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Effect of Tobracef on biofilms of aminoglycoside resistant *Pseudomonas aeruginosa*

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Abstract: *The present study was undertaken with the aim to find prevalence of biofilm forming ability among Pseudomonas aeruginosa clinical isolates. A total of 129 clinical isolates were identified from clinical specimens by standard microbiological techniques. Biofilm formation ability among these isolates was studied using in vitro bifilm formation assay. Minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) were studied using procedure as described in Clinical and Laboratory Standards Institute.*

Among 129 isolates, the prevalence of aminoglycoside resistance was 75.2 % out of which 70.1% were found to be biofilm producers. The MIC for Tobracef in aminoglycoside resistant strains ranged from 8 to 16 µg/ml and for other antibacterial drugs it ranged from 16 to 256 µg/ml. The MBEC for Tobracef in biofilm producing isolates was ranged from 32 to 64 µg/ml and for other antibacterial agents, it ranged from 1024 to 8292 µg/ml. When the effect of half of MBEC of drugs were evaluated on preformed biofilms, Tobracef significantly reduced viable counts of bacterial cells presented in biofilms of P. aeruginosa with log reduction values ranging 5.17 to 5.46 logs.

Our data showed that Tobracef has significantlly enhanced activity against aminoglycoside resistance biofilm producing isolates. Therefore, use of this antibiotic should be considered to treat the infections caused by aminoglycoside resistant biofilm producing isolates.

Keywords: *Bifilm, Clinical isolate, MIC, MBEC,Tobracef.*

I. Introduction

Pseudomonas aeruginosa is a gram negative opportunistic human pathogen frequently causing life-threatening infections including septicemia, cystic fibrosis, and urinary tract infections [1-3]. Infections due to *P. aeruginosa* has been implicated as the major cause of morbidity and mortality in cystic fibrosis patients [4]. *P. aeruginosa* is accounting for 10-15% of the nosocomial infections world wide [5]. Among gram negative organisms, *P. aeruginosa*, has been identified as a principal biofilm-producing opportunistic pathogen [6-7]. Biofilms are a group of bacteria which are formed when bacteria adhere to any solid surface and enclosed themselves in a self-produced extracellular polymeric substance (EPS) which consists of polysaccharides, nucleic acids and proteins [8]. EPS is responsible to attach biofilms to surface and protect them in harsh condition [9].

In the past few decades, biofilm producing *P. aeruginosa* have been implicated in various types of infections such as biomaterial associated infections, pulmonary infections and catheter associated urinary tract infections [10-11]. It has been estimated around 65 % to 80% of all chronic human infections caused by organisms in biofilm state which are difficult to treat [4,12]. The prevalence of biofilm producing *P. aeruginosa* vary from 65 % to 83 % [13,14] which are higher in multidrug resistant isolates [15].

One important feature of biofilm producing bacteria is increased resistance to antibiotics [15], which increases up to thousand times in comparison to planktonic bacteria of the same species [16,17]. Resistance of biofilm producing bacteria to antibiotics may be due to a number of factors including retardation of diffusion of antimicrobial agents through polysaccharide matrix that surrounds the cells in the biofilm [18], decreased growth rate and expression of resistance genes. Furthermore, negatively charged exopolysaccharide is very effective in protecting cells from positively charged aminoglycoside antibiotics by restricting their permeation, possibly through binding [19].

The efficacy of drugs including aminoglycosides is reduced when *P. aeruginosa* exist within a biofilm [20,21]. A number of reports demonstrated up to 70 to 85.2 % resistance to *P. aeruginosa* in biofilm state against various drugs including aminoglycoside [22,23].

In view of steadily increasing incidence of difficult to treat infections caused by biofilm producing *P. aeruginosa* and reducing efficacy of drugs there is a dire need of alternate therapies. Tobracef, is one such novel drug (under patent protection) which is a combination of third-generation beta lactam antibiotic ceftazidime and tobramycin in a ratio of 8.33:1, supplied with solvent. Tobracef has been demonstrated to have noticeable antibacterial activity [24]. Its antibacterial activity has also been proved in animal model [25]. To authors knowledge, the effect of Tobracef on *P. aerugionsa* biofilm has not been reported earlier. Therefore the

current investigation was undertaken to determine the prevalence of biofilm formation among *P. aeruginosa* isolated from different clinical specimens. Susceptibility studies in terms of minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) were studied. Moreover, we evaluated the effect of half of MBEC of drugs on reduction of viable bacteria in biofilm.

II. Materials And Methods

2.1. Bacterial strains

A total of one twenty nine multidrug resistant *P. aeruginosa* isolated from clinical specimens of pus, blood, sputum and catheter tips were obtained from –a multispeciality hospital of Gurgaon Haryana, India. The identity of clinical isolates was confirmed by standard microbiological techniques [26]. Prior to use, clinical specimens were spread onto Mueller-Hinton Agar medium (MHA; Hi-Media, Mumbai, India), and incubated at 35°C for 24 hrs. Further, single colony grown on MHA was inoculated into 10 ml of Müller-Hinton broth (MHB; Hi-Media, Mumbai, India) and grown to mid log phase on the shaker. Aminoglycoside resistance in these isolates was tested by disc diffusion method as per Clinical and Laboratory Standards Institute guidelines (CLSI) [27]. The bacterial suspension, at a concentration of 10^6 colony-forming units (cfu/ml), were used for susceptibility studies and biofilm formation.

2.2. Selection of clinical isolates of *P. aeruginosa*

To evaluate the biofilm forming ability of aminoglycoside resistant *P. aeruginosa* isolates, 100 µl of each isolate at 10^6 cfu/ml was seeded into wells of 96-well microtiter plate containing 100µl sterile MHB. The wells with MHB served as control. The plate was then incubated at 37ºC for 48 hrs. Following incubation, wells were washed once with phosphate buffer saline (PBS; pH 7.2) and stained with crystal violet (1%). The stained crystal violet was solubilized in 95% ethanol and absorbance was measured at 575 nm and specific biofilm forming index (SBF) was calculated using the formula: biofilm formation (AB) – control (CW) / growth (G). The degree of biofilm production was classified in three categories: weak (SBF $\leq$ 0.5), moderate (0.5 > SBF $\leq$ 1) and strong (SBF > 1) [28].

2.3. Antibacterial agents

A novel antibiotic combination comprising ceftazidime and tobramycin herein after referred to as Tobracef (Venus Remedies Limited, India), ceftazidime (Fortum, GSK Limited, India), tobramycin (Tobraneg; Elder Pharmaceuticals India), amikacin (Alfakim; Ranbaxy Laboratries Limited, India), gentamicin (Ranbiotic Ranbaxy Laboratories, Gurgaon India), levofloxacin (Lebact; Piramal Healthcare, India) piperacillin plus tazobactam (Zosyn; Wyeth Pharmaceuticals, Mumbai, India) were used in the study. Tobracef, Zosyn and Fortum were reconstituted according to instructions of manufacturer, rest all were ready to use. Working solutions were prepared using Mueller Hinton broth (MH, Hi-Media, Mumbai, India).

2.4. Antimicrobial Susceptibility

Minimum inhibitory concentration (MIC) of each drug was determined by the broth dilution method according to the CLSI [27]. The MIC value represents the lowest dilution at which bacteria fail to grow.

2.5. Minimum Biofilm eradication Concentrations (MBECs) Determination

MBECs of drugs were determined by a broth dilution method in MHB according to the procedure of CLSI [27]. Briefly, after development of biofilms on pegs, pegs were washed with phosphate buffer saline (PBS; pH 7.0) to remove non-adherent bacteria and thereafter treated with different concentrations of antibiotics ranging from 2 µg/ml to 8192 µg/ml. Concentration of antibiotic at which no viable cell-counts in biofilms were obtained was taken as MBEC.

2.6. Analysis of pre-formed biofilms

Biofilms developed on the calgarry biofilm device (CBD) were further analysed in term of viable bacterial counts.

2.7. Bacterial cell counting

The number of adhering bacteria within the biofilm was determined by breaking four pegs of the CBD under aseptic conditions. The broken pegs were submerged in sterile PBS and sonicated mildly at 60 hz for 10 min. The disrupted biofilms were subsequently serially diluted in PBS and plated on MHA plates for viable cell counting. The viable counts were recorded as total colony forming units (cfu/ml). All samples were run in triplicate.

2.8. Effect of drugs on biofilm

To study the effect of drugs on biofilm, after development of biofilms on pegs, the lid was placed on the plate having half concentrations of MBECs of drugs.

III.Results

3.1. Identification of clinical isolates

Out of 129 *clinical isolates*, all were confirmed to be *P. aeruginosa* as all of them produced pyocyanin in Pseudomonas isolation agar and observed to be oxidase positive. Maximum *P. aeruginosa* isolates were recovered from pus 34.1% (44/129) followed by sputum 27.1% (35/129), catheter 26.3 % (34/129) and blood 12.4% (16/129). Among these isolates, the maximum (82.8%) aminoglycoside resistant isolates were recovered from sputum followed by pus (81.8%), catheter (73.5%) and blood 43.7% (Table 1).

3.2. Selection of biofilm producing *P. aeruginosa*

Of 97 aminoglycoside resistant clinical isolates, 68 isolates were biofilm producers out of which 64.7% (44/68) were strong biofilm producers as evident by strong staining around the wells; 19.1% (13/68) were moderate biofilm producers and 14.7 % (11/68) were weak biofilm producers. The highest strong biofilm producer was recovered from sputum 48.3 % (14/29) followed by pus 44.4% (16/36), catheter 44% (11/25) and blood 42.8 % (3/7) (Table 1).

3.3. MIC and MBEC

As shown in Table 2, Tobracef emerged as the most active antibacterial agents with MIC value 8-16 µg/ml. The MIC values for piperacillin and tazobactam+ amikacin, levofloxacin, ceftazidime+levofloxacin were 16-128 µg/ml. Remaining drugs ceftazidime, tobramycin, amikacin and gentamycin showed MIC values 128-256 µg/ml. The MBEC of Tobracef for *P. aeruginosa* biofilms was approximately 3X of MIC, indicating that 3 times more Tobracef is required to kill the bacteria in biofilms than that was required to inhibit planktonic bacterial cells. The other drugs showed higher MBECs against *P. aeruginosa*. The MBEC of levofloxacin, piperacillin and tazobactam+amikacin, ceftazidime+levofloxacin was approximately 6X to 7 X of MIC indicating that 6 to 7 times more antibacterial agents required to kill bacteria in biofilms. On the other hand, ceftazidime, tobramycin, amikacin and gentamycin showed 8X to 9X times higher MBEC values than MIC.

When the effect of half of MBEC of drugs were evaluated on preformed biofilms, the data presented in Table 2 shows that only Tobracef significantly reduced viable counts of bacterial cells presented in biofilms of *P. aeruginosa* with log reduction values ranging 5.17 to 5.46 logs. The combination of ceftazidime+levofloxacin was the second most effective drugs with log reduction value 2.62 to 2.98. The other drugs showed only 1.12 to 2.76 log reduction in bacteria after treatment. The staining of pegs following treatment with half of MBEC of drugs also supports these results. The pegs treated with Tobracef show maximum removal of biofilm as evident by least staining with crystal violet followed by ceftazidime plus levofloxacin (Figure not shown).

IV. Discussion

Biofilm forming bacteria caused a variety of infections in humans and treatment of these infections imposes a major threats for clinicians as these are very hard to treat. Bacteria in biofilm are protected from antibiotics due to the presence of large amount of exopolysaccharide. The prevalence of aminoglycoside resistant strains observed to be 75.2 % was in accordance with earlier study where the incidence of aminoglycoside resistance was reported 55 % to 83 % [29]. There are a number of methods available for evaluating the biofilm ability of *P. aeruginosa* which includes microtitre plate, tube method, radiolabelling, congo red agar plate method, confocal laser scanning microscopy. Among these, crystal violet microtitre plate is the most popular [26], hence was used in current study.

Biofilm formation is one of the important mechnisms of drug resistance [23]. In biofilm, bacterial cells aggregate in multicellular communities encapsulated by EPS, therefore a strategy that disrupts this structure might be able to play a beneficial role and the efficacy of the antibiotic might be restored. Moreover, chemicals that prevents polysaccharide synthesis as well as interfere with cell to cell communication required for normal biofilms formation [30], might be helpfull in biofilm disruption. The results of our study showed that only 70.1% aminoglycoside resistant strains were found to be biofilm producers which is in accordance with a study conducted in India reporting 65 % [14] biofilm producing *P. aeruginosa*. Although, yet another study, reported 85.2% biofilm producing clinical isolates [23].

Monotherapy of antibiotics are effective only on planktonic cell [14]. However, monotherapy failed to respond against planktonic cells when they acquire other phenotypic behaviour such as biofilm forming ability. A large number of antibacterial agents have also been used against biofilm producing isolates but none of them were observed to be effective [31]. However, our results showed that Tobracef seems to be more efficacious in both planktonic and biofilm cells when compared with other drugs. Tobracef offers a new strategy for the treatment of infections caused by bacteria in biofilm state.

The present data add support to clinicians for the treatment of bacteria in biofilm state. The exact mechanism how Tobracef demonstrated enhanced efficacy in both planktonic as well as biofilm cells is not known. However, this combination in addition to antibacterial activity, it may have distorted the surface charge on bacterial cell membrane, thereby impairing nutrient uptake, translocation, adherence and biofilm formation. From the above results, it is clear that Tobracef is the only product which has the efficacy to eradicate the biofilm very efficiently as compared to other antibacterial agents.

V. Conclusion

In conclusion this study has shown that Tobracef has significantly enhanced activity against aminoglycoside resistant as well as biofilm producing isolates which is evident by lowest MIC and MBEC values compared to other drugs. Moreover, half of MBEC of Tobracef effectively kill the bacteria in biofilm and reducing the viable biofilm cell number by > 5 logs. Therefore, use of this antibiotic should be considered to treat the infections caused by both aminoglycoside resistant and biofilm producing isolates in comparison to commonly used combinations of third generation cephalosporin and quinolone or piperacillin tazobactam with aminoglycosides.

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Table 1: Specimen wise distribution of biofilm producing isolates.

Clinical isolates	No. of isolates	Aminoglycoside resistance	Biofilm producers		
			Strong	Moderate	Weak
Sputum	35	29	14	3	4
Blood	16	7	3	1	1
Pus	44	36	16	6	4
Catheter	34	25	11	3	2
Total	129	97	44	13	11

Table 2: MIC and MBEC of antimicrobial agents against planktonic and biofilms cells.

Drug Name	MIC (µg/ml) against *P. aeruginosa*	MBEC (µg/ml) against *P. aeruginosa*
Ceftazidime	128 - 2 56	4096-8192
Levofloxacin	16-128	1024-2048
Tobramycin	128 - 256	2048-4096
Amikacin	128 - 256	2048-4096
Gentamicin	128 - 256	2048-4096
Piperacillin+tazobactam + amikacin	16 - 128	1024-2048
Ceftazidime + levofloxacin	16 - 128	1024-2048
Tobracef (ceftazidime and tobramycin)	8--16	32-64

Table 3. Effect of antibacterial agents on biofilms.

Drug Name	Time (h)	Biofilm concentration (cfu/peg)[a] before treatment (A)	Biofilm concentration (cfu/peg)[b] after treatment (B)	Log reduction (logA-logB)	Log reduction range against all *P. aeruginosa*
Ceftazidime	12	$6.5x10^8(8.81)^a$	$3.6x10^7(7.55)^a$	1.26	1.12 – 1.56
Levofloxacin	12	$6.3x10^8(8.79)^a$	$1.4x10^6(6.14)^a$	2.65	2.42 – 2.88
Tobramycin	12	$5.2x10^8(8.71)^a$	$2.3x10^6(6.36)^a$	2.35	2.21 – 2.51
Amikacin	12	$6.1x10^8(8.78)^a$	$1.8x10^6(6.25)^a$	2.53	2.46 – 2.72
Gentamicin	12	$5.6x10^8(8.74)^a$	$2.9x10^6(6.46)^a$	2.28	2.18 – 2.44
Piperacillin+tazobactam + amikacin	12	$6.4x10^8(8.80)^a$	$2.6x10^6(6.41)^a$	2.39	2.13 - 2.76
Ceftazidime + levofloxacin	12	$6.2x10^8(8.79)^a$	$1.2x10^6(6.07)^a$	2.72	2.62 - 2.98
Tobracef (ceftazidime + tobramycin)	12	$5.9x10^8(8.77)^a$	$3.2x10^3(3.50)^a$	5.27	5.17 – 5.46

Only strong biofilm producing *P. aeruginosa* isolates were taken for this study.