

Effect of Bacteriophage and Sub-inhibitory concentration of Imipenem on Biofilm Production by *Pseudomonas aeruginosa* on Endotracheal tubing - An *in-vitro* model system

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ABSTRACT

Aim: To study biofilm production by *Pseudomonas aeruginosa* on endotracheal tubing in the presence of bacteriophage and sub-inhibitory concentration of imipenem. **Methods and Results:** A total of 20 clinical isolates of *P.aeruginosa* obtained from endotracheal samples were taken for this study. Bacteriophages were obtained from sewage samples from sewage water treatment plant. Biofilm assay was performed by modified O'Toole and Kolter method wherein a piece of sterile 0.5cm² ETT was incorporated into the microtitre plate wells. Student's unpaired t test, Kruskal Wallis, ANOVA and HSD Tukey's test were used to analyse data with SPSS 17.0

software. *P* value of <0.05 was considered statistically significant. Of the 20 isolates, only four (20%) of the isolates were resistant to imipenem. In the presence of bacteriophage, the biofilm production was very less in comparison to biofilm production in the presence of sub-inhibitory concentration of imipenem or in plain broth ($p = 0.004$). **Conclusions:** Bacteriophages effectively reduced the biofilm production by *P. aeruginosa* on catheter tip. **Significance and Impact:** The present study demonstrates the effectiveness of using bacteriophage as a biofilm reducing agent. Further studies are required to prove the use of bacteriophage coated ETT to curb hospital acquired infections.

Key words: Antibacterial agents, Bacteriophages, Biofilms, Intratracheal, Intubation, *P. aeruginosa*

INTRODUCTION

The potential for contracting a microbial pathogen is highest within a hospital environment [1]. One of the most important organisms is *Pseudomonas aeruginosa* mainly because of its ability to resist antibacterial agents and disinfectants [2].

Indwelling devices like endotracheal tubes (ETT), have been associated with hospital acquired infections and are the most important risk factors for ventilator associated pneumonia (VAP) [3,4]. The remarkable ability of *P. aeruginosa* to produce biofilms in various environmental conditions makes them resistant to antibiotic treatment by providing a diffusion barrier which limits penetration of the antibiotics and therefore promotes chronic infectious disease [5,6].

The use of antiseptic-coated ETT or the uses of devices to remove the mucus from the ETT lumen have been proposed to prevent colonization of ETT by *P. aeruginosa* [7,8].

Bacteriophages have been shown to prevent biofilms by lysing single and mixed species of bacterial biofilms in experimental studies [9-11]. Bacteriophages have been used to act as antibiofilm agents in many settings mainly because of their ability to degrade extracellular polymeric substance present in the biofilm and cause bacterial lysis [12-14].

Aims and objectives

We aim to study biofilm production by *Pseudomonas aeruginosa* on endotracheal tubing in the presence of bacteriophage and sub-inhibitory concentration of imipenem.

MATERIALS AND METHODOLOGY

Collection and identification of bacterial isolates: The study was conducted in Microbiology laboratory attached to a tertiary care hospital. Clinical isolates of *P. aeruginosa* isolated during a period of six months from endotracheal secretions were taken for the study. Only those samples which yielded a significant count of > 100000 cfu/ml were considered. The isolates were identified and antibiotic susceptibility testing was done by VITEK 2 Compact (C) system (bioMerieux, North Carolina, USA). *P. aeruginosa*, ATCC 27853, a reference strain was included in this study as control. Institutional ethics committee clearance has been obtained for this study.

Determination of Sub-inhibitory concentration of antipseudomonal drug: Minimum inhibitory concentration (MIC) of antipseudomonal drug imipenem was determined by tube dilution method

as per CLSI guidelines [15]. Phosphate buffered saline (pH 6.4) was used as the solvent. Suitable minimum inhibitory range of imipenem for *Pseudomonas* spp. is 0.06-16mg/l, these dilutions were taken to determine MIC and sub MIC. The concentration of antibiotic in just the next dilution to the MIC was considered as the sub-inhibitory concentration of the antibiotic.

Preparation of bacteriophage lysate: Sewage sample from sewage water treatment plant was collected and after centrifugation at 10000 g for 10 min, 1ml of the supernatant was added to 5ml of 16 - 18 h old *P.aeruginosa* ATCC 27583 broth cultures. The mixture was incubated at 37°C for 18-24h and 1.5ml of the mixture was centrifuged to pellet out bacterial cells. Supernatant was filtered through 0.2µm membrane filter. In order to confirm the presence of bacteriophages 10 fold dilutions of the filtrate was done [1:10 to 1:100000] after which 100 µl of each dilution was added to 1ml of 18 h old *P. aeruginosa* culture. This was added to 5ml of molten soft agar [Tryptone agar]. Pour plating was done and the plates were incubated in upright position for 24h at 37°C. Then the plates were observed for clear plaques [16].

Biofilm assay on endotracheal tubings: Biofilm formation was performed in 3 different sets i.e., plain brain heart infusion (BHI) broth, broth with sub inhibitory concentration of imipenem and broth with the crude coli phage. Bacterial culture in brain heart infusion broth with turbidity corresponding to 0.5 Mac Farland (1.5×10^8 cfu/ml) was prepared.

Biofilm formation with plain broth: Sterile 0.5cm² ETT piece was dispensed into microtitre wells and 200µl of bacterial culture in BHI broth was added.

Biofilm formation in the presence of bacteriophage: Equal volumes of bacterial culture in BHI broth and bacteriophage suspension, making a total volume of 200µl was dispensed into the wells. Sterile 0.5cm² ETT piece was placed in each well.

Biofilm formation in the presence of sub-inhibitory concentration of imipenem: Sterile 0.5 cm² ETT piece was dispensed into the wells and 200µl of bacterial culture in BHI broth containing the sub-inhibitory concentration of imipenem was added.

After incubation of the plates at 37°C for 24h, the biofilm formed inside the ETT piece was subjected to scanning electron microscopy. The ETT pieces were washed with phosphate buffered saline (pH 7.4), vortexed for 2 minutes and viable cells were quantified using surface plating method.

The microtitre plates were processed as per O'Toole and Kolter method and read with Micro ELISA plate reader at 570nm. Tests were run in duplicate and a mean of the two readings were taken [17-20].

Statistical analysis: The results of the duplicate tests were tabulated and analysed using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA) and by using Students unpaired t test, Kruskal Wallis, ANOVA and Tukey HSD with P<0.05 considered statistically significant.

RESULTS:

Antibiogram pattern of the isolates:

A total of 20 strains of *P. aeruginosa* were isolated from endotracheal tube samples received in the Microbiology laboratory. Of these 18 (90%) were sensitive to cefoperazone-sulbactam and

piperacillin-tazobactam, 17(85%) to cefoperazone and ceftazidime, 16 (80%) to amikacin and aztreonam. Of the 20 isolates, 4 (20%) were resistant to imipenem and 16 (80%) were sensitive to imipenem.

Among the aminoglycosides, 16 (80%) and 15 (75%) strains were sensitive to amikacin and gentamicin respectively; among antipseudomonal penicillin, the isolates were highly sensitive 18 (90%) to piperacillin-tazobactam and 15 (75%) were sensitive to piperacillin; among carbapenems 16 (80%) *P.aeruginosa* strains were sensitive to imipenem and 17 (85%) to meropenem; among cephalosporins, the isolates were highly sensitive to cefoperazone-sulbactam 18 (90%) and 17 (85%) of strains were sensitive to cefoperazone and ceftazidime respectively; among monobactams, 16 (80%) of the clinical isolates were sensitive to aztreonam; among quinolones, 5 (25%) of the isolates exhibited resistance to ciprofloxacin (Fig. 1)

Comparison between biofilm produced by *P. aeruginosa* isolates in plain broth and in the presence of sub-inhibitory concentration of imipenem

Including the standard strain *P.aeruginosa*, ATCC 27853, 17 (85%) of the isolates showed sub-inhibitory concentration ranging from 0.02mg/l to 0.08mg/l. Ten (50%) of the *P.aeruginosa* strains showed increase and ten (50%) showed a decrease in biofilm formation in the presence of sub-inhibitory concentration of imipenem in comparison to biofilm in broth ($p = 0.812$). (Fig. 2, 4). Fig 5b shows an increase in the biofilm production in the presence of sub-inhibitory concentration of imipenem. There was not much correlation between imipenem resistant strains and biofilm production in plain broth ($p = 0.278$), with sub-inhibitory concentration of imipenem ($p = 0.891$) or phage ($p = 0.829$). The surface plating of washed ET tubings which were subjected to biofilm formation in plain broth and in the presence of sub-inhibitory concentration of imipenem showed $>10^5$ CFU/ml colonies. The electron micrograph of the endotracheal tubings showed similar results (Fig. 5 a, 5 b).

Effect of phage on biofilm formation: On treating the *P.aeruginosa* clinical isolates with the bacteriophage, there was a sharp decrease in the production of biofilm, 16 (80%) of the strains produced less amount of biofilm than the plain broth (Fig. 3, 4). ($p = 0.005$) In comparison to biofilm in plain broth or with sub-inhibitory concentration of imipenem, biofilm formation in the presence of crude coli phage was much less ($p = 0.004$). The imipenem resistant strains were 100% sensitive to the phage treatment with marked decrease in the production of biofilm. All the four imipenem resistant strains showed reduction in biofilm production in the presence of phage, in comparison to biofilm in plain broth. The surface plating of washed ET tubings subjected to biofilm formation in plain broth showed $>10^5$ CFU/ml colonies. The surface plating of washed ET tubings subjected to biofilm formation in the presence of crude coli-phage showed 1×10^2 CFU/ml colonies. The electron micrograph of the endotracheal tubing showed similar results (Fig. 5 a, 5 c)

DISCUSSION

P.aeruginosa causes opportunistic bacterial health care associated infections which include pneumonia, cystic fibrosis, urinary tract infection, blood stream and surgical site infections [1,2]. *P.aeruginosa* effectively grows in moist conditions and is commonly found in various places

of hospital environment because of its ability to resist antibacterial agents and disinfectants. The prevalence of hospital acquired infection depends on the factors like prolonged treatment in hospital, the immune status of the patient, invasive procedures used and the resistance of microorganism to antibiotics [1,2].

P.aeruginosa has a marked ability to induce biofilm based infection. Biofilm produced are less susceptible to host immune and inflammatory responses [22,23]. Biofilm production is mainly associated with the antibiotic resistance due to the difficulty of antibiotics to penetrate into the cells which secrete the polymeric matrix which contains polysaccharide, proteins and DNA [24].

Indwelling catheters have been identified as risk factors for hospital acquired infections. Endotracheal tubes (ETT), a device inserted into the patient's trachea through the mouth or nose to maintain an open airway are the most important risk factor for causing ventilator associated pneumonia (VAP) by impairing mucociliary clearance, disrupting the cough reflex, and promoting the accumulation of tracheobronchial secretions in the lungs [3,4]. The inner side of the endotracheal tube can be a reservoir for microorganisms and provide them a surface to adhere and produce *biofilm* [3,4].

There are many studies in the past where herbs, chemicals like silver sulfadiazine, specific bacteriophages and cocktail bacteriophages were used to prevent bacterial biofilm formation [25-31]

Certain researchers studied the Chinese traditional herb which inhibited biofilm formation of *P.aeruginosa*. The extract of *Herba patriniae* showed significant inhibitory effect on most biofilm-associated genes. *H.patriniae* extract decreased the exopolysaccharide produced by *P.aeruginosa*. This extract helped in promoting inventions of new drugs against biofilm-associated infections [25].

A study attempted to prevent the colonization of bacteria using several antibacterial coated endotracheal tubing (ETT) which was tested in an in-vitro as well as animal studies. Silver sulfadiazine (SSD) in polyurethane was the antibacterial coating used against the growth of *P.aeruginosa*. In both studies, the SSD-ETT remained bacteria-free compared to Standard-ETT [26]. In a study, *P.aeruginosa* phage M4 was used for the prevention of biofilm formation by *P.aeruginosa*. The hydrogel-coated catheters coated with Pseudomonas phage M4 were inoculated with *P.aeruginosa* cultures. It was found that the mean viable biofilm count on untreated catheters was more than the catheters which were treated with the bacteriophage. The regrowth of biofilms on phage treated catheters occurred but re-treatment with the phage significantly reduced the biofilm growth [27].

In a study, an engineered bacteriophage was used to express a biofilm degrading enzyme during the infection to concomitantly attack the bacterial biofilm and the biofilm matrix. This study showed that the efficacy of enzymatic bacteriophage was greater than that of the non-enzymatic bacteriophage [28].

The interaction of bacteriophage with bacterial biofilms has the beneficial effect in the prevention of biofilms [9]. Phages are capable of lysing host bacteria in biofilms produced by using single and

mixed species of bacteria in experimental studies [9,10]. Bacteriophages have been used as antibiofilm agents in industrial and clinical settings including phage therapy, biofilm infected medical devices, filtration membranes [12,13]. The strategy adopted by phages during the phage-bacterium interaction is the production of polysaccharide depolymerases to degrade extracellular polymeric substance present in the biofilm and allow phages to access encased bacterial cells and cause their lysis [14].

In an in vitro study, electrochemical scaffoldings were used to generate a constant concentration of hydrogen peroxide in order to enhance the tobramycin susceptibility of preformed biofilms produced by the *P.aeruginosa* PA01 [29].

A lytic P2 bacteriophage isolated from Ganges river water was used to study the impact of these bacteriophage on different stages of biofilm (initial biofilm formation, establishment of matrix and maturation of biofilm) produced by *P.aeruginosa*. This study showed that the P2 lytic bacteriophage has ability to kill cells at the initial stage of biofilm formation [30].

In a study, the cocktail bacteriophages were used against the biofilm produced by different strains of *P.aeruginosa* which showed a drastic decrease in the biofilm followed by rise in the bacteriophage titer. The study indicates that the successful phage infection and replication and the good complementation between the four phages constituting the cocktail CT-PA [31].

In the present study, on treating the *P.aeruginosa* clinical isolates with the bacteriophage, there was a sharp decrease in the production of biofilm in endotracheal tubes, 80% of the strains produced less amount of biofilm than the plain broth without phage. In comparison to biofilm in plain broth or with sub-inhibitory concentration of imipenem, biofilm formation in the presence of crude coli phage was much less in the endotracheal tubings ($p = 0.004$). The imipenem resistant strains were 100% sensitive to the phage treatment with marked decrease in the production of biofilm. The electron micrograph of the endotracheal tubings also showed similar results.

Till date the use of phage coated endotracheal tubing has found success in reduction of pseudomonas biofilm only in vitro [32]. We did not find any clinical trials on phage coated endotracheal tubing being used on patients. Unfamiliarity with such treatment strategies, presence of bacterial toxin producing gene in phages, narrow specific host range of phages may be some disadvantages of phage therapy [33]. Probably this concept may come into use in future.

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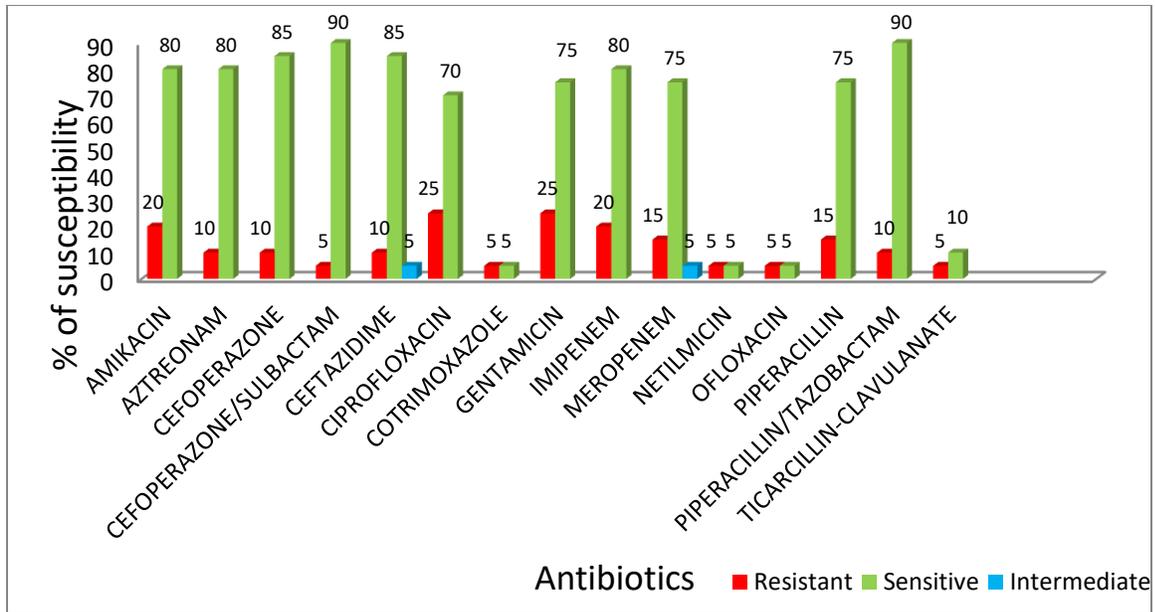


Figure 1 : The antibiotic susceptibility pattern of *P.aeruginosa* to various antibiotics

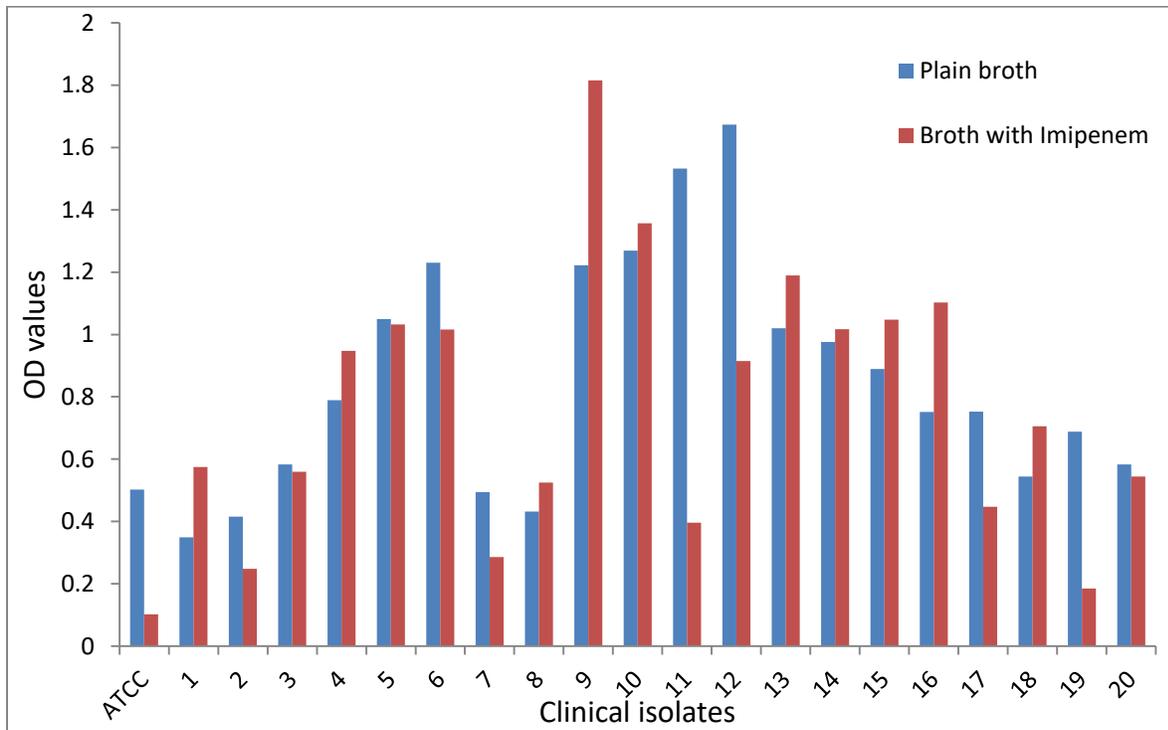


Figure 2: Comparison between OD₅₇₀ of biofilm produced by *P.aeruginosa* isolates in plain broth and in the presence of sub-inhibitory concentration of imipenem

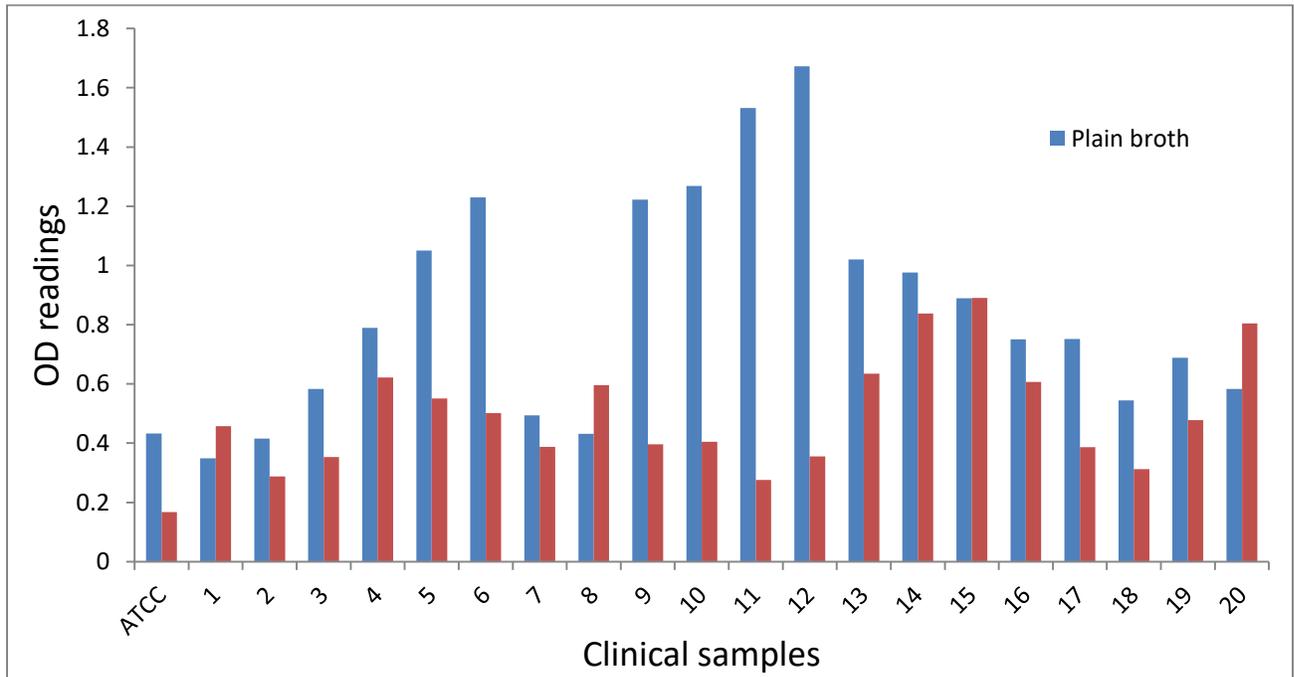


Figure 3: Comparison between OD₅₇₀ of biofilm produced by *P.aeruginosa* isolates in plain broth and in the presence of bacteriophage

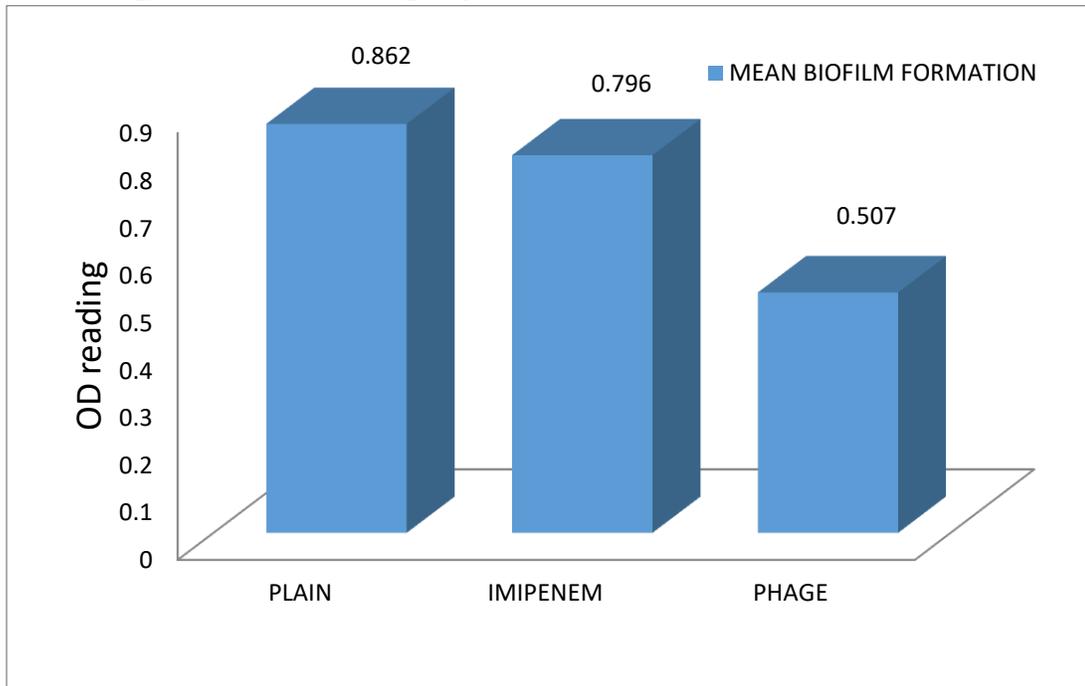


Figure 4: Comparison of biofilm produced by *P.aeruginosa* isolates in different conditions

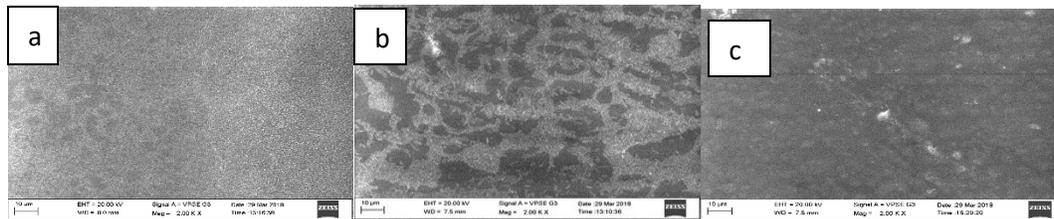


Figure 5: Scanning electron microscopic image of *P. aeruginosa* biofilm on endotracheal tubing pieces [a] Biofilm produced in plain broth; [b] Biofilm produced in the presence of sub-inhibitory concentration of imipenem; [c] Biofilm produced in the presence of bacteriophages