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Antibiotic-EDTA Combination Induced Dispersal of *Pseudomonas aeruginosa* Biofilm

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The present study was under taken to detect biofilm production in nosocomial isolates of *Pseudomonas aeruginosa* and EDTA- antibiotic combination induced dispersal and killing of Pseudomonas cells in biofilm. Different clinical samples were collected from site of infection judged on the basis of clinical findings. *P. aeruginosa* was isolated and identified by standard conventional methods. Biofilm production was detected by Congo Red Agar (CRA) method and further antibiogram was analyzed in presence of antibiotics as well as in combination with EDTA by disc diffusion method and Agar well diffusion technique respectively.

Amongst the 100 samples, 80 Pseudomonas isolates were obtained and screened for biofilm detection. Maximum isolates (85%) were found able to grow within slime by CRA method. Most of the isolates exhibited resistance towards Penicillin suggesting that its further use may lead enhancement of drug resistance disaster. Increased in diameter of zone of inhibition was recorded in presence of combination revealing its better utility for treatment purpose. The combination of EDTA-Gentamicin and EDTA-Tobramycin significantly inhibited Pseudomonas growth. The present study demonstrated that EDTA is a potent *P. aeruginosa* biofilm disrupter. EDTA treatment of Pseudomonas biofilm results in 1000 fold greater killing than treatment with antibiotics alone. A combination between proper amounts of EDTA with antibiotics will improve the control of *Pseudomonas aeruginosa* biofilm.

Key words: Biofilm, *Pseudomonas,* Drug resistance, EDTA.

Biofilm is a structural community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surfaces. Bacterial biofilm are abundant in environment and are involved in the several human bacterial infections. Biofilm can withstand host immune response and are much more resistant to antibiotics treatment than their non-attached individual free living counterparts. For these reasons biofilm infections are persistent and individual often show recurring symptoms following antibiotic therapy. Biofilm are also often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and an extra cellular matrix of polymeric substances[1].

One of the best studied models for biofilm formation is the bacterium *Pseudomonas aeruginosa*. Pseudomonas has several virulence factors like extracellular toxins, proteases, and exopolysaccharide (EPS) which adapt specific host tissue infections. The EPS layer secretes around provide extra resistance to the bacterium and facilitate its easy survival in number of disinfectants. The outer membrane become less permeable impacting it, an inherent resistance and

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along with that the capability of bacteria to grow within biofilm protects them from adverse environmental factors[2,3].

Pseudomonas aeruginosa, a virulent microorganism and well know opportunistic bacterium which infects various tissues and organs causing severe problems. Ability of bacteria to grow within biofilm, allows the bacteria to adapt resistance mechanism for antibiotics, drugs, disinfectants and in general for other toxic compounds. The frequency of outbreaks associated with multi-drug resistance *Pseudomonas aeruginosa* enforce investigators to have an in-depth study of the bacterium and make efforts to trace some alternative approaches. Several non molecular techniques have been studied all taking advantage of the enzymes zinc dependence by using chelating agents. The metal chelator EDTA causes lysis, loss of viability, and increased sensitivity to a variety of antimicrobial agents. This has led to the use of EDTA as a potent *P. aeruginosa* biofilm disrupter[4].

EDTA has a detrimental effect on the outer membrane permeability of Pseudomonas by chelating divalent cations from their binding sites in lipopolysacharide . EDTA facilitate the release of a significant proportion of EPS from the cell, although prolong treatment with EDTA are lethal. The short treatment of EDTA increases the permeability of outer membrane to antimicrobial agents, thus there can be synergy between EDTA and antibiotics to combat biofilm producing *Pseudomonas aeruginosa*[5,15].

MATERIAL AND METHODS

Bacterial Isolation

In this study 100 samples taken from clinical materials (pus, blood, urine etc.) were collected from site of infection considered on the basis of clinical findings. Total 80 isolates of *Pseudomonas aeruginosa* were examined. All samples were transferred in a trypticase soya broth for enrichment. Prior incubation each enriched culture was streaked on MacConkey Agar and Blood Agar plates. All suspicious screened colonies of relevant pathogen was analyzed for their morphological, cultural and Biochemical characters. Further identification was done as per standard literature[6].

Biofilm detection

The obtained isolates of Pseudomonas were exposed to detection of biofilm production by Congo Red Agar (CRA) method developed by Freeman *et. al.* [7,16] was used in this study. Plates of CRA were prepared, inoculated and incubated aerobically at 37°C for 24h. Isolates that produced black colonies with dry crystalline consistency were regarded as slime positive; where as those showing pink colonies were slime negative.

Susceptibility test

Antibiotics susceptibility test was performed with the agar disc diffusion method (8). Isolates were categorized as susceptible, moderately susceptible and resistant based upon interpretive criteria developed by the clinical and laboratory standards institute (CLSI). The antibiotics used were , Penicillin G (10mcg), Ampicillin (10mcg), Kanamycin (10mcg), Tobramycin (10mcg), Gentamicin (30mcg), Chloramphenicol (50mcg), Tetracycline (30mcg) and Ciprofloxacin (5mcg) etc.

Antibiotic-EDTA combination

Antibiotic resistant isolates of biofilm producing Pseudomonas were further exposed to EDTA-antibiotic combination so as to analyze the change in susceptibility pattern of organism. The combination was prepared by incorporating different concentration of EDTA (15mM, 20mM and 25mM) in sterile distilled water containing respective antibiotic [Gentamicin (10mcg), Tobramycin (10mcg)]. The antibiotics were selected depending on their frequency of occurrence in regular prescription. Conventional Agar well diffusion method was performed for determination of potentiating ability of Antibiotic-EDTA combination to induced dispersal of biofilm producers[9]. To determine the stability and optimal pH at which maximum dispersion found antibiotic-EDTA combination were treated at various range of pH i.e. 3, 6, 8, 12 and 14.

RESULTS

In present study total 100 clinical samples (viz. Blood, Pus, and Urine etc.) were analyzed to isolate *Pseudomonas aeruginosa*. Isolates were identified by morphological, biochemical and cultural characteristics. Total 80 isolates of Pseudomonas were further evaluated for biofilm

detection by CRA method. Percentage of biofilm production in *Pseudomonas aeruginosa* measured as positive with CRA method is 85%.

The percentage of antibiotic resistance exhibited by biofilm producers as well as non-producers was shown in Table 1. Maximum

Table 1. Antibiotics resistance (%) of biofilm producers (n=60) and Non-producers (n=20) isolates

S. No	Antibiotics	Non-biofilm producers		Biofilm producers	
		n	%	n	%
1.	Penicillin	11	55	58	96.6
2.	Ampicillin	8	40	56	93.3
3.	Kanamycin	6	30	48	80
4.	Tobramycin	5	25	51	85
5.	Gentamicin	7	35	53	88.3
6.	Chloramphenicol	6	30	48	80
7.	Tetracycline	7	35	47	78.3
8.	Ciprofloxacin	5	25	45	75

Table 2. Effect of Antibiotic-EDTA combination on Biofilm Producing *Pseudomonas*

Number of Pseudomonas isolates	Inhibition zone diameter (mm)					
	Gentamicin-EDTA			Tobramycin-EDTA		
	G15	G20	G25	T15	T20	T25
1	16	19	26	15	18	20
2	15	20	25	14	17	23
3	17	19	24	15	18	20
4	16	20	25	16	19	21
5	15	21	26	14	17	21
6	17	23	24	15	19	24
7	15	20	23	16	18	23
8	16	21	25	15	17	24
9	17	19	24	16	19	21
10	16	20	23	15	18	21

Table 3. Effect of pH on antibiotic-EDTA combination

Antibiotic-EDTA	Activity present/absent (+/-) at different Ph				
	3	6	8	12	14
G15	-	+	++	+	-
G20	-	+	++	+	-
G25	-	+	++	+	-
T15	-	+	++	+	-
T20	-	+	++	+	-
T25	-	+	++	+	-

++: optimum pH showing largest zone of inhibition

resistance was noted against Penicillin Ampicillin, Gentamicin and Tobramycin.

The inhibition zone diameter of antibiotic-EDTA combination against multi-drug resistant *Pseudomonas aeruginosa* on an average was recorded and shown in Table 2. The combination of gentamicin and tobramycin with various concentrations of EDTA shows significant increase in the sensitivity against *Pseudomonas aeruginosa*.

The stability of antibiotic-EDTA biofilm dispersal activity was studied at different range of pH shown in Table 3. Maximum activity was obtained within a range of 3-12 pH while antagonistic activity was lost below 3 and above 12 pH.

DISCUSSION

This study was designed to be a model to evaluate the interaction between antibiotics and divalent cations during the sensitivity test. *P. aeruginosa* was selected because it resists antibiotics, and its ability to grow within biofilm. Total 100 clinical samples were analyzed to isolate *Pseudomonas aeruginosa*. 80 isolates of Pseudomonas were identified and screened for biofilm detection by CRA method. The percentage of biofilm producing isolate was measured as 85%, it means maximum isolates (n=68) has ability to grow within slime.

The comparative antibiotic sensitivity test of obtained biofilm producers and non-producers Pseudomonas was made by agar disc diffusion method[8]. In the present study results of antibiotic susceptibility test showed multiple resistances against usually recommended antibiotics. Variability in sensivity and resistant patterns were similar to those reported by Arun Jyothi *et. al.*[10]. Antibiotic resistance pattern exhibited by non-biofilm producers were found to be against penicillin (55%), Tobramycin (25%), Gentamicin (35%) and Ciprofloxacin (25%). Biofilm production in Pseudomonas significantly enhanced antibiotic resistant with respects to non-biofilm producers. Maximum resistance was observed against Penicillin in all biofilm producers isolates (96.6%) followed by Ampicillin (93.3%), Gentamicin (88.3%) and Chloramphenicol (80%).

Our study also showed an association between biofilm production and multi-drug resistance in Pseudomonas. Reason behind this may be the delayed penetration of antimicrobial agents inside the bacterial cell. Suci *et. al.*[11] demonstrated a delayed penetration of ciprofloxacin into *Pseudomonas aeruginosa* biofilm; what normally required 40 seconds for a sterile surface while biofilm containing surface required 21 minutes. Previous studies suggest that the selective pressure from the use of antimicrobial agents is a major determinant for the emergence of resistant strain[12]. Also inappropriate use of antimicrobials results in subinhibitory concentration and favour an outbreak of infection by *P. aeruginosa* resistant to most available antimicrobials.

Intervention strategies are of great economic relevance because traditional antibiotic therapy is usually not sufficient to eradicate biofilm based infections. One of the strategies may be to penetrate the antibiotics through biofilm matrix and kill the biofilm associated cells. Keeping this in mind the potentiating effect induced by antibiotic-EDTA combination was analyzed. Exposure of *P. aeruginosa* biofilm to EDTA significantly increased antibiotic inhibition zone diameter and triggered detachment of cells from biofilm. In present study combination of gentamicin and Tobramycin with EDTA increased the sensitivity of biofilm producers increased upto great extent.

Chen *et. al.*,[13] reported that EDTA (10mM) treatment results in a 49% reduction in cell count, and they presented some evidences that this was due to dispersal of biofilm bacteria. Other studies have also suggested a role for calcium in stabilizing biofilm of bacteria. This type of killing or detachment pattern has been observed at different range of pH to determine its stability. The pH values between 3 to 12 shows effective activity of antibiotic-EDTA combination while pH 8 was found to be optimum.

The use of EDTA to treat biofilm-related infections is being evaluated by several groups, with promising results. Kilinger *et. al.*[14] describe that EDTA-antibiotic toxicity was selected on the base that EDTA alone shown no significant effect against biofilm producing *Pseudomonas aeruginosa*. The treatment done using simple ointment preparation and the result of various treatments give a clear conclusion about the

significant of using EDTA in combination with antibiotics specially protein inhibitor antibiotics. The result of this study suggested that the activity of EDTA against biofilm cells is mediated by chelation of several divalent cations that are required to stabilize the biofilm matrix. This dispersal process and the increased cell permeability facilitated by EDTA may also explain the enhanced killing observed in gentamicin, tobramycin-EDTA combination.

In conclusion biofilm production play a major role in antibiotic resistance mechanism and chelating activity of EDTA significantly increased the sensitivity of antibiotics. A combination between EDTA and antimicrobial agents can successfully be used in inhibition of pathogens especially in superficial treatment and can be used in general disinfectant and sanitization purposes.

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