430, *P. aeruginosa* NCIM 5029 and *E. coli* MTCC 739. This implies that ciprofloxacin induces biofilm formation in all the tested strains in a concentration dependent manner. Cefotaxim concentration has a strong positive linear relationship with the percentage of biofilm persistence of *S. aureus* 1430. However there is no significant effect on the biofilm persistence of the other *Staphylococcus*, *Pseudomonas* and *E. coli strains*.

DISCUSSION

The increased antibiotic resistance of biofilm bacteria was in agreement with the previous studies [9, 16, 17]. Minimum inhibitory concentration, the standard used for the antibiotic susceptibility testing for the treatment of many acute infections is ineffective in case of biofilm mediated chronic and persistent infections. *P. aeruginosa* urinary tract infections were reported to persist even after ciprofloxacin treatment for 7 days [18]. Since the margin between MIC and MBIC is huge, the poor predictive value of MIC for treatment can be understood. Therefore, for the treatment of biofilm infections MBIC will be more ideal rather than the MIC.

The aminoglycosides did not promote biofilm formation in any of the bacterial strains tested. Instead they were found to inhibit biofilm persistence of *S. aureus* MTCC 1430. Earlier reports show that the sub inhibitory concentration of aminoglycoside antibiotics induces biofilm formation in *P. aeruginosa* and *E.coli.* In *P. aeruginosa* the presence of aminoglycoside antibiotic tobramicin was found to induce biofilm formation through the gene, the aminoglycoside response regulator (*arr*) [1]. The aminoglycoside activity in this study may be due to the absence of *arr* gene in the tested strains.

There are reports showing subminimal inhibitory

concentrations of antibiotics acts as agonists of biofilm formation. Sub inhibitory concentrations of tetracycline and streptogramin antibiotic quinupristin-dalfopristin can enhance initial attachment and intercellular adhesion of certain bacteria [19].The minimum inhibitory concentration againstbacteria living as biofilm is known as minimum biofilm inhibitory concentration (MBIC). In comparison with MIC of a planktonic bacteria, the MBIC value of same bacteria in its biofilm mode of growth is much higher. Thus dose calculatiuons based on MIC values will provide only a subinhibitory concentration to the bacterial cells living in the biofilm community.Hence, biofilm mediated infections should be treated with a dose determined based on MBIC. The concept of loading dose also has to be checked, whether by using that, we are promoting a robust/ efficient biofilm which is very suitable for the adverse environment.

CONCLUSION

The use of ineffective antibiotics increases the biofilm persistence and only the highly resistant variants are selected by this process for survival. Antibiotics should be used against any biofilm mediated infection only after checking its MBIC and finding its effectiveness in vitro. Otherwise it may lead to the development of further differentiated biofilms and new phenotypic variants.

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