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SCREENING FOR BIOFILM-FORMING EFFICACY OF CLINICAL ISOLATES

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ABSTRACT: Seven pathogenic isolates with clinical history were procured from the Department of Microbiology, RMMCH, Annamalai University, Chidambaram, Tamil Nadu. Among them, five isolates (S1-S5) showed multi-drug resistance to the selected antibiotics. Maximum multi-drug resistance was exhibited by four Gram negative isolates (S1, S2, S4 and S5). Initial qualitative analysis of these four isolates for biofilm forming efficacy by the Congo-Red Agar method revealed that three of these isolates (S1, S2 and S4) were biofilm producers. Maximum biofilm formation was exhibited by S4. Further qualitative analysis of biofilm formation by S4 using the Tube Adherence method yielded a positive result. Biofilm forming efficacy of S4 was then quantitatively analyzed by a spectrophotometric assay. An optical density of 0.38 was recorded at a wavelength of 540 nm, which was significantly higher than the O.D. value of the control (0.05), confirming biofilm formation by the isolate.

INTRODUCTION: Developing drugs against prokaryotic cells like bacteria was comparatively easier than developing drugs against eukaryotic cells, like fungi, protozoa and helminths. This was because, at the cellular level, eukaryotic cells resemble human cells in structure, while prokaryotic or bacterial cells were markedly different. The lip polysaccharide outer layer of Gram-negative bacteria and the porins, forming water channels across this layer, play a pivotal role in the selective toxicity of antibacterial action. Generally, small, hydrophilic drugs pass through the porin channels while larger lipophilic drugs do not.

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Hence, such drugs were not as effective against Gram-negative bacteria compared to their effectiveness against Gram-positive bacteria. The principal objectives of the present study were to procure the pathogenic isolates with clinical history and to determine the antibiotic resistance pattern of the collected isolates towards selected antibiotics. Qualitative methods analyzed the biofilm-forming efficacy of the selected multi-drug resistant isolates.

MATERIALS AND METHODS:

Sample Collection: Pathogenic isolates with clinical history were procured as slants from the Department of Microbiology, Rajah Muthiah Medical College Hospital (RMMCH), Annamalai University, Chidambaram.

Culture Methods: The collected test organisms were cultured in a nutrient agar medium.

Kirby Bauer Sensitivity Assay: 9.5g of Mueller Hinton Agar powder was dissolved in 250ml of

distilled water and autoclaved. The sterilized medium was then poured into 12 sterile petri plates. The plates were then inoculated with different bacterial cultures using the spread plate technique. Sterile swabs were used to make uniform lawns. Discs of the selected antibiotics were then placed on the plates, following which the plates were incubated at 37°C for 24 h [1]. Determination of Multiple Antibiotic Resistance (MAR) Index

From the antibiotic susceptibility test results, the Multiple Antibiotic Resistance (MAR) index was using the following formula [2].

MAR Index = Number of antibiotics to which the isolate was resistant / Total number of antibiotics

Biofilm Assay:

Qualitative Analysis:

Congo-Red Agar Method: The medium was prepared by adding 7.4 grams of Brain-Heart Infusion (BHI) broth, 1 gram of sucrose and 2 grams of agar dissolved in 200ml of distilled water and autoclaved for 15 minutes at 121 °C.

Congo red stain was prepared separately as concentrated aqueous solution (0.16 grams) in 3ml of distilled water) and autoclaved for 15 min at 121 °C. The stain was then added to BHI agar at 55 °C. The BHI agar medium was then poured into 9 sterile petri plates. The plates were inoculated with the test organisms and incubated at 37°C for 24-48 h [1, 3].

Tube Adherence Method: A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried.

Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains [4].

Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times [5].

Quantitative Analysis:

Tube Adherence + Spectrophotometric Assay: A loopful of *Pseudomonas* isolates was sub-cultured in 2mL of Tryptic Soy Broth and incubated at 37°C for 24 hours. To each tube an additional amount of 2 ml of Tryptic Soy broth with 2% glucose was added, and tubes again incubated at 37°C for 24 h. After incubation, the growth medium was discarded. Each tube was washed with Phosphate Buffer Saline (PBS) to eliminate the unbound bacteria. To evaluate the formation of biofilm, remaining attached bacteria were fixed with 2 ml of 99% methanol.

After 15 min the tubes were emptied and left to dry. The attached film was stained for 5 min with 2 ml of 2% crystal violet. Excess stain was rinsed by placing the tubes under running tap water. Tubes were air-dried, and the dye attached to cells was dissolved in 1.5 ml of 33% glacial acetic acid. The optical density (OD) of each tube was determined at 540 nm. The blank was determined by measuring OD of a tube filled with PBS, and positive control was determined by measuring OD of the tube with pure culture [6]. All isolates were tested in triplicate. The absorbance value (OD) of the test organism was interpreted [7].

RESULTS AND DISCUSSION:

Authentication of Collected Clinical Isolates: **Table 1** summarizes the authentication details of the collected clinical isolates. Seven pathogenic isolates with clinical history were procured from the Department of Microbiology, RMMCH, Annamalai University, Chidambaram, Tamil Nadu. The isolates collected were *Escherichia coli*, *Klebsiella*, *Citrobacter*, *Pseudomonas*, *Proteus*, *Enterococcus*, and *Staphylococcus*. Among the isolates, five were Gram-negative (*Escherichia coli*, *Klebsiella*, *Citrobacter*, *Pseudomonas*, and *Proteus)*, and two were Gram-positive (*Enterococcus* and *Staphylococcus)* in nature.

The isolates were obtained from the urine samples of patients of various age groups, suffering from urinary tract infection (UTI), admitted at RMMCH. The isolates of *Escherichia coli*, *Klebsiella*, *Citrobacter*, *Pseudomonas*, *Proteus*, *Enterococcus*, and *Staphylococcus*, have been authenticated as S1, S2, S3, S4, S5, S6 and S7, respectively.

TABLE 1: AUTHENTICATION OF COLLECTED CLINICAL ISOLATES

S. no.	Isolate name	Nature (Gram stain)	Source	Authentication
1	*Escherichia coli*	Gram negative	Urine	S1
2	*Klebsiella*	Gram negative	Urine	S2
3	*Citrobacter*	Gram negative	Urine	S3
4	*Pseudomonas*	Gram negative	Urine	S4
5	*Proteus*	Gram negative	Urine	S5
6	*Enterococcus*	Gram positive	Urine	S6
7	*Staphylococcus*	Gram positive	Urine	S7

Kirby Bauer Sensitivity Assay: The antibiotic susceptibility pattern of the procured pathogenic isolates with clinical history can be observed in Figures 8-16. The same has been summarized in **Table 2.** S1-S5 constituted the Gram-negative isolates, while S6 and S7 were Gram-positive isolates. The Gram-negative isolates, S1-S5, were treated with five antibiotics: Amoxicillin / Clavulanic acid, Cefixime, Ciprofloxacin, Streptomycin and Tetracyclin. The absence of clear zones was prominent in S1 under the action of all antibiotics except Cefixime, which induces a small zone of 10 and 11. While one subculture of S2 exhibited lack of clear zone formation against all the antibiotics used, another subculture exhibited the formation of clear zones measuring 18, <10 and 17 against Ciprofloxacin, Streptomycin and Tetracyclin respectively, maintaining a lack of zone formation against Amoxicillin / Clavulanic acid and Cefixime.

S3 exhibited a lack of zone formation under the impact of Amoxicillin / Clavulanic acid, Cefixime and Tetracyclin while forming small zones of diameter 12 and 14 against Ciprofloxacin and *Streptomycin*. S4 forms clear zones of diameter 15 and 16 against Ciprofloxacin and diameter 12 against Cefixime (one subculture), while showing no clear zones against the other antibiotics used. S5 exhibited a complete lack of clear zones against Amoxicillin/Clavulanic acid and Cefixime, while forming minute clear zones of diameter 12, 11 and <<10 against the other antibiotics-Ciprofloxacin, Streptomycin and Tetracyclin, respectively. The Gram-positive isolates, S6 and S7, were treated with five antibiotics: Ciprofloxacin, Streptomycin, Tetracyclin, Oxacillin and Vancomycin. Both isolates exhibit the formation of clear zones against all the antibiotics. S6 exhibited clear zones measuring 12, 17, 21, 11 and 16 against Ciprofloxacin, Streptomycin, Tetracyclin, Oxacillin, and Vancomycin, respectively.

The first subculture of S7 exhibited clear zones measuring 26, 20, 21, 12 and 17, while the second subculture showed clear zones measuring 22, 13, 22, 13 and 17 against Ciprofloxacin, Streptomycin, Tetracyclin, Oxacillin and Vancomycin, respectively. In this study, Amoxicillin / Clavulanic acid and Cefixime were used exclusively against Gram-negative isolates (S1-S5), while, Oxacillin and Vancomycin were used exclusively against the Gram-positive isolates (S6 and S7). Based on the zone measurements summarized in **Table 2**, the resistance pattern of all the pathogenic isolates with clinical history was tabulated in **Table 3**. It was noted that, with the exception of the second subculture of S2, which showed susceptibility towards Tetracyclin, all other Gram-negative isolates were resistant to all the antibiotics used against them.

However, mixed results were observed when the Gram-positive isolates were treated with the selected antibiotics. S6 was susceptible to the action of Streptomycin and Tetracyclin while being resistant to Ciprofloxacin, Oxacillin and Vancomycin. S7 showed susceptibility towards Ciprofloxacin and Tetracyclin while being resistant to Streptomycin, Oxacillin, and Vancomycin. It was observed that both the Gram-positive isolates were susceptible to Tetracyclin, resistant to Oxacillin while showing moderate or intermediate level resistance to Vancomycin. In the present study, all the Gram-negative isolates (S1-S5) with clinical history were resistant to the action of Amoxicillin / Clavulanic acid, Cefixime, Ciprofloxacin, Streptomycin, and Tetracyclin and hence, considered to be multi-drug resistant. This was found to be in agreement with the results of previous studies conducted by various researchers, which have indicated that Gram-negative bacteria were more prone to developing multi-drug resistance, compared to their Gram-positive counterparts, due to their multilayered cell

envelope, which acts as a barrier to broad-spectrum antibiotics. The envelope seems to be designed to restrict the penetration of all molecules, allowing entry only to nutrients, which pass through porins and specialized transporters. Most antibiotics, being amphipathic, were blocked by the outer membrane of the cell envelope of Gram-negative bacteria [8].

TABLE 2: ANTIBIOTIC SUSCEPTIBILITY PATTERN OF COLLECTED CLINICAL ISOLATES

Antibiotics / Isolates	Amoxicillin/ Clavulanic acid	Cefixime	Ciprofloxacin	Streptomycin	Tetracyclin	Oxacillin	Vancomycin
Gram-negative							
S1	Ncz	10	Ncz	Ncz	Ncz	_	_
	Ncz	11	Ncz	Ncz	Ncz		
S2	Ncz	Ncz	Ncz	Ncz	Ncz	_	_
	Ncz	Ncz	18	<10	17		
S3	Ncz	Ncz	12	14	Ncz	_	_
S4	Ncz	Ncz	15	Ncz	Ncz	_	_
	Ncz	12	16	Ncz	Ncz		
S5	Ncz	Ncz	12	11	<<10	_	_
Gram-positive							
S6	_	_	12	17	21	11	16
S7	_	_	26	20	21	12	17
			23	13	22	13	17

Key: Ncz = No clear zone visible.

TABLE 3: RESISTANCE PATTERN OF COLLECTED CLINICAL ISOLATES

Antibiotics / Isolates	Amoxicillin/ Clavulanic acid	Cefixime	Ciprofloxacin	Streptomycin	Tetracyclin	Oxacillin	Vancomycin
Gram-negative							
S1	R	R	R	R	R	_	_
	R	R	R	R	R		
S2	R	R	R	R	R	_	_
	R	R	I	R	S		
S3	R	R	R	I	R	_	_
S4	R	R	R	R	R	_	_
	R	R	R	R	R		
S5	R	R	R	R	R	_	_
Gram-positive							
S6	_	_	R	S	S	R	I
S7	_	_	S	I	S	R	I
			S	R	S	R	I

Key: R = Resistant I = Intermediate S = Susceptible.

Multiple Antibiotic Resistance Index (Mar-I): The Multiple Antibiotic Resistance (MAR) Index was evaluated for all the isolates under study. It has been tabulated in **Table 4**. It was observed that the Gram-negative isolates, S1-S5, exhibited a MARI of 1, while the Gram-positive isolates, S6 and S7, exhibited a MARI of 0.6. Multi-drug resistance was most prominent in the isolates S1, S2 and S4. The absence of a clear zone was conspicuous. The MAR Index also confirmed the remarkable MDR exhibited by the Gram-negative isolates.

In a 2016 study conducted by Nachammai *et al.* [1], *E. coli* was found to be the major uropathogen, constituting 69% of the isolates. Furthermore, 84% of these *E.coli* isolates were found to be multi-drug resistant. Recently, Shelenkov *et al.* [9] reported the presence of thirty-two MDR isolates of *Klebsiella*, three of which belonged to a very rare ST377 type [2]. Thus, our findings were in agreement with previous reports and trends in multi-drug resistance patterns.

TABLE 4: MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEX OF THE COLLECTED CLINICAL PATHOGENIC ISOLATES

Isolate	Mar-I
S1	1
S2	1
S3	1
S4	1
S5	1
S6	0.6
S7	0.6

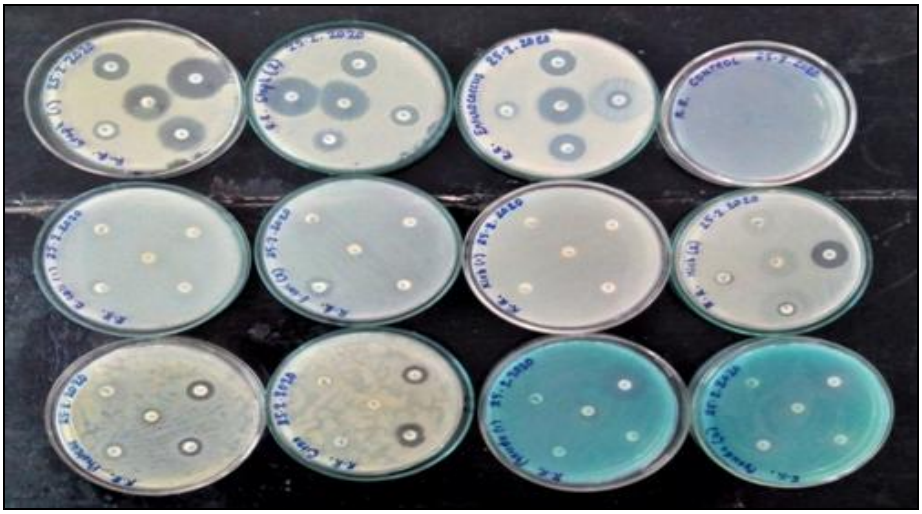

FIG. 1: ANTIBIOTIC SENSITIVITY ASSAY

FIG. 2A: S1 - PLATE 1 **FIG 2B: S1 - PLATE 2**

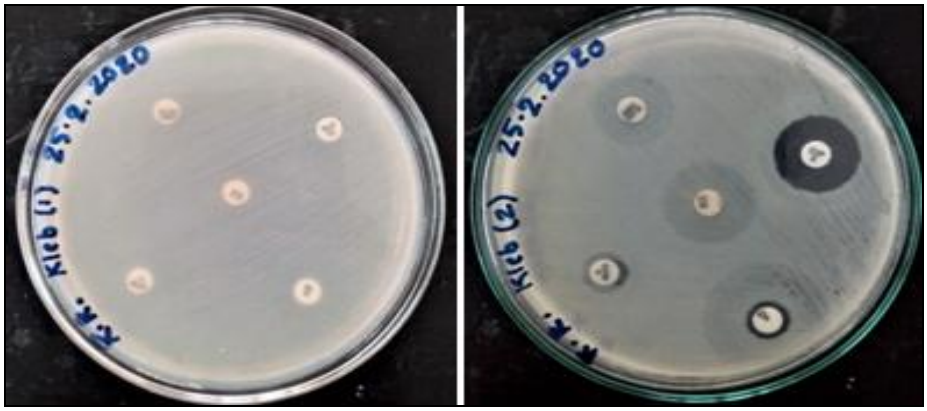

FIG. 3A: S2 - PLATE 1 **FIG. 3B: S2 - PLATE 2**

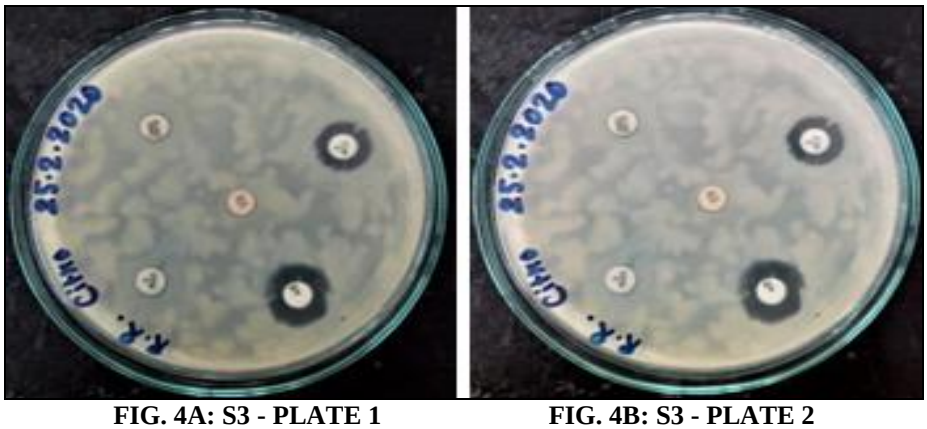

FIG. 4A: S3 - PLATE 1 **FIG. 4B: S3 - PLATE 2**

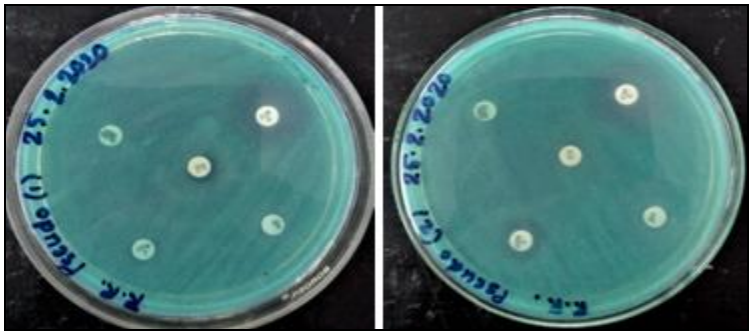

FIG 5A: S4 - PLATE 1 **FIG. 5B: S4 - PLATE 2**

FIG. 6A: S5 - PLATE 1 **FIG. 6B: S5 - PLATE 2**

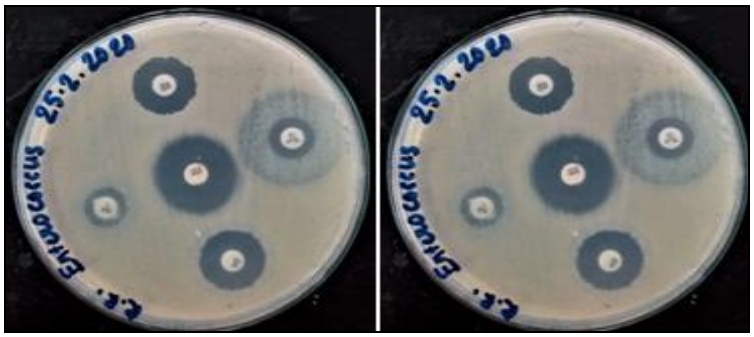

FIG. 7A: S6 - PLATE 1 **FIG. 7B: S6 - PLATE 2**

FIG. 8A: S7 - PLATE 1 **FIG. 8B: S7 - PLATE 2**

FIG. 9: CONTROL

Biofilm Assay: Based on the observations recorded in **Table 3**, the isolates exhibiting maximum antibiotic resistance or multi-drug resistance were selected for further testing for biofilm production. Thus qualitative analysis for biofilm production by Congo-Red Agar (CRA) method was carried out for S1-S4. The results of the assay are displayed in **Fig. 10-16**. On the basis of the CRA method, the isolate showing maximum biofilm-forming efficacy, S4, was then further analyzed by the Tube Adherence Method. The result has been displayed in figures **16A** and **B**. The biofilm-forming efficacy of S4 was then quantitatively analyzed using a combination of the simple tube method and spectrophotometry. An OD value of 0.38 at a wavelength of 540nm was obtained, where OD value of the control (only broth) was evaluated to be 0.05 at the same wavelength. The higher O.D. value exhibited by the isolate indicates a confirmatory result for biofilm production. In comparison to the Gram-negative isolates in this study, the Gram-positive isolates - S6 and S7 were relatively susceptible to the selected antibiotics, with the formation of clear zones around the antibiotic discs. S6 was susceptible to the action of Streptomycin and Tetracyclin while being resistant to Ciprofloxacin, Oxacillin and Vancomycin. S7 showed susceptibility towards Ciprofloxacin and Tetracyclin while being resistant to Streptomycin, Oxacillin and Vancomycin. It was observed that both the Gram-positive isolates were susceptible to Tetracyclin, resistant to Oxacillin while showing moderate or intermediate level resistance to Vancomycin. Thus, Tetracyclin may be considered, subject to further studies, effective against these Gram-positive isolates.

Qualitative Analysis:

Congo red Agar Method: Table 5 summarized the qualitative analysis observations of biofilm-forming efficacy of the selected isolates by CRA method. Biofilm production was most significant in S4 and completely absent in S5. S1 exhibited a moderate level of biofilm production and S2 showed low levels of biofilm formation. The qualitative analysis of biofilm formation was performed using the Congo Red Agar (CRA) method on the Gram-negative isolates exhibiting significant multi-drug resistance, namely, S1, S2, S4 and S5.

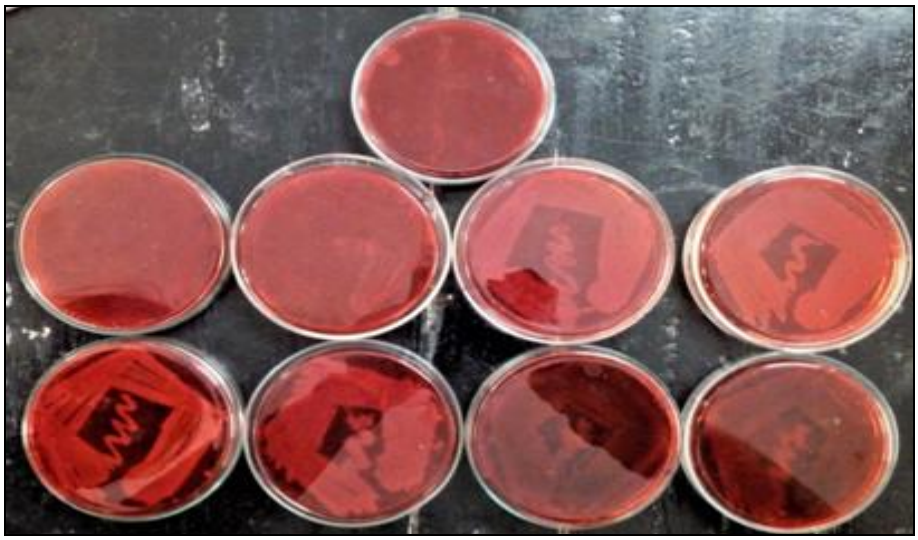

FIG. 10: QUALITATIVE BIOFILM ASSAY CONGO RED AGAR METHOD

In a recent study undertaken by Sunayana Raya *et al.*, biofilm-forming *E.coli* were found to be more resistant to quinolones, cotrimoxazole, and third-generation cephalosporin, ceftriaxone.

Additionally, most biofilm-forming strains were found to be multi-drug resistant [10]. Thus, biofilm production and multi-drug resistance were closely associated.

The results of CRA assay (displayed in **Fig. 10-16** indicated that among the collected clinical isolates, S4 was the most potent biofilm-producing MDR strain. While biofilm formation to a lesser extent was observed in S1 and S2, it was completely absent in S5. In a study, 70% of the uropathogenic *E. coli* isolates exhibited biofilm production by CRA method [1].

TABLE 5: QUALITATIVE ANALYSIS OF SELECTED ISOLATES BY CRA METHOD

S. no.	Isolate	CRA
1	S1	A few black colonies were observed at the edges and center of the culture, indicating moderate biofilm formation
2	S2	A few black colonies were observed only at the edges of the culture, indicating a low level of biofilm formation
3	S4	A high number of black colonies with dry crystalline consistency was observed, indicating significant biofilm production
4	S5	No black colonies were observed, indicating the absence of biofilm formation

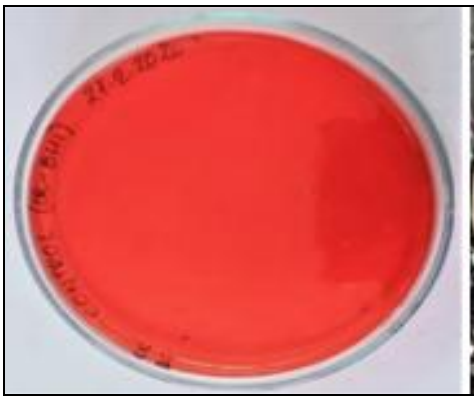

FIG. 11: CONTROL

FIG. 12: S5

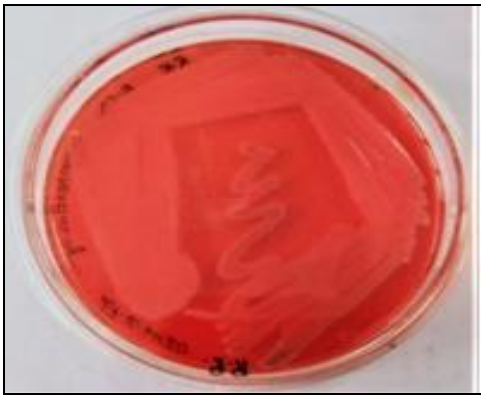

FIG. 13: S1

FIG. 14: S2

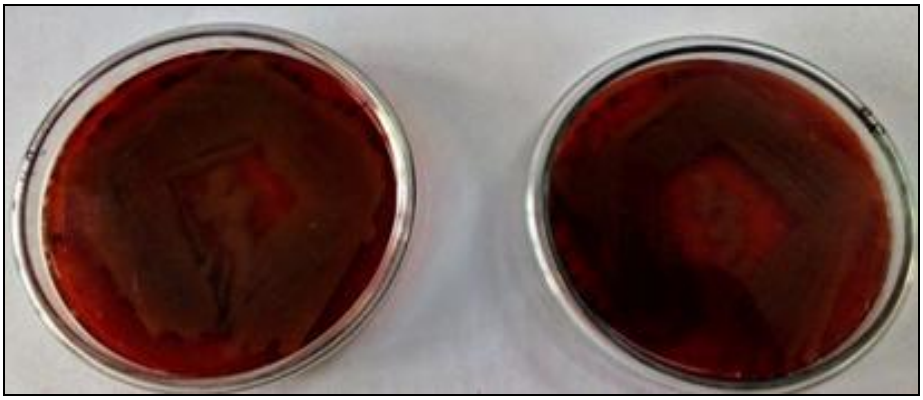

FIG. 15: S4

Tube Adherence Method: Table 6 summarizes the results of the qualitative analysis of biofilm-forming efficacy of S4 using the Tube Adherence method. A visible purple film lining the walls and bottom of the test tube was found, indicating a positive result for biofilm formation. No purple film was observed in control, which only contained the broth. Further qualitative analysis of S4 by Tube Adherence Method followed by quantitative analysis by a combination of the tube method and spectrophotometric assay confirmed the superior biofilm-forming efficacy of S4.

Detection of bacterial biofilm production using this method has been previously performed by Rakhshanda Baqai *et al*, for uropathogenic isolates [5]. *Pseudomonas* was known to be one of the leading causes of nosocomial infections. A recent study quantitatively analyzed biofilm-forming efficacy of Imipenem-resistant *P. aeruginosa* isolates and found a high propensity of IRPA to form biofilm, which was strongly associated with higher drug resistance [11]. Thus, the conclusion of the present study that *Pseudomonas* (Isolate S4) was the most potent biofilm-forming MDR isolate among the seven procured pathogenic isolates with clinical history was backed by previous studies.

TABLE 6: QUALITATIVE ANALYSIS OF S4 BY TUBE ADHERENCE METHOD

Sl. no.	Isolate	TA method
1	Control	Absence of visible purple film on the test tube wall.
2	Test isolate (S4)	Presence of a visible purple film lining the walls and bottom of the test tub, indicating positive result.

FIG. 16A: S4 SUBCULTURED IN TRYPTICASE SOY BROTH AFTER 24 H INCUBATION

FIG. 16B: PURPLE FILM ON TEST TUBE WALL INDICATING POSITIVE RESULT FOR BIOFILM FORMATION

Quantitative Analysis Spectrophotometric Assay: Table 7 summarizes the results of the quantitative analysis of biofilm formation by the pathogenic isolate S4. The optical density of the test isolate (S4) was recorded at a wavelength of 540nm using a spectrophotometer. An O.D. value of 0.38 was recorded. The control gave an O.D. value of 0.05. The notably higher O.D. value exhibited by S4 indicates a positive result for biofilm formation.

TABLE 7: QUANTITATIVE ANALYSIS OF BIOFILM PRODUCTION

Sl no.	Isolate	O. D. value
1.	Control	0.05
2.	Test isolate (S4)	0.38

CONCULUSION: Among the collected pathogenic isolates, S4 was found to be the most potent biofilm-forming MDR strain. Future work would entail combating the biofilm formation observed in S4 by treatment with green synthesized nanoparticles. Halting biofilm formation may in turn help in reducing the multi-drug resistance exhibited by the pathogen.

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CONFLICTS OF INTEREST: The authors declared no conflicts of interest.

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