INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

SCREENING FOR BIOFILM FORMING EFFICACY OF CLINICAL ISOLATES

Roopshali Rakshit, D. Sangeetha and P. Ramya
Department of Microbiology
Faculty of Science, Annamalai University
Annamalainagar, Tamilnadu, India

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. Thus, 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.

Keywords: Biofilm Formation, Bacteriostatic, Clinical Isolates.

INTRODUCTION

Developing drugs against prokaryotic cells like bacteria are comparatively easier than developing drugs against eukaryotic cells, like fungi, protozoa and helminthes. This is because, at the cellular level, eukaryotic cells resemble human cells in structure, while prokaryotic or bacterial cells are markedly different. The lipopolysaccharide 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 sized lipophilic drugs do not. Hence, such drugs are not as effective against Gram negative bacteria, when compared to their effectiveness against Gram positive bacteria.

An antibiotic may directly kill the pathogen (bacteriocidal) or arrest its growth (bacteriostatic). In case of bacteriostatic antibiotics, the pathogen is usually then destroyed by the host's own defence mechanisms like phagocytosis and antibody production. An antibiotic may act on bacteria in various ways. It may inhibit cell wall synthesis, protein synthesis, nucleic acids synthesis, synthesis of essential metabolites or even injure the plasma membrane.**Invalid source specified.**

Overuse of antibiotics has given rise to a phenomenon known as antibiotic resistance. Bacteria respond to the life and death situation imposed on them by antibiotics by facilitating certain mutations in their genome. These mutations equip the bacterial cell with arms to survive the toxic effects of the antibiotic, bestowing upon them and their descendants, a selective growth advantage over non-resistant bacteria.**Invalid source specified.**

It has been proposed that there is a sequential relationship between bacterial tolerance and bacterial resistance. This has been backed experimentally when ancestral, tolerant strains gave rise to resistant E. coli strains in an experiment exploring the effect of periodic ampicillin treatment that resembled medical practice.**Invalid source specified.** Tolerant bacteria display slower growth or have a longer lag phase. This results in lower activity of targets, reducing the efficiency of an antibiotic, which enables the bacteria to evade killing. Compared to non-tolerant strains, tolerant bacteria have longer minimum duration of killing by an antibiotic. This becomes especially advantageous during transient antibiotic treatments.**Invalid source specified.**

Tolerance mutations have a larger target and hence a greater chance of successful occurrences, when compared to resistance mutations. However, after the establishment of a tolerant mutant in the population, resistance mutations are more prone to occur. **Invalid source specified.Invalid source specified.**

1.4: BIOFILMS

"A biofilm is defined as the community of microbes embedded in an organic polymer matrix, adhering to surface. In short, the basic ingredients of a biofilm are, microbes, glycocalyx and surface." **Invalid source specified.** The glycocalyx is an important component of a biofilm, where it is often referred to as extracellular polymeric substance (EPS). It is produced by the microbial community itself and helps the cells in the biofilm to attach irreversibly to the surface and to each other. The target surface may be

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

biological or non-biological. The EPS provides protection to the microbial cells in the biofilm, facilitates inter-cellular communication and anchors the microbial community to a suitable surface.**Invalid source specified.**

While individual planktonic bacteria cause acute bacterial infection, a biofilm comprising of a multicellular microbial community, typically causes chronic infections. A biofilm functions as whole, like a single organism, giving rise to chronicity and persistence. Each individual cellular organism in the biofilm is analogous to an individual cell in our body. The presence of persister cells, along with the ability to trade genes between each other, gives rise to antibiotic resistance. Inside a biofilm, bacteria exist in multiple forms and metabolic states, as different phenotypes, having multiple defenses. A biofilm grows slowly, making it a difficult target for antibiotics. A higher concentration of antibiotics is often required (500 to 5000 times the normal concentration). Additionally, the matrix, comprising of the extracellular materials produced by the microbial community, helps in defending them against the human immune cells and antibiotics.

METHODOLOGY

3.1: 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.

3.2: CULTURE METHODS

The collected test organisms were cultured in nutrient agar medium.
0.98g of nutrient agar medium was suspended in 35mL of distilled water in a clean, sterilized conical flask. The suspension was autoclaved for 15 minutes at 121°C to obtain optimum sterility. After the medium cooled down to a hand-bearable temperature, it was poured into seven sterile test tubes. The test tubes were then placed in a slanting position and left undisturbed until the media solidified. The seven collected clinical isolates were then inoculated on the surface of the media (one test organism in each slant) using an inoculation loop. The slants were then incubated at 37°C for 24 hours.

3.3: 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 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 hours.**Invalid source specified.**

3.4: DETERMINATION OF MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEX

From the results of the antibiotic susceptibility test, the multiple antibiotic resistance (MAR) index was using the following formula:

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics}}$$

Invalid source specified.

3.5: BIOFILM ASSAY

3.5.1: QUALITATIVE ANALYSIS

3.5.1.1: 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 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 (approx.) in 3ml of distilled water) and autoclaved for 15 minutes 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 hours.**Invalid source specified.Invalid source specified.**

3.5.1.2: TUBE ADHERENCE METHOD

As described by Christensen et al., (1982), 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.

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

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. **Invalid source specified.**

3.5.2: QUANTITATIVE ANALYSIS

3.5.2.1: TUBE ADHERENCE + SPECTROPHOTOMETRIC ASSAY

A loopful of Pseudomonas isolate 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 hours. 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 minutes the tubes were emptied and left to dry. The attached film was stained for 5 minutes 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. Optical density (OD) of each tube was determined at 540 nm. The blank was determined by measuring OD of tube filled with PBS and positive control was determined by measuring OD of tube with pure culture. **Invalid source specified.** All isolates were tested in triplicate. The absorbance value (OD) of the test organism was interpreted according to the criteria proposed byStepanovic et al. **Invalid source specified.**.

RESULTS

4.1: 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 theisolates, 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 havebeen authenticated as S1, S2, S3, S4, S5, S6 and S7 respectively.
Table 1: Authentication of collected clinical isolates.

Sl. 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

4.2: 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 have been summarized in Table 2. S1-S5 constitute the Gram-negative isolates while S6 and S7 are Gram positive isolates. The Gram-negative isolates, S1-S5, are treated with five antibiotics, namely, Amoxicillin/Clavulanic acid, Cefexime, Ciprofoxacin, Streotomycin and Tetracyclin.

The absence of clear zones is prominent in S1 under the action of all antibiotics except Cefexime, which induces a small zone of 10 and 11. While one subculture of S2 exhibits lack of clear zone formation against all the antibiotics used, another subculture exhibits 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 Cefexime. S3 exhibits a lack of zone formation under the impact of Amoxicillin/Clavulanic acid, Cefexime and Tetracyclin, while forming small zones of diameter 12 and 14 against Ciprofloxacine and Streptomycin. S4 forms clear zones of diameter 15 and 16 against Ciprofloxacin and diameter 12 against Cefexime (one subculture), while showing no clear zones against the other antibiotics used. S5 exhibits a complete lack of clear zones against Amoxicillin/Clavulanic acid and Cefexime, while forming minute clear zones of diameter 12, 11 and <<10 against the the other antibiotics – Ciprofloxacin, Streptomycin and Tetracyclin respectively.

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

The Gram-positive isolates, S6 and S7, are treated with five antibiotics, namely, Ciprofloxacin, Streptomycin, Tetracyclin, Oxacillin and Vancomycin.

Both isolates exhibit the formation of clear zones against all the antibiotics. S6 exhibits clear zones measuring 12, 17, 21, 11 and 16 against Ciprofloxacin, Streptomycin, Tetracyclin, Oxacillin and Vancomycin respectively. The first subculture of S7 exhibits clear zones measuring 26, 20, 21, 12 and 17, while the second subculture shows clear zones measuring 22, 13, 22, 13 and 17 against Ciprofloxacin, Streptomycin, Tetracyclin, Oxacillin and Vancomycin, respectively.

In this study, Amoxicillin/Clavulanic acid and Cefexime are used exclusively against Gram negative isolates (S1-S5), while, Oxacillin and Vancomycin are 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 the 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.

Table 2: Antibiotic susceptibility pattern of collected clinical isolates

Antibiotics → / **Isolates** ↓	Amoxicillin/ Clavulanic acid	Cefexime	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

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

Table 3: Resistance pattern of collected clinical isolates.

Antibiotics → / **Isolates** ↓	Amoxicillin/ Clavulanic acid	Cefexime	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 (MARI)

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.

Table 4: Multiple Antibiotic Resistance (MAR) Index of the collected clinical pathogenic isolates.

ISOLATE	**MARI**
S1	1
S2	1
S3	1
S4	1
S5	1
S6	0.6
S7	0.6

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

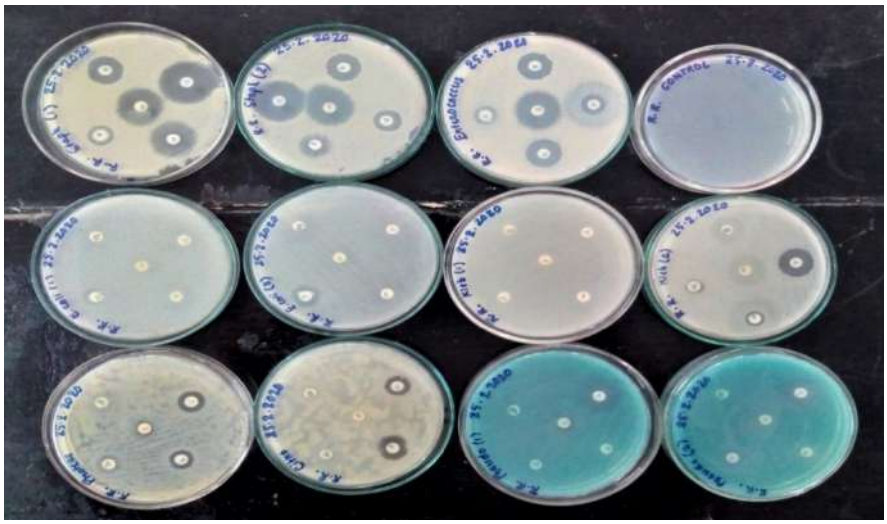

Figure 8: Antibiotic sensitivity assay

Figure 9a: S1 - plate 1Figure 9b: S2 - plate 2

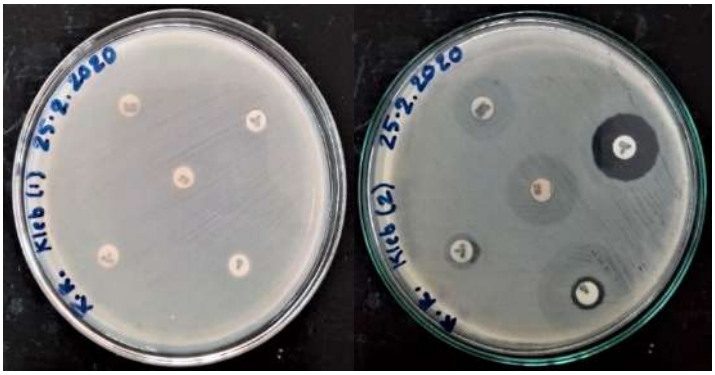

Figure 10a: S2 - plate 1Figure 10b: S2 - plate 2

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

Figure 11a: S3 - plate 1 Figure 11B: S3 - plate 2

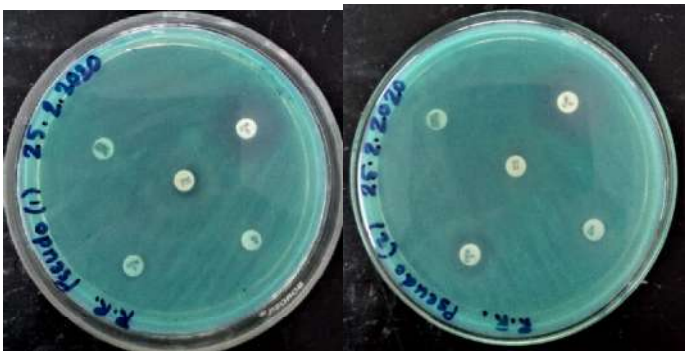

Figure 12a: S4 - plate 1Figure 12b: S4 - plate 2

Figure 13a: S5 - plate 1Figure 13b: S5 - plate 2

Figure 14a: S6 - plate 1Figure 14b: S6 - plate 2

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

Figure 15a: S7 - plate 1Figure 15b: S7 - plate 2

Figure 16: Control

4.3: 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 Figures 17-22. 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 23a 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.

4.3.1: QUALITATIVE ANALYSIS

4.3.1.1: CONGO RED AGAR METHOD

Table 5 summarizes the observations of qualitative analysis of biofilm forming efficacy of the selected isolates by CRA method. Biofilm production was most sigificant in S4 and completely absent in S5. S1 exhibited moderate level of biofilm production and S2 showed low levels of biofilm formation.

Table 5: Qualitative analysis of selected isolates by CRA method:

Sl. No.	Isolate	CRA
1.	S1	A few black colonies were observed at the edges and centre of the culture, indicating moderate biofilm formation.
2.	S2	A few black colonies were observed only at the edges of the culture, indicating 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 observed, indicating absence of biofilm formation.

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

Figure 17: Qualitative biofilm assay (Congo Red Agar method)

Figure 18: ControlFigure 19: S5

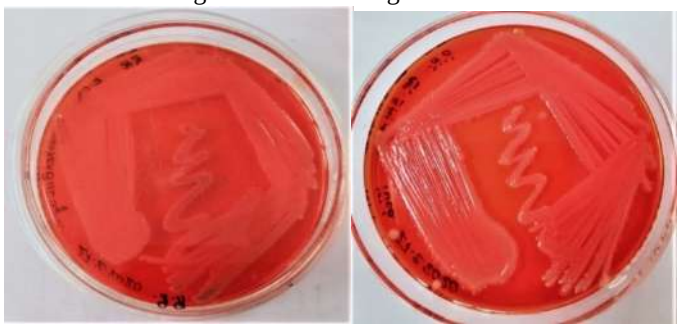

Figure 20: S1Figure 21: S2

Figure 22: S4

4.3.1.2: TUBE ADHERENCE METHOD

Table 6 summarizes the results of the qualitative analysis of biofilm forming efficacy of S4 using the Tube Adherance 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 the control which only contained the broth.

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

Table 6: Qualitative analysis of S4 by Tube Adherance Method:

Sl. No.	Isolate	TA Method
1.	Control	Absence of visible purple film on 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.

Figure 23a: S4 subcultured in Trypticase Soy Broth (after 24 hours incubation)

Figure 23b: Purple film on test tube wall indicating positive result for biofilm formation.

4.3.2: 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.

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

Table 7: Quantitative analysis of biofilm production:

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

CHAPTER 5: DISCUSSION

In the present study, all the Gram-negative isolates (S1-S5) with clinical history, were found to be resistant to the action of Amoxicillin/Clavulanic acid, Cefexime, Ciprofloxacin, Stretomycin 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 are 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 in a way to restrict the penetration of all molecules, allowing entry only to nutrients, which pass through porins and specialized transporters. Most antibiotics, being amphipathic in nature, are blocked by the outer membrane of the cell envelope of Gram-negative bacteria. **Invalid source specified.**

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, **Invalid source specified.**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. reported the presence of thirty-two MDR isolates of Klebsiella, three of which belonged to a very rare ST377 type. **Invalid source specified.** Thus, our findings are in agreement with previous reports and trends in multi-drug resistance pattern.

In comparison to the Gram-negative isolates in this study, the Gram-positive isolates - S6and S7 were found to be 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 Grampositive 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.

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. 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. **Invalid source specified.** Thus, biofilm production and multi-drug resistance are closely associated.

The results of CRA assay (displayed in Figures 17-22) 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 S1and S2, it was completely absent in S5. In the study conducted by Nachammai et al, 70% of the uropathogenicE. coli isolates exhibited biofilm production by CRA method. In another study conducted by Shne Whelan et al, 93.6% of the E. coli isolates believed to be the causative agent of UTI, were found to be biofilm producers. **Invalid source specified.** In this study, however, although a certain level of biofilm production was observed in isolates S1 and S2, it was surpassed by far, by the amount of biofilm produced by S4. Now, although biofilm production of S1 and S2 did not meet the expectations garnered from previous studies, the high biofilm forming efficacy of S4 came as no surprise. It was noted by Clayton.W Hall et al, that, historically, P. aeruginosahas served as the prototypic model organisms for the study of antibiotic resistance and tolerance in bacterial biofilms. **Invalid source specified.** The expression of the ndvB gene in P. aeruginosa seems to confer biofilm tolerance and also acts as a determinant of resistance. The ndvBgene has been historically describe as the intrinsic biofilm antibiotic resistance gene. **Invalid source specified.** Thus, our findings are in alignment with previous reports.

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 RakhshandaBaqai et al, for uropathogenic isolates. **Invalid source specified.**Pseudomonas is known to be one of the leading causes of nosocomial infections. A recent study quantitatively analysed biofilm forming efficacy of Imipenem-resistant P. aeruginosa isolates and found high propensity of IRPAto form biofilm, which is strongly associated with higher drug resistance. **Invalid source specified.**

Thus, the conclusion of the present study that Pseudomonas (Isolate S4) is the most potent biofilm forming MDR isolate among the seven procured pathogenic isolates with clinical history, is backed by previous studies.

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April :2021
Online Copy Available: www.ijmer.in
Digital certificate of publication:http://ijmer.in/pdf/e-Certificate%20of%20Publication-IJMER.pdf

CONCLUSION

It can be concluded from the present study that multi-drug resistance developed by a pathogen is closely related to its biofilm forming efficacy.

The emergence and increasing prevalence of multi-drug resistant strains of bacteria pose a grave threat to society today and should be treated with utmost urgency. Most MDR strains have emanated from nosocomial settings. This study revealed the continued prevalence of multi-drug resistant strains of bacteria in nosocomial set-ups and the association of multi-drug resistance with biofilm forming efficacy of the pathogenic isolates. Multi-drug resistance was particularly prominent in the Gram-negative pathogenic isolates, which also exhibited significant biofilm formation.

Biofilm formation contributes significantly to the development of antibiotic resistance in bacterial strains. The success of biofilms in thwarting the action of antibiotics is a result of a combined effect of various mechanisms. Better understanding and analysis of the mechanisms underlying biofilm-specific antibiotic resistance and tolerance, is essential for the development of treatments to enhance the susceptibility of biofilms to antibiotics.

Green synthesized nanoparticles may be considered in this aspect, as a potential treatment option.

REFERENCES

There are no sources in the current document.

Filename:	22
Directory:	C:\Users\DELL\Documents
Template:	C:\Users\DELL\AppData\Roaming\Microsoft\Templates\Normal.dotm
Title:	
Subject:	
Author:	Windows User
Keywords:	
Comments:	
Creation Date:	4/16/2021 4:41:00 PM
Change Number:	5
Last Saved On:	5/4/2021 10:27:00 PM
Last Saved By:	Murali Korada
Total Editing Time:	22 Minutes
Last Printed On:	5/6/2021 3:03:00 PM

As of Last Complete Printing

Number of Pages:	12
Number of Words:	4,209 (approx.)
Number of Characters:	23,996 (approx.)