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Synergistic and antagonistic action of antibiotics against biofilm forming *Staphylococcus aureus*

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ABSTRACT

12 Clinical isolates of Staphylococcus aureus from various specimens of infectious bodily sites having ability of biofilm formation were screen in this study. On the basis of their ability to attach to polymeric surfaces, the formation of biofilm was determined in 6 wild type clinical isolates. Minimum biofilm inhibitory concentration (MBIC) of seven antibiotics (ampicillin, azithromycin, ciprofloxacin, erythromycin, gentamycin, oflaxicin, and penicillin) was estimated against the established biofilm on polystyrene microtiter plates. Biofilms were observed to be less susceptible to antibiotics by comparing the MBIC with MIC. The synergism result was investigated by the comparison of MBIC and FBIC. Synergy was demonstrated against the combination of beta lactam antibiotics (ampicillin + penicillin and ampicillin + cloxacillin) and their combination with macrolide antibiotics (ampicillin + azithromycin and penicillin + azithromycin). The observed values of partial synergistic, indifferent and antagonistic result were 12.5%, 16.67% and 55.55% respectively.

Keywords: Biofilm**;** *Staphylococcus aureus*; MIC; MBIC; FBIC; Σ FBIC.

Abbreviations *S.aureus: Staphylococcus aureus, MIC: Minimum Inhibitory Concentration; MBIC: Minimum Biofilm Inhibitory Concentration; FBIC: Fractionate Biofilm Inhibitory Concentration; Σ FBIC: Summation of FBICs; TSB: Tryptone Soya Broth; MH: Muller- Hinton (Broth); Amp: Ampicillin, Azt: Azithromycin, Cip : Ciprofloxacin, Clx: Cloxacillin, Ert: Erythromycin, Gnt: Gentamicin, Ofl: Ofloxacin, Pcn: Penicillin, Acx: Ampicillin + Cloxacillin.*

INTRODUCTION

Today, the therapy of biofilms is a problem. Current treatment paradigms for biofilm associated infections of semi permanent indwelling devices typically involve surgical replacement of the device combined with long term antibiotic therapy and incur high heath care costs. Biofilm infections of certain indwelling medical devices by common pathogens such as *Staphylococci* are not only associated with increased morbidity and mortality but are also significant contributors to the emergence and dissemination of antibiotic resistance traits in the noscomial setting.

S.aureus is an adaptable, pathogenic and opportunistic pathogen and can infect humans resulting in a myriad of infections such as skin lesions, scalded skin syndrome, impetigo, osteomyelitis, pneumonia, endocarditis, wound infections etc. About 20% of the populations are long term carriers of *S. aureus*. It is often resistant to many antibiotics used in causative therapy; moreover, *S.aureus* is also able to form biofilms.

Microorganisms are able to adhere to various surfaces, encase themselves in a hydrated matrix of polysaccharide and protein and form a slimy layer known as “biofilm” [1]. The infectious microbes have evolved various mechanisms to evade antimicrobial therapy and the most important among them is the ability to either form or live

within a biofilm. Bacteria that adhere to implanted medical devices, such as catheters, contact lenses or pacemakers, and to the other surfaces such as a dental plaque, can become a cause of persistent infections. The bacteria harbored inside biofilms are less exposed to the host's immune response and less susceptible to antibiotics [2].

In this study the *in vitro* affect of 12 antibiotic combinations was investigated in biofilms formed by *S.aureus* using biofilm-susceptibility testing. To discern the synergistic and antagonistic effects of the antibiotics, MBICs and FBICs were calculated.

MATERIALS AND METHODS

Collection of strains. Clinical isolates of *S.aureus* (12 strains) were obtained from a local pathology centre of Lucknow. These strains were isolated from various infectious bodily sites and were identified depending on biochemical or enzyme based tests.

Screening for biofilm production. To investigate the biofilm characteristics of *S.aureus*, cells were incubated in tryptone soya broth (TSB) at 37° C for 24 hours [3]. After 24 hours incubation period, the broth mediums of culture tubes were withdrawn carefully. Surface adhering cells were stained with crystal violet for 20 minutes at room temperature (modified Christensen method) [4,5]. Cells were grown in in small test tubes, according to. After 1 day incubation, the culture tubes were emptied of their contents and the tube adhering cells were stained with crystal violet for 20 minutes Then the tubes were washed three times with water and allowed to dry in inverted position. A well visible film lining on the walls of tubes were considered to be positive.

Biofilm formation. The biofilm positive strains of *S.aureus* were grown in U-wells microtiter plates (Laxbro, India) containing 75 µL TSB at 37° C for 24 hours. After 24 hours incubation, the microtiter plates were washed three times with phosphate- buffered saline (PBS) under aseptic conditions to eliminate unbound cells and dried in inverted position [6].

Biofilm susceptibility testing. (i) Antibiotic Preparation. The appropriate dilutions of the respective antibiotics in Muller Hinton broth (MHB) were prepared. The strains were tested against the susceptibility to Amp (500mg; Ranbaxy, India), Azt (500mg; Cipla, Protec, India), Cip (500mg; Cipla, India), Ert (250mg; Alembic, India), Gnt (40mg; Nicolas, India), Ofl (200mg; Cipla, Protec, India) and Pcn (500mg; Pfizer, India).

100 µL of the prepared dilutions were transferred into the dried wells containing pre-established biofilms. The plates were incubated for 18-20 hrs at 37°C. After this MBICs were evaluated.

Synergy and Antagonistic testing. The synergistic effects were determined against the combination of antibiotics (50% antibiotic 'A' and 50% antibiotic 'B'). The antibiotic combinations were prepared in MHB.100 µL volumes of the prepared antibiotic combination were transferred into the pre- established biofilm containing wells of microtiter plate and incubated at 37°C for 18-20 hrs. Then the MBICs were determined [7].

FBIC of each agent was calculated as follows [8]:

$$FBIC_A = MBIC_{A(c)} / MBIC_{A(a)},$$
$$FBIC_B = MBIC_{B(c)} / MBIC_{B(a)}$$
$$\Sigma\ FBIC = FBIC_A + FBIC_B$$

where subscripts A and B denote antibiotics A and B, subscripts in parentheses c and a denote the activity measurements in combination and alone, respectively. The summation of both FBICs was used to array the combination of antimicrobial agents as synergistic ($\Sigma FBIC = 0.5$), partially synergistic ($0.5 < \Sigma FBIC = 1$), indifferent ($1 < \Sigma FBIC = 4$), or antagonistic ($\Sigma FBIC > 4$).

S.aureus strains were tested against the susceptibility to the combination of antibiotics Amp + Pcn, Amp + Clx, Azt + Ert, Amp + Azt, Pcn + Azt, Amp + Cip, Amp + Ofl, Cip + Pcn, Ofl + Azt, Cip + Azt, Art + Gnt, Gnt + Azt.

MIC (the lowest concentration of antibiotic which inhibits the growth of a planktonic bacterial population) was determined according to the guidelines of *NCCLS* [9].

MBICs were statistically compared with MIC using a paired Student's *t*- test, as significant results being defined those at $p < 0.05$; p values were calculated with VassarStats, website for statistical computation.

Table I. Comparison of MIC and MBIC (means and medians; both in mg/L) of 6 *S.aureus* biofilm forming strains

Antibiotic	MIC		MBIC	
	Mean	Median	Mean	Median
Amp	20.8	80.0	101.8	400.0
Ofl	41.4	166.67	205.3	825.0
Cip	52.2	53.3	107.4	110.23
Pcn	53.6	80.0	119.6	161.87
Azt	54.8	53.33	240.5	107.83
Gnt	728.0	500.0	38.0	200.72
Ert	35.6	53.33	69.1	59.37

Table II. Antibiotic susceptibility of *S.aureus* strain-18 as a planktonic (MIC) and a biofilm population (MBIC, both in mg/L)

Antibiotic	MIC	MBIC
Amp	20*	100
Ofl	50*	250
Cip	60*	100
Pcn	60*	100
Azt	60*	100
Gnt	750*	250
Ert	40*	40

** Susceptible according to conventional MIC evaluation*

Table III. Synergistic, partially synergistic, indifferent or antagonistic actions of antibiotic combinations

ANTIBIOTIC COMBINATION	SYNERGESTIC		PARTIALLY SYNERGESTIC		INDIFFERENT		ANTAGONISTIC	
Amp + Pcn	5	83	2	33	0	- -	1	16
Amp + Clx	3	50	2	33	0	--	1	16
Azt + Ert	0	- -	0	- -	2	33	1	16
Amp + Azt	1	16-	3	50	2	33	1	16
Pcn + Azt	2	33	2	33	1	16	1	16
Amp + Cip	0	- -	0	- -	1	16	5	83
Amp + Ofl	0	- -	0	- -	4	66	2	33
Cip + Pcn	0	- -	0	- -	1	16	5	83
Ofl + Azt	0	- -	0	- -	0	--	6	100
Cip + Azt	0	- -	0	- -	1	16	5	83
Ert + Gnt	0	- -	0	- -	0	--	6	100
Gnt + Azt	0	- -	0	- -	0	--	6	100
TOTAL NUMBER	11	15.27	9	12.50	12	16.67	40	55.55

RESULTS

Out of 12 clinical isolates only 6 isolates (1, 6, 8, 11, 18, and 28) (50%) were biofilm positive.

Antibiotic susceptibility of attached cells. Biofilm positive *S.aureus* of 6 strains 1- fold to 6- fold higher MBIC than MIC values (Table: I) were obtained ($p<0.05$) for antibiotics. *S. aureus* strain-18 shows gave MBIC 1- fold to 5- fold higher values than the MIC. Values for Gnt differed only 0.33 fold, i.e. MBIC value for Gnt was insignificant in comparison to the corresponding MIC value (Table II).

Synergy affects has been shown by four combinations of antibiotics comprising of two beta lactams and beta lactam with macrolides. The synergistic and partial synergistic effect of four combinations Amp + Pcn, Amp + Clx, Pcn + Azt, Amp + Azt were shown as 15.27% and 12.50% respectively (Table III). The two beta lactam antibiotics, Amp + Pcn, have shown synergistic effect against 5 strains of *S. aureus*.

Three combinations have shown antagonistic effect on all the 6 strains of *S. aureus* (Table III). Antagonistic affects were shown by the combinations of fluoroquinolone antibiotics.

The indifferent result was found for the combination of Amp + Ofl against 4 strains of *S. aureus* (Table III).

DISCUSSION

The present study demonstrates that *S. aureus* have ability to easily grow in a biofilm. The *S. aureus* biofilms developed in an unmodified small polystyrene plates without the necessity of tissue culture plates as reported by Walnecka *et al*. [10]. This shows that microbial community of *S. aureus* as a biofilm can easily grow in an adaptable environment and can cause threatening diseases. Many several diseases including endocarditis, osteomyelitis and foreign body related diseases appear to be caused by biofilm associated *S. aureus* [11].

To investigate the required therapy, this study first aims to check the susceptibility of *S. aureus* biofilms against individual antibiotics. The current study reveals that that the singular dose of antibiotic is unable to perform effectively against biofilms. The individual antibiotic used against *S. aureus* biofilms have shown up to 5-fold higher value of MBIC in comparison to MIC, resulting from planktonic cells. Gnt was found to be an exception being non effective against planktonic state but against biofilms similar results were obtained by Amorena *et al*. [2]. The comparison of MIC and MBIC indicates that two fluoroquinolone antibiotics required up to 5- fold higher concentration to inhibit the biofilms. Macrolides antibiotics were much effective with respect to fluoroquinolone antibiotics against *S. aureus* biofilms. Beta lactam antibiotics were required up to 2- fold to 5-fold higher concentration for biofilms. Thus, these results designate that higher effective treatment then antibiotics is required to inhibit the biofilm.

To investigate the effective treatment against *S. aureus* biofilms combination of antibiotics are used in the present study. The synergistic effects of these combinations were evaluated with FBICs (Table III). Beta lactam and macrolide antibiotics have given effective response against biofilms at the primary stage in comparison to the fluroquinolone antibiotics, which inhibits the reproduction and DNA repair mechanism. This shows that the mechanism of beta lactam and macrolide antibiotics are effective in inhibiting the biofilms. This suggests that not only the overall morphology of the biofilm but also transcriptional profile (phenotype) of the constitutional bacteria plays an important role in antibiotic resistance of biofilms.

The population density in the biofilm is influenced by the quroum sensing of the microbes. Several serious diseases are caused by biofilm-associated *S. aureus*, infections in which the accessory gene regulator quorum sensing system is thought to play an important role [12]. The synergistic effect by the combinations of beta lactam and macrolide antibiotics can be explained on the account of the inhibition in cell wall synthesis and protein synthesis within the biofilm. The quorum sensing of *S. aureus* involving the accessory gene regulator thought to be hindered by the combinations of antibiotics which results in the inhibition of biofilms.

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