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Research article

Liquid infused silicon based urinary catheters: A simple laboratory model to study bacterial biofilm formation

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ABSTRACT

Biofilms are organized communities of micro-organisms buried firmly on an extra polymeric matrix attached to the living surface. The matrix which is formed surrounding the bacteria makes them tolerant to harsh environmental conditions as well as resistant to antibacterial treatment. The development of biofilms in clinical or hospital settings is the major reason for several infections. They have a negative impact when they are formed on medical devices as it is difficult to eradicate them using disinfectants or antibiotics available. Several *in vitro* models have been implemented in the last decade to evaluate antimicrobial activity on bacterial biofilms. Although well-established static biofilm methods are available; there is a lack of simple dynamic biofilm models to replicate the clinical settings of biofilm development. In this scenario, considering the importance of this research area, we planned to design laboratory-scale working models to study biofilms. We used *Pseudomonas aeruginosa* as a model organism. We divided the biofilm models into two categories namely static and dynamic models. The dynamic model of biofilm, that we used for mimicking the clinical setting, was a silicone-based catheter model. This new model will contribute to deeper knowledge about the physiology, structure, formation, and composition of biofilms. This method can be used as an appropriate and up-to-date technique to study biofilms in laboratory settings.

Keywords: Biofilms, Antibiotic resistance, Urinary tract infection, Nosocomial infection

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INTRODUCTION

Biofilm infections are estimated to be responsible for up to 80% of all infections in humans and animals, posing a major health challenge [1]. Planktonic bacteria are individual micro-organisms, whereas biofilms are well-connected organizations of millions of such microorganisms. Bacterial biofilms are highly resistant to antibiotics when they are formed inside the matrix of a biofilm [2,3]. The National Institutes of Health (NIH) reported that about 65% of microbial infections are associated with Biofilm formation [4].

Pseudomonas aeruginosa is a member of genus Pseudomonas, called *Pseudomonads*. It is a gram-negative facultative anaerobe, non-spore-forming rod-shaped, motile, oxidase-positive, and lactose non-fermenters, notorious opportunistic bacteria that is found along with Pseudomonas species as normal flora in gut and skin [5]. Infections caused by *P. aeruginosa* are difficult to treat because of the resistance they exhibit against a wide range of antibiotics, especially, β-lactams, penem group of antibiotics, aminoglycosides, and fluoroquinolones. Horizontal gene transfer, quorum sensing, and enzyme promiscuity are techniques employed by microorganisms to thrive in biofilms, and this genomic plasticity aids survival under harsh environments [6]. *P.aeruginosa* has a strong ability to form biofilms in clinical equipment and medical devices [7].

The catheters routinely employed in clinical practice are among the medical devices that are susceptible to microbial contamination. Pathogens that are present at the site of catheter insertion attach easily to the surface and colonize to establish a biofilm. The bacterial cells encased in the biofilm are a thousand times more resistant to conventional antibiotic treatment when compared with planktonic cells[8] and hence bacterial biofilms have to be inhibited [9]. At the research level, the development of biofilm working models will help to mimic real environmental conditions [10]. Considering the importance of this research area, we planned to design a simple, economical, and reliable laboratory-scale working model to study biofilms. We used silicon tube catheters for biofilm formation and testing. We also tested the ability of selected antibiotics to prevent the

formation of biofilm.

MATERIALS AND METHODS

Materials and reagents

Medical/clinical-grade silicone tube urinary catheters were used in this study. Biofilm-forming bacterium *Pseudomonas aeruginosa* (ATCC 15442) was used as the test organism. Dehydrated media like Nutrient broth, Tryptic soy broth (TSB), Glucose, Brain heart infusion broth, Blood base agar, and Mueller Hinton Agar were procured from HI media, India. Crystal violet (0.1%), Congo red (0.8 g/L), and Acridine orange (1%) dyes, all chemical consumables used were research grade procured from Sigma Aldrich.

Experiments to confirm the formation of biofilms, quantification, and structural assessment

Tube method

The tube method is a qualitative test for the detection of biofilm-producing micro-organisms which form a visible lining surrounding the walls of the tubes [11]. 10mL of Tryptic soy broth (TSB) was served with 1% glucose and the test organism was inoculated and incubated at 37°C for 24 hours and the tubes were decanted and washed with phosphate buffer saline solution (pH7.2) and stained with 1% Crystal violet for 15-20 minutes and the excess stain was removed with deionized water and dried. The test was carried out in triplicate. T1-single loop of culture, T2-double loop of culture, T3-triple loop of culture C-control-without any organism. The tubes which showed visible lining around the tubes were recorded [12].

Congo red agar test (CRT)

It is a qualitative test to detect biofilm-forming organisms, which produce a color change in the colonies when they are inoculated on the Congo red agar (CRA) medium [13]. It is a chromatic evaluation for the detection of biofilms as black colonies are formed by biofilm-producing organisms and non-biofilm produces reddish pink colored colonies on the CRA plates. The plates were poured with the composed media allowed to solidify and then streaked with concerned organisms to check the biofilm-producing organism.

Modified Congo red agar test (MCRT)

We used a modification of the CRA method where the composition of sucrose, glucose, sucrose, and NaCl with blood base agar is used. The medium was autoclaved and poured into dry plates and allowed to solidify after which the test organism was streaked and incubated at 37° C for 24 h for two days. The plates which showed black colonies with dry crystalline consistency are slime-producer and the colonies which were pinkish red are non-slime producers [14].

Biofilm formation using 96 well plate method

This is a quantitative method that is used for the quantitative method for detection of biofilm where the polystyrene plate was inoculated with the bacterial suspension present in Tryptic soy broth and incubated for 48h at 37°C [15]. After incubation, the biofilm formation takes place as the wells around the walls and the plates were decanted and gently washed with Phosphate buffer saline and after which the fixation is done using sodium acetate. The plate was stained with 0.1% crystal violet and allowed to stand for 15-20 minutes at room temperature, the excess stain was washed with deionized water. The plates were dried and again resolubilized with methanol/glacial acetic acid and read spectrophotometrically using an ELISA microplate reader at 570 nm. The TSB with 1% glucose serves as blank which is used to identify whether biofilm is formed or not. The OD value greater than blank is determined as a biofilm producer [12]. Plate 1: The culture was loaded as 100µl, 200µl, and 300µl of test organisms and Control with no organism & incubated at 37°C; Plate 2: The broths containing organisms with different concentrations of antibiotics such as 100µg,200µg,300µg,400µg and the broth containing only the organism without adding any antibiotic was loaded and incubated. When (i) the mean OD value is <0.120, then, no adherence and weak biofilm formation; (ii) the mean OD value is 0.120-0.240, then, moderate adherence and biofilm formation; (iii) the mean OD value is >0.240, then, strong adherence and high biofilm formation.

Antibiotic susceptibility testing

Antibiotic sensitivity testing or Antibiotic susceptibility testing is the measurement of the susceptibility of a bacterium to a particular antibiotic. This is used because bacteria can have resistance to some antibiotics. If the antibiotics inhibit microbial growth, it produces clear ringed zones around them. Antibiotic discs are used to check the extent of bacteria that gets affected by the antibiotic. After measuring the zone diameter, it is compared with the database of the zone of the standard to determine whether the bacterium is Resistant (R), Susceptible(S), or Intermediate (I). The measurement is compared to the criteria set by the Clinical and Laboratory Standards Institute (CLSI)**.** The medium used for this test is Mueller Hinton agar where the test organism is swabbed uniformly at 90° (lawn culture) and the disc impregnated with antibacterial substances is placed on the medium aseptically using forceps and pressed slightly and the plates were incubated at 37°C for 24 hours. The organism which is sensitive to the particular antibiotics produces a clear zone around them (zone of inhibition) and if the organism is resistant no zones are formed around the disc.

Fluorescence assay

This method is used to quantitatively observe the morphology of microorganisms adhered to the surfaces and semi-quantitatively estimate the number of micro-organisms attached to the surface. The organism is loaded into 6 well plates placing the

coverslip at the bottom of the plate and was incubated for 48h at 37°C. After incubation, the plates were decanted and washed with Phosphate buffer saline and after drying it was stained with 1% Acridine orange and allowed to stand for 20 minutes at room temperature. The plates were washed and the excess stain was removed using deionized water. The organism adhered to the surface is observed, the observation requires a clear, transparent, and planar surface on which the microorganisms attach and does not create a 3D vision of biofilm. Dyes can be used such as epifluorescence and fluorescent to enhance the image quality of microorganisms. This helps in the comparison of sessile and planktonic forms of microorganisms [12]. The coverslips which were placed inside the plates were viewed under a Fluorescence microscope. For observation of biofilms using a Fluorescence Microscope, biofilms should be fluorescent as a result of the green fluorescent molecule, of protein which is expressed by the organism's forming biofilms or either by staining these biofilms producing organism using fluorescent-labeled dye. Here 1% Acridine orange is used for staining and was visualized under a fluorescence microscope. Acridine orange staining helps in the direct investigation of biofilm produced on catheters by microscopy.

Silicon tube catheters-based model for biofilm formation

Medical-grade silicon-based catheter tubes were used for this study. In 20ml of TSB, 10 mg of Gentamicin was added after which it was inoculated with 1 loopful (1.46×10^{-5} cells/ml) of *Pseudomonas aeruginosa* culture loaded in one catheter. In another tube TSB with a loopful of culture without adding any antibiotic was loaded into the catheter.

Testing of the sample after catheter treatment using spectrophotometer analysis

The culture of *P. aeruginosa* which was inoculated overnight was loaded into the tubes and incubated for a week. After aspirating all the contents of the tube without leaving any droplets hanging on the inner walls of the tubes were gently washed with sterile water. Then they were loaded with 0.1% of CV stain and the tubes were thoroughly rotated and left to stand for about 15-20 minutes. The stain was aspirated and the tube was washed again with sterile water to remove the excess stain. Then 0.5ml of ethanol was added to the tube after which the tubes were swirled around for proper decolorization and elution of the tube was done using ethanol. The intensity of the color of the eluent was then measured using Spectrophotometer at 550 nm and the OD value was recorded. The sample showing the OD value of the tubes more than the control is considered strongly positive more adherent to the surface and slime-producing.

Testing of the sample after catheter treatment using the tube method

The samples which were aspirated from the tubes were carried out for tube method detection. A loopful of samples from the tubes containing the antibiotic with and without organism is inoculated and incubated for 24 h at 37°C. After which the growth was observed and tubes were washed with PBS buffer and allowed to dry at room temperature. The tubes were checked for intensity formed by the biofilm.

Testing of the sample after catheter treatment using the CRT method

The samples which are aspirated from the tubes were carried out for the CRT method. A loopful of samples from the tubes containing the antibiotic with and without organism is inoculated into freshly prepared Congo red agar medium (Continuous and V-quadrant streaking). And the inoculated plates were incubated for 24 h at 37°C. After this, the growth of the plates was observed for slime producers and non-slime producers. If the organism showed black dry crystalline colonies, they are biofilm producers. If the organism does not show any growth on the freshly prepared agar plates after inoculation by streaking, they are the non-biofilm producer.

Testing of the sample after catheter treatment using the MCRT method

The samples which are aspirated from the tubes were carried out for the MCRT method. A loopful of samples from the tubes containing the antibiotic with organism and antibiotic without organism is inoculated into freshly prepared modified Congo red agar medium (Continuous and V-quadrant streaking), And the inoculated plates were incubated for 37°C and at room temperature for 2 subsequent days. After this, the growth on the plates was observed for slime producers and non-slime producers. If the organism showed black dry crystalline colonies, they are biofilm producers. If the organism does not show any growth on the freshly prepared agar plates after inoculation by streaking, they are non-biofilm producers.

Testing of the samples loaded into the catheter tubes daily using CRA and MCRA method

The samples were aspirated on daily basis from the catheter tubes containing antibiotics with and without organism and then inoculated into CRA plates and MCRA plates (V-quadrant streaking) and incubated at 37°C for 24 hours and 37°C for 24 h followed by subsequent incubation for 2 days respectively. The growth of the organism with antibiotic was checked for black dry crystalline colonies in the CRA and MCRA plate. This was done to check the effectiveness of the antibiotic against the organism.

Docking antibiotics against LecA and LecB proteins

The X-ray crystallographic structure of the *P. aeruginosa* lectins LecA (Protein Data Bank accession no. 1L7L) and LecB (Protein Data Bank accession no. 1GZT) was used for docking the antibiotics that the biofilm-forming *P. aeruginosa* cells were susceptible to, using Auto dock Tools (ADT) 1.5.6 [16]. The computation of the grid was performed using Auto grid 4.2 programs in ADT. The ligand and target were blindly docked. The Lamarckian

Genetic Algorithm with 10 independent runs per ligand with an initial population of 150 randomly positioned ligands on the receptor binding site was used to perform the docking [17].

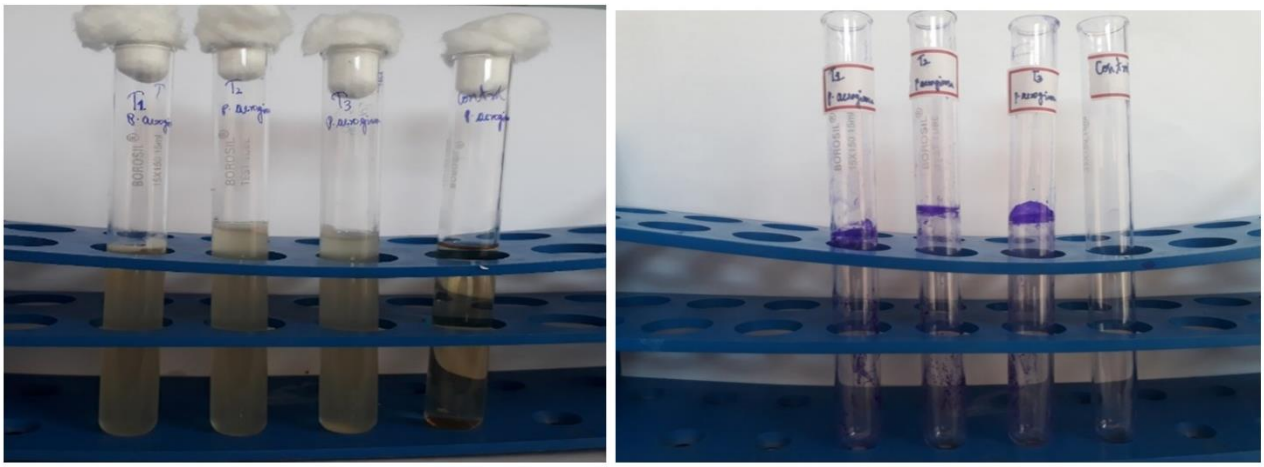
RESULTS AND DISCUSSION

The tube method, CRT, MCRT, 96 well plate method, and fluorescence assay were performed in the static model to confirm the biofilm formation and compare it with our proposed dynamic model.

Tube method

The tube method is a qualitative assay used for the detection of biofilm-producing organisms because of the occurrence of visible biofilm. The crystal violet used here stains the biofilm formed by the organism so that the results can be easily visualized. The third polystyrene test tubes indicate biofilm formation as compared with the first two tubes (Figure 1).

Figure 1: (A) Test tubes showing growth of the organism after incubation; and (B) Test tubes stained with Crystal violet to check the intensity of biofilm formation. The tubes from the left indicate high biofilm production rather while the first two test tubes indicate a lack of biofilm production.

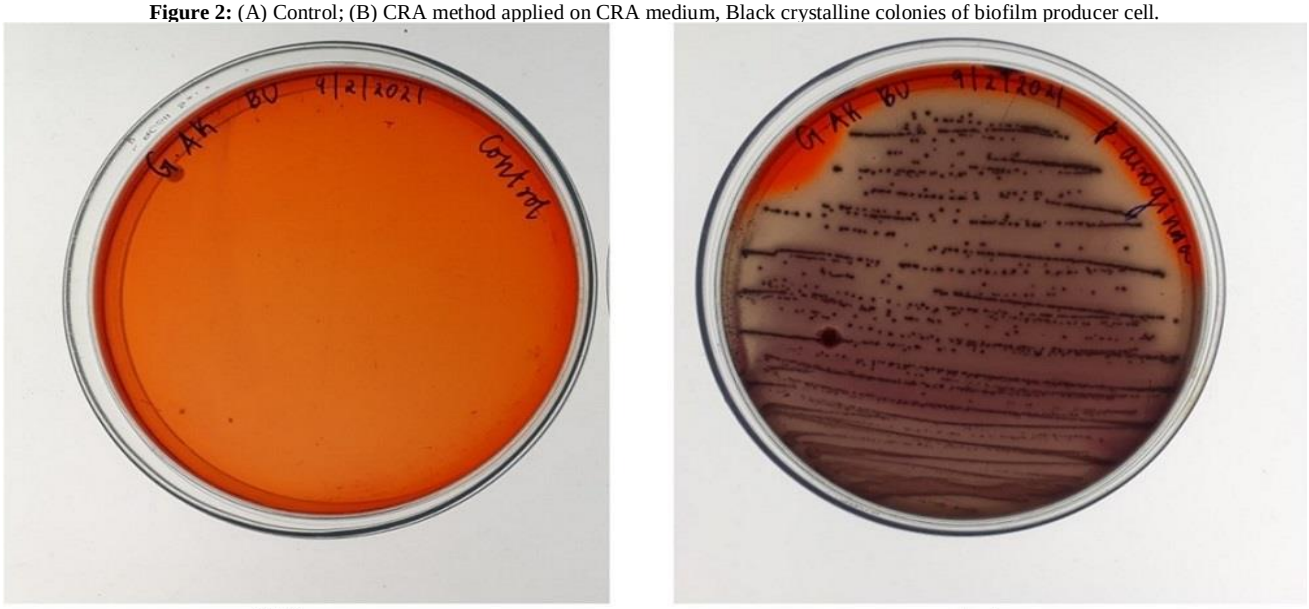


(A) (B)

Congo red agar test (CRT)

The CRA plate test uses a solid medium namely Congo red agar. This method allows for direct analysis of the colonies and the identification of slime-forming strains (which appear as black colonies on the Congo red agar) and non-slime-forming strains (red-colored colonies). Black colonies denote that the organism can form biofilms (Figure 2).

Figure 2: (A) Control; (B) CRA method applied on CRA medium, Black crystalline colonies of biofilm producer cell.

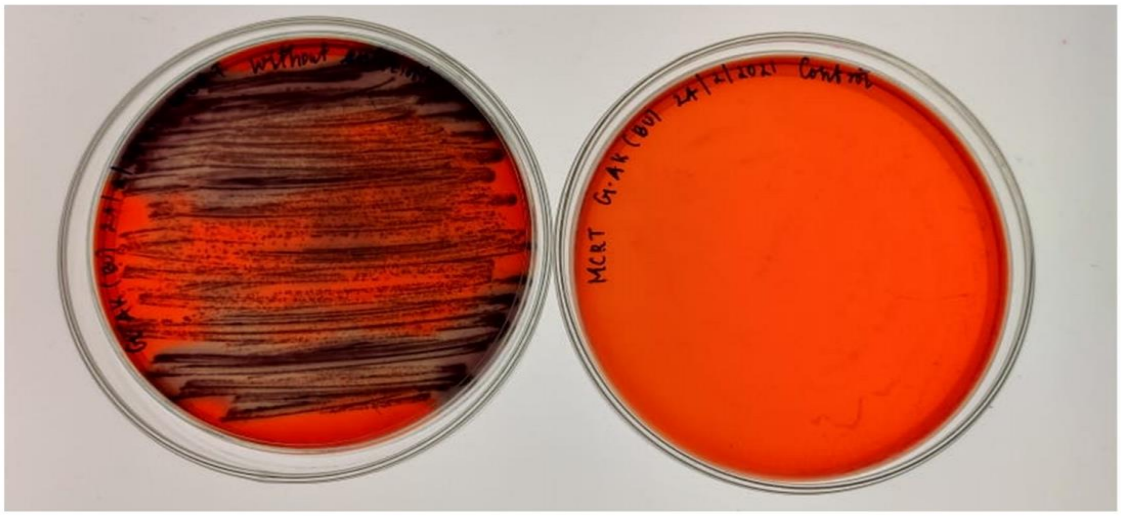


(A) (B)

Modified Congo red agar test (MCRT)

Modified Congo red agar test a modification of CRA Plates is done. The MCRT plate test uses a solid medium namely Blood base agar, sucrose, glucose, agar, and NaCl is used. This method allows for direct analysis of the colonies and identification of slime-forming organisms (which appear as dry crystalline black colonies on Modified Congo red agar) and non-slime-forming strains that form red-colored colonies. Dry crystalline Black colonies denote that the organism can form biofilms. The non-dry crystalline black colonies denote that the organism is non-biofilm former (Figure 3).

Figure 3: (A) MCRA method applied on MCRA medium. Black crystalline colonies of biofilm producer cell; (B) Control.



96 well plate method:

The 96-well Microtiter plate is a quantitative analysis of biofilm formation. After incubation, for 2 days at 37°C the plates were decanted, washed with Phosphate buffer saline, and stained for 15-20 minutes using 1% crystal violet, decolorized using acetic acid, and read spectrophotometrically at 570 nm (Figure 4). The individual will picture taken by microscope at 10X magnification is shown in Figure 5.

Figure 4: 96 well Microtiter plate is loaded with an organism with different concentrations of antibiotics. The untreated wells showed strong biofilm formation. Whereas the well-loaded with a high concentration of antibiotics showed mild biofilm producer.

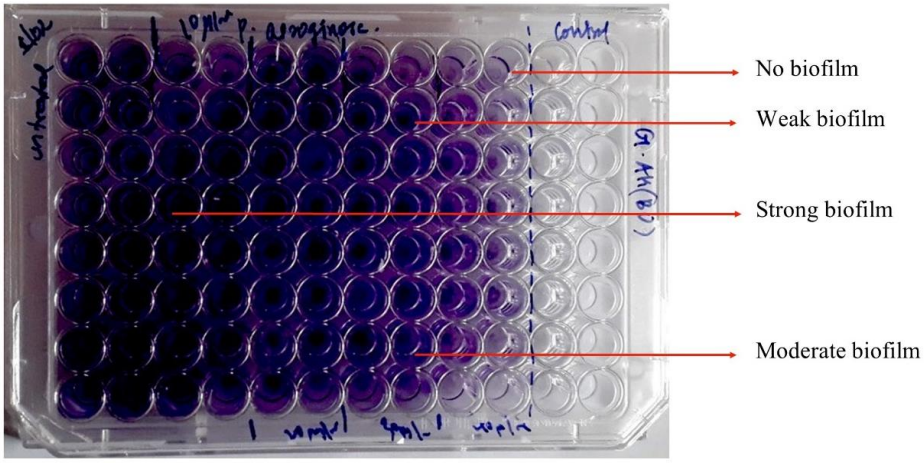
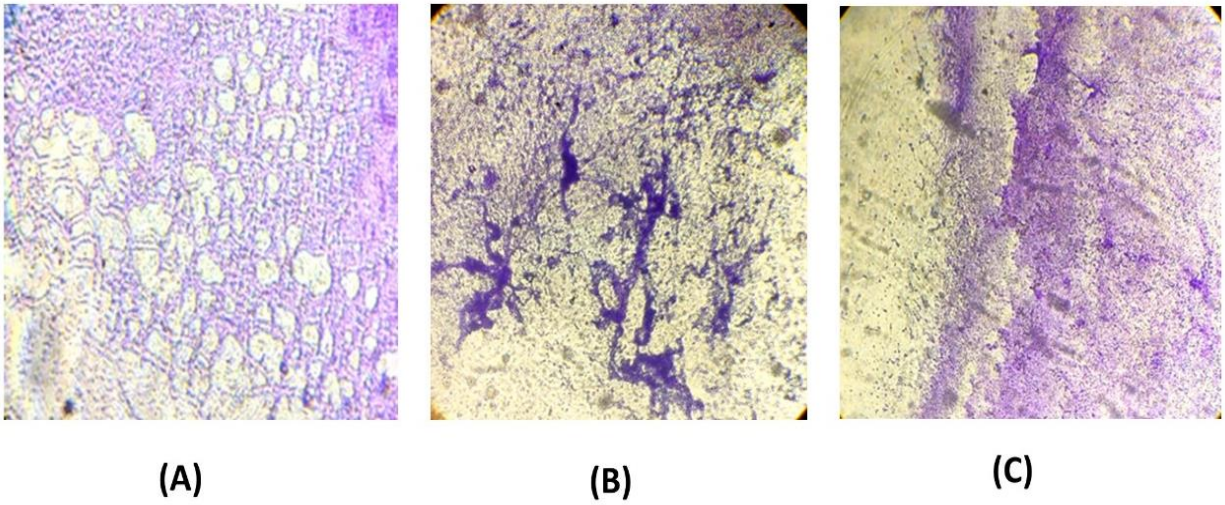


Figure 5: Individual good picture was taken by microscope at 10X magnification. (A) Strong biofilm producer(untreated); (B) Mild biofilm producer; (C) Weak biofilm producer (treated).



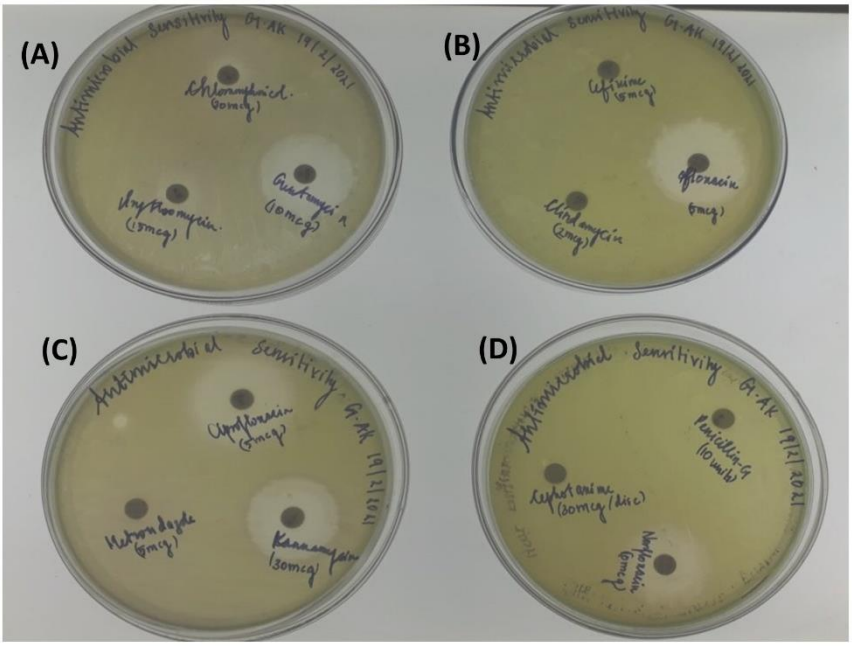
Antibiotic susceptibility testing

Antibiotic susceptibility testing was used to understand the resistance and sensitivity of *P. aeruginosa* (1.46×10^{-5} cells/ml) to different antibiotics. The organism was found to be more sensitive to the antibiotics like gentamicin with a zone of inhibition of 37mm, ciprofloxacin with a zone of inhibition of 35mm, and ofloxacin with a zone of inhibition of 34mm, as shown in Table 1, and Figure 6. Due to the variation of the drug targets, i.e., ciprofloxacin and ofloxacin act on DNA gyrase whereas gentamicin acts on the protein synthesis of the cell wall. Hence, ciprofloxacin and ofloxacin were used with the dose of 5 mcg separately and with gentamicin of dose 10 mcg.

Table 1: Measurement of zones produced by the organism (zone of inhibition)

Antibiotics (µg/disk)	Measurement of zone
GEN (10mcg)	37(S)
K(30mcg)	34(S)
CIP(5mcg)	35(S)
OF(5mcg)	34(S)
NX (10mcg)	22(S)
Chloramphenicol	14(I)
Erythromycin	15(I)

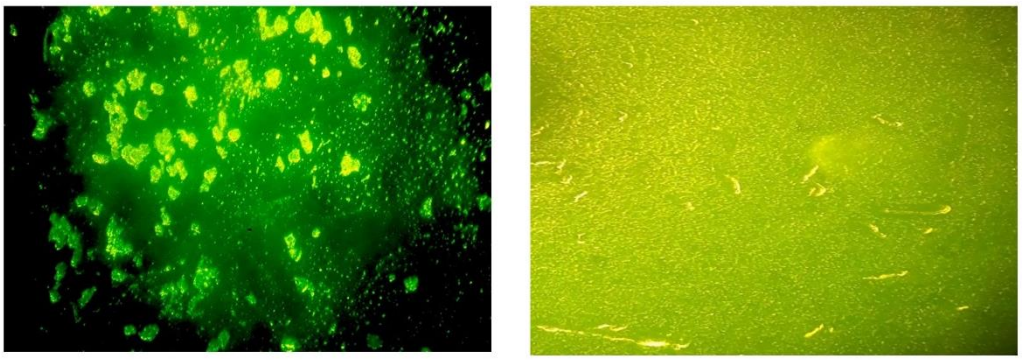
Figure 6: Antimicrobial Susceptibility Testing, is used to determine the **"**zone of inhibition" correlates with the resistance and sensitivity of the antibiotics used against the organism.



Fluorescence assay

Acridine orange dye aided in differentiating sessile and planktonic bacteria. We used fluorescence assay to observe the biofilm formed by *P. aeruginosa* as shown in Figure 7.

Figure 7: Fluorescence Microscopy of biofilms formed by *Pseudomonas aeruginosa* (A) Biofilm formation; (B) Control.



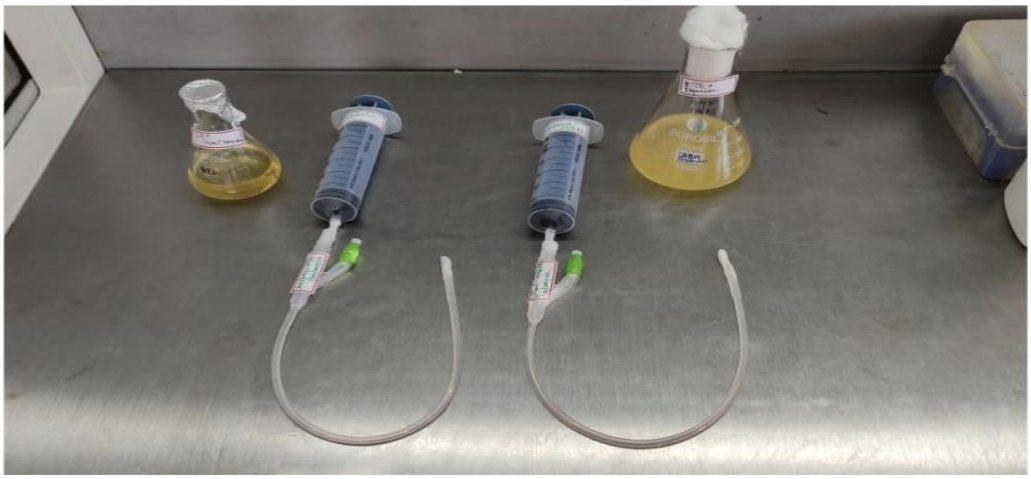
Dynamic Model

Testing of the sample after catheter treatment using the tube method

After incubating the catheters for 7-8 days the samples from the catheter were collected and tested (Figure 8 and Figure 9). Tubes containing samples withdrawn from the catheter after 8 days of incubation with antibiotic and without antibiotics were tested using a loopful of sample aspirated from the tube inoculated in the TSB medium and incubated for 24 h at 37°C and then stained with 1% crystal violet. The tubes inoculated with the sample containing only organisms showed visible lining along the walls of the tubes and at the bottom, whereas the tubes inoculated with a sample containing

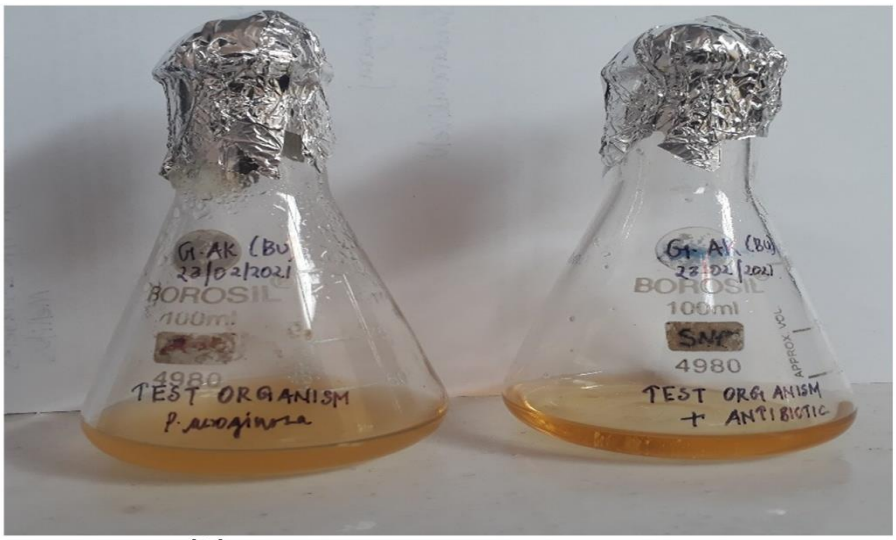
the test organism with the antibiotic did not show any visible lining along the walls or bottom of the tubes as shown in Figure 10.

Figure 8: (A) Culture with organism; and (B) Culture with organism and antibiotics were loaded into the catheters.



(A) (B)

Figure 9: Flasks containing (A) test organism; and (B) test organism and antibiotic after incubation for up to 8-10 days.



(A) (B)

Figure 10: (A) Staining of the catheter containing antibiotic and without antibiotic with 0.1% crystal violet for 15-20 minutes; (B) The catheter after staining and ethanol wash to read spectrophotometrically.

(A) (B)

Testing of the sample after catheter treatment using spectrophotometer analysis

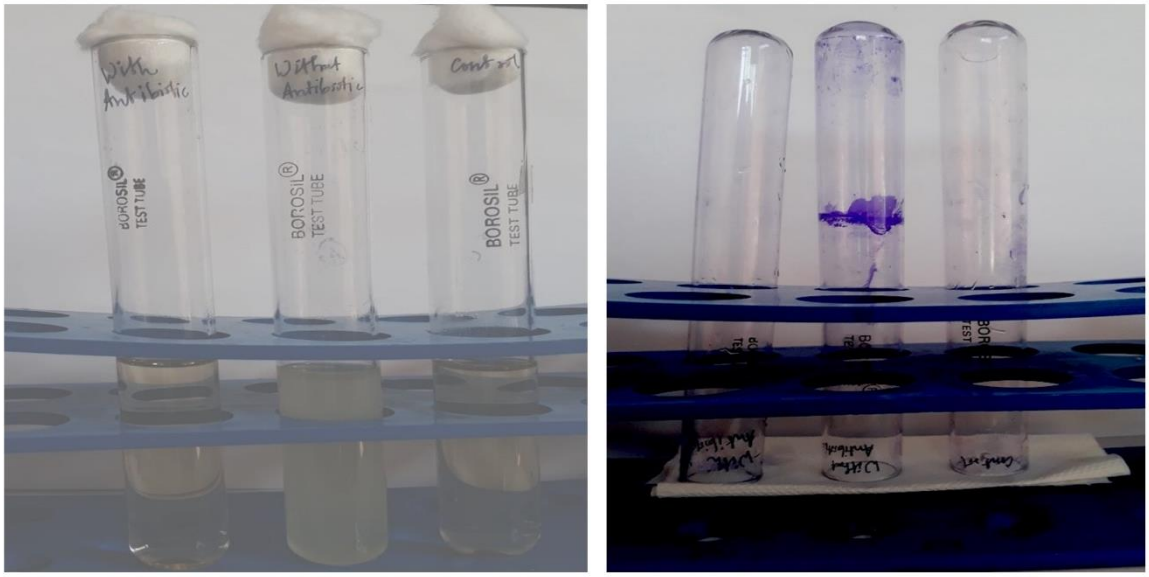
The sample aspirated from the tube was stained with 1% crystal violet and allowed to stand for 15-20 minutes and was washed with

0.5 ml of 70% ethanol and swirled around for decolorization. The intensity of the color of the eluent was measured using a spectrophotometer at 570 nm. Table 2 shows the obtained OD values and since the OD values of the samples are more than that of the control, the adherence of the biofilm is strong (Figure 11).

Table 2: OD values for the samples aspirated from the catheters spectrophotometrically

SAMPLES	OD VALUE (Gentamicin)	OD VALUE (Ciprofloxacin)	OD VALUE (Ofloxacin)
TSB(CONTROL)	0.200A	0.200A	0.200A
With Antibiotic	0.785A	0.747A	0.679A
Without Antibiotic	1.409A	1.409A	1.409A

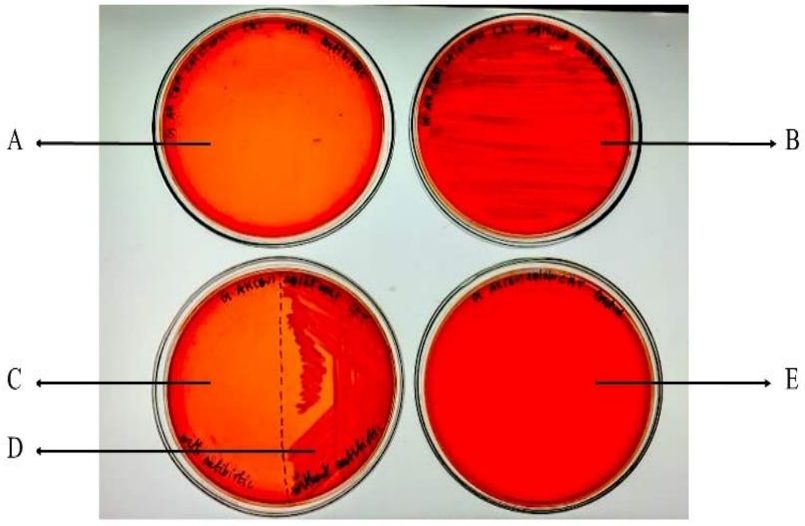
Figure 11: (A) Tubes showing growth after incubation; (B) Test tubes stained with Crystal violet to check the intensity of biofilm formation. The tubes inoculated with organism and antibiotic did not show any growth and no visible lining was formed on the walls of the tubes, whereas the tubes inoculated with organism showed growth and on staining it formed a visible lining around the walls of the tubes



Testing of the sample after catheter treatment using the CRT method

The aspirated sample from tube 1 (with antibiotic) and tube 2 (without antibiotic) was inoculated by streaking on the freshly prepared congo red agar plate and incubated. The samples from tube 1 showed no colony growth on the agar plate. The samples from tube 2 produced black-colored colonies, which is an indication of biofilm formation, as shown in Figure 12.

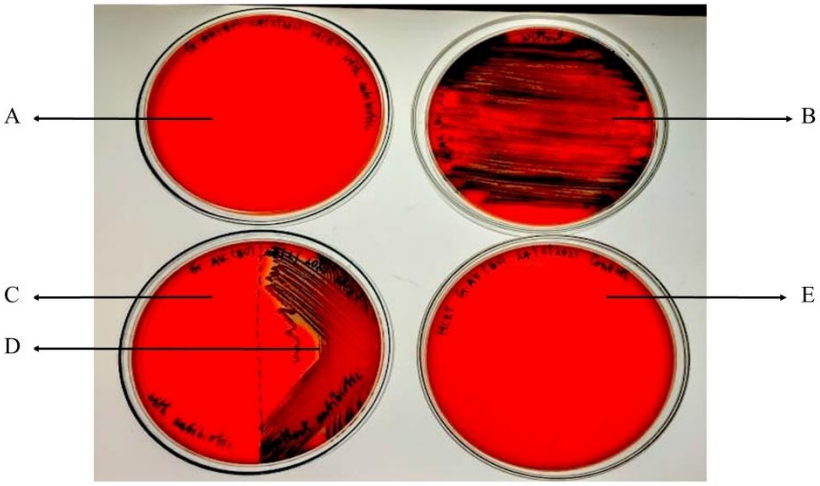
Figure 12: Congo red agar test (A) with antibiotic (no growth); (B) without antibiotic (black crystalline colonies); (C), (D) with and without antibiotic respectively; (E) Control.



Testing of the sample after catheter treatment using the MCRT method

The aspirated sample from tube 1 (with antibiotic) and tube 2 (without antibiotic) was inoculated by streaking on the freshly prepared modified congo red agar plate and incubated. The samples from tube 1 showed no colony growth on the agar plate. The samples from tube 2 produced black-colored crystalline colonies, which is an indication of biofilm formation, as shown in Figure 13.

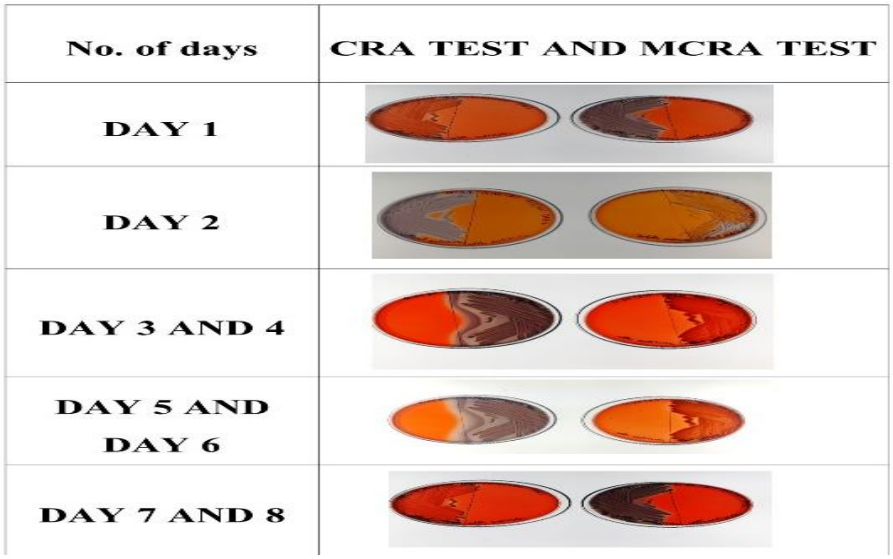
Figure 13: Modified Congo red agar test (A) with antibiotic (no growth); (B) without antibiotic (dry black crystalline colonies); (C), (D) with and without antibiotic respectively; (E) Control.



Testing of the samples loaded into the catheter tubes daily using CRA and MCRA method

The samples tested using CRA and MCRA method has been tabulated in Figure 14. No growth was found for more than 8 days of catheter incubation.

Figure 14: CRA and MCRA plates from the samples aspirated from the catheter tube daily. Results - The dosage or concentration of antibiotics may vary to treat the samples which are obtained from the hospitals or clinics directly.



Docking antibiotics against LecA and LecB proteins

From the above results, gentamicin and ciprofloxacin showed as a potential candidates but due to the acquired resistance of ciprofloxacin by P. aeruginosa from other organisms is increasing currently, the next potential candidate is gentamicin which has a low resistance profile currently and has good docking score with lectin proteins. To explore the possibility that the susceptible antibiotics may directly interact with the lectins, we performed in silico docking of the five antibiotics with the LecA and LecB proteins. The binding energies of the antibiotics with LecA and LecB are tabulated in Table 3. This suggests that the four antibiotics do not directly interact with the lectin proteins, but gentamicin directly interacts, as the mechanism of it is to disintegrate protein synthesis and cell membrane. We understand that gentamicin can be effective in preventing the formation of biofilm but not able to act against the developed biofilm.

Table 3: Binding energies (in kcal/mol) of the antibiotics docked to the LecA and LecB proteins in silico

Antibiotics	Binding energy (Kcal/mol)	
	LecA	LecB
Gentamicin	-2.94	441.75
Kanamycin	-0.64	356.05
Ciprofloxacin	297.45	79.21
Ofloxacin	-5.73	181.1
Norfloxacin	221.71	51.53

CONCLUSION

Treatment of biofilm-related infections is difficult, due to the protective layer built by the cells. In this study, we used perfused silicone tube catheters as a dynamic model to study biofilm formation by *P. aeruginosa.* We successfully developed and tested a simple, economical, and reliable lab silicone-based tube catheter as a dynamic model. Similar biofilm testing models have been reported

for both bacteria and fungi in a few earlier publications [17, 18], but the present proposed model is much more economical and simpler to design, validate and replicate. Thus, this model will be of more advantage with minimum requirements. The liquid medium can be perfused through the silicon tube in a controlled environment mimicking the clinical settings. The molecular mechanism behind the formation of biofilm inside the silicon tubing needs to be further explored. Fabrication of silicon tubes using advanced polymer chemistry techniques and coating of tubes with antimicrobial agents are other ways to prevent the formation of biofilms.

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