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Assessment and Comparative Study of Biofilm Formation with Frequency of Multi Drug Resistance in Strains of *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration between both authors. Author KF managed the literature searches, wrote the protocol, performed the experiments and wrote the first draft of the manuscript. Author KA designed the study and managed the analyses of the study. Both the authors read and approved the final manuscript.

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ABSTRACT

Background: The study was conducted to identify the role of biofilms in the antibiotic susceptibility in the strains of *Staphylococcus aureus*. A total of 19 non-repeated pus/wound swab samples from different anatomic locations and 17 samples that were previously identified as *Staphylococcus aureus* and preserved in the labs were included in the study. The *Staphylococcus aureus* was identified based on colony morphology, Gram's stain, biochemical tests (catalase and coagulase tests) and molecular identification through PCR amplification. Methodology: A total of 26 samples were recovered out of the 31 samples. Kirby-Bauer disk diffusion susceptibility test was used to determine the sensitivity or resistance of *Staphylococcus aureus* to methicillin. Out of the 26 strains, 4 were highly resistant, 10 were moderately resistant and 12 strains were sensitive. Three different protocols (Tube Method, Congo Red Agar Method and Tissue Culture plate method) were used for the detection of biofilm formation for both resistant and sensitive strains. Result: Comparative analysis of the antibiotic susceptibility and biofilm formation by different protocols showed that 70% strains that are resistant to antibiotic methicillin produced moderate-strong biofilms. 50% have produced the moderate-strong biofilms in all 3 protocols. In case of sensitive, 50% strains had produced none-weak biofilms in all 3 protocols. Decisions: The strains that had

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zone of inhibition of close to resistance produced weak-strong biofilms but they all produced weak biofilms in CRA method. It can be concluded that the strains of *Staphylococcus aureus* that have the ability to produce biofilms become methicillin resistant.

Keywords: Biofilm; antibiotic susceptibility; congo red agar; tube method; tissue culture plats.

1. INTRODUCTION

Staphylococcus aureus is a gram-positive, microorganism species that colonizes the anterior nares of around 20–25% human population, and 75–80% are inter-mittently colonized [1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important infective agents. It is the cause for several diseases from skin to serious invasive infections like respiratory disorder, infections of soft tissues, bones, heart valves, and even fatal blood disorder in humans [2].

Numerous pathogenic microorganisms are able of evolution among their lifecycle within the situation and all have able to change abruptly and attached with surface and available accessibility along with to desire and auxiliary resistances[3].

Formation of a Biofilm consist of five steps:

Step 1: Attachment to the surface and it is a reversible stage of bacteria.
Step 2: It is unalterable attachment facilitated by the formation of extra cellular polymers material.
Step 3: To start formation of biofilm and mature by the grow micro colonies.
Step 4: Three Dimensional structure is formed containing cells in a groups with having connection inside the group of clusters for the supply of cell needs like water and nutrients and waste removal.
Step 5: After the maturation time to detachment and scattering of cells from the based attachment of biofilm and start new cycle of biofilm formation; new dispersed cells are more like to planktonic [4].

The genetic and molecular basis of biofilm formation in Staphylococci is varied [5].The flexibility to create a biofilm affords a minimum of 2 properties: the adherence of cells to a surface and accumulation to form multilayered cell clusters. A trade mark is that the production of the slime substance polysaccharide intracellular adhesion (PIA), a carbohydrate composed of beta - one,6 - linked N-acetyl glucosamine with part diacetylated residues, in which the cells are embedded and guarded against the host's immune defense and antibiotic treatment. *Staphylococcus aureus* is an opportunistic infective agent that produces biofilms on medical equipment and causes respiratory disorder, meningitis, carditis, osteitis and blood poisoning. The biofilm formation by *Staphylococcus aureus* involves complicated processes. The biofilm cells are controlled along and exhibit an altered composition with relevance microorganism physiology, metabolism and gene transcription.

Staphylococcus aureus is a most pathogenic strain in nosocomial and environmental – acquired infection related with life threating disease worldwide. [3] In 1961, first infected case of MRSA was reported at England [6] however, in 1980, MRSA was reported in United States .MRSA is intervened by *mecA* gene and that gene is encoded by PBP penicillin-binding protein due it less efficacy of β-lactam antibiotics towards MRSA except Sparfloxacin and ciprofloxacin. MRSA is in light because of it is resistance with beta lactams, quinolones and aminoglycosides classes of antibiotics. Biofilm production among the strains of *Staphylococcus aureus* is a very hard to treat because of Antibiotic resistance [7]The expanding medicate resistance at the side inducible clindamycin resistance, methicillin resistance and biofilm generation among the strains of *Staphylococcus aureus* are show as the genuine issues to the successful treatment of the contaminations caused by S. aureus. So, the most targets of this think about were to decide the antimicrobial helplessness designs at the side the rates of inducible clindamycin resistance, methicillin resistance and biofilm generation among the strains of *S. aureus* disconnected from pus/wound swab tests. [7].

The study mainly focuses on the assessment and comparative of Biofilm Formation with frequency of multi drug resistance in strains of *Staphylococcus aureus* in the different department of Shaheed Zulfikar Ali Bhutto Institute of Science & Technology (SZABIST).

2. MATERIALS AND METHODS

2.1 Isolation and Identification of *Bacterial strains*

Staphylococci are gram-positive organisms reside in the nasal cavity and other mucous membranes as well as skin of the humans. 19 skin samples were collected by gently rubbing the tip of sterile cotton swab on the face of students at Shaheed Zulfikar Ali Bhutto Institute of Science & Technology (SZABIST) 100 and 154 campuses. 17 samples that were preserved at campus lab were acquired.

2.2 Bacterial Growth on Selective Media

MSA is thought to be a selective media for *Staphylococcus aureus.* Staphylococci can survive the high salt concentrations of MSA and thus grow without any problem. When mannitol is incited, the acid formed turns the phenol red pH indicator from red (base) to yellow (acid). All of the 19 swabs were streaked onto a small (one forth) section MSA plate using aseptic technique. The plates were left in incubator at 37°C for 24 hours [8].

2.3 Biochemical Characterization

2.3.1 Catalase Test

Staphylococci (which are catalase positive) were differentiated through catalase test. On a microscope slide small number of colonies grown on MSA were placed followed by few drops of 3% H_2O_2. Bubbles are produced at once if the sample contains Staphylococci. The ones that were catalase negative (did not produced the bubbles) were safely discarded [9].

2.3.2 Coagulase Test

Coagulase test was performed to differentiate the *Staphylococcus aureus* from other species of Staphylococci as only *Staphylococcus aureus* has the ability to clot the blood plasma. Hence isolating the *Staphylococcus aureus* from other species of Staphylococci [10].

2.4 Screening of Antibiotic

Kirby-bauer disk diffusion susceptibility test was used to determine the sensitivity or resistance of *staphylococcus aureus* to methicillin. The inhibition zone diameters were measured in millimeters using plastic (transparent) meter rule [11].

2.5 To Determine the Relationship between the Nature of the Strains and Biofilm Production the following Protocols were Performed

2.5.1 Protocol 1: Tube method

Trypticase Soy Broth (TSB) was prepared with 1% glucose and poured in to the test tube. After the media was autoclaved, colonies of each strains were transferred in to separate tubes and tubes placed in incubator. After 24 hours, the cultures of tubes were discarded and tubes were washed with phosphate buffer saline (PBS) pH 7.3. Tubes were left to dry. 0.1% crystal violet stain was prepared and tubes were stained with it. Excess stain was discarded and tubes were washed with deionized water. The strains that produced biofilms formed a visible line on the side (wall) and bottom of the tube.

2.5.1.1 Tube Method Results

Tubes were examined and amount of biofilm formation was scored as absent, 1-weak, 2-moderate, 3-strong [12].

2.5.2 Protocol 2: Congo Red Agar Method (CRA)

A special medium which was mixture of Brain Heart infusion agar (37 gm/l), sucrose (5gm/l), agar no 1 (10 gm/l) and Congo red dye (0.8 gm/l) was produced. After the medium was autoclaved it was poured into the plates and strains were streaked onto the plates. Plates were incubated for 24 to 48 hours. The strains that produced strong biofilms formed black colonies and the ones that remained pink indicated weak biofilm production [13].

2.5.3 Protocol 3: Tissue Culture Plate Method (TCP)

Tryptic soy broth (TSB) was prepared with 1% glucose and poured in to the test tube. After the media was autoclaved, colonies of each strains were transferred in to separate tubes and tubes placed in incubator. After 24 hours the cultures from tubes were poured into the 96 wells flat bottom micro-titre plate. Aluminum foil was used to cover the plate and plate was placed in the incubator for 24 hours. The cultures from wells were then discarded and wells were washed with PBS. 0.1% crystal violet stain was prepared and wells were stained with it. Excess stain was discarded and wells were washed with deionized

water. Optical density of each well was measured at 570 nm using an automated ELISA plate reader [13,14].

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Bacterial strains*

17 samples that were preserved at campus lab were acquired Shaheed Zulfikar Ali Bhutto Institute of Science & Technology (SZABIST) 100 and 154 campuses.

3.2 Bacterial Growth on Selective Media

MSA is thought to be a selective media for *Staphylococcus aureus.* Staphylococci can survive the high salt concentrations of MSA and thus grow without any problem. 8] Yellow & Pink pin pointed colonies appeared on Manitol Salt Agar and yellow-Creamish colonies appeared on Tryptic Soya Agar (TSA) Staphyloxanthin is a carotenoid pigment that is produced by some strains of Staphylococcus aureus.

3.3 Identification of Biochemical Characterization

3.3.1 Catalase Test

Staphylococci (which are catalase positive) were differentiated through catalase test. After the treatment with H_2O_2 Produced Bubbles and catalase test is positive [9] This therefore, differentiated between *streptococci* and Staphylococci. 31 samples were catalase positive and were separated and sub cultured.

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
Mannitol fermentation	*Positive*	*Negative*	*Usually Positive*

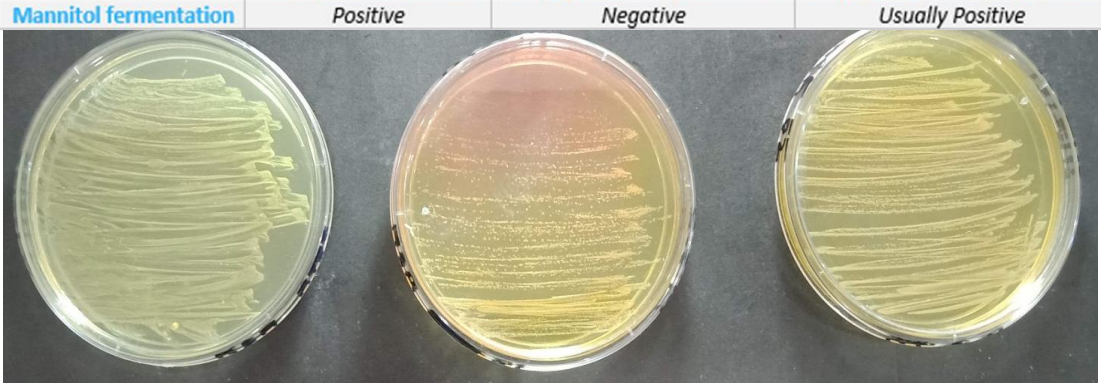

Fig.1. Growth of Staphylococcus aureus on MSA

Catalase Test Result

Test	*Staphylococci*
Bubble Formation	*Positive*

Fig. 2. Staphylococci species forming bubbles when reacted with Hydrogen peroxide

3.3.2 Coagulase Test

Coagulase test the ability to clot the blood plasma. Hence isolating the *Staphylococcus aureus* from other species of Staphylococci. Out of the 31 samples that were tested for coagulase test 26 were coagulase positive [10]. *Coagulase Test Result:*

3.4 Screening of Antibiotic

The results were interpreted according to CLSI guidelines. An inhibition zone diameter of ≤ 13 mm was reported as methicillin resistant and ≥ 14 mm was reported as methicillin sensitive [11].

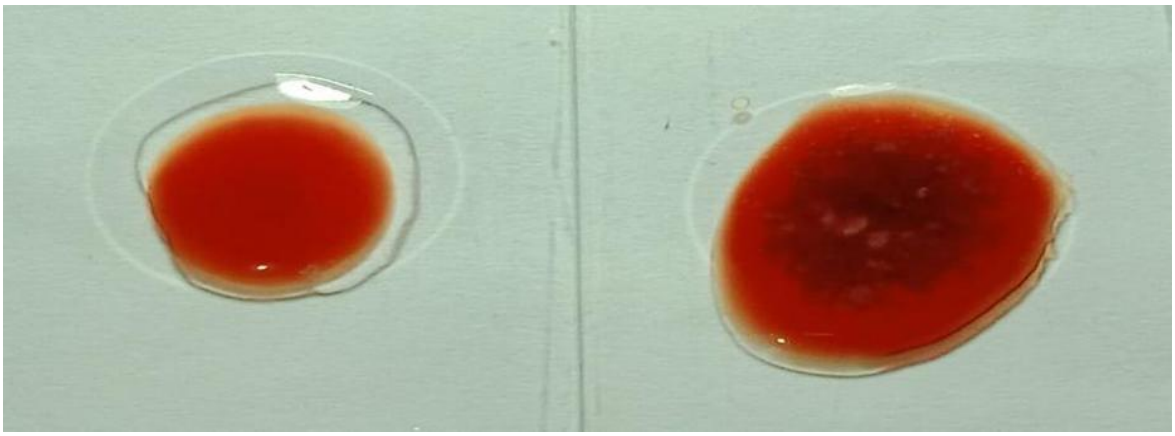

Fig. 3. Coagulase test

Table 1. Nature of strains tested for Methicillin resistance

Nature	Quantity
Highly resistant (9 mm or less)	4
Moderately resistant (greater than 10mm ≤ 13 mm)	10
Sensitive (greater than 13 mm)	12
Total	26

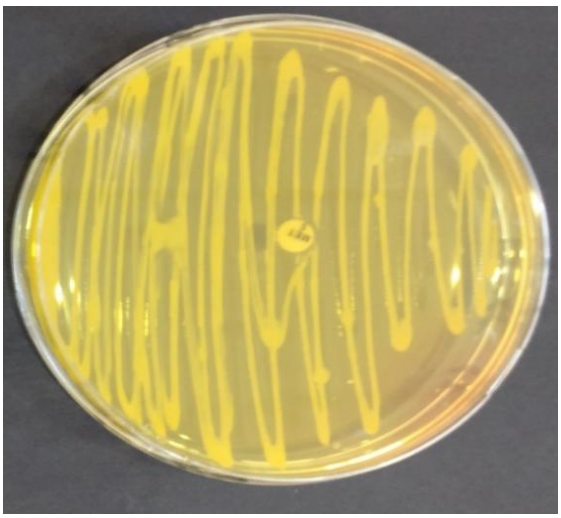

Fig. 4. Methicillin Resistant Strain with no inhibition zone

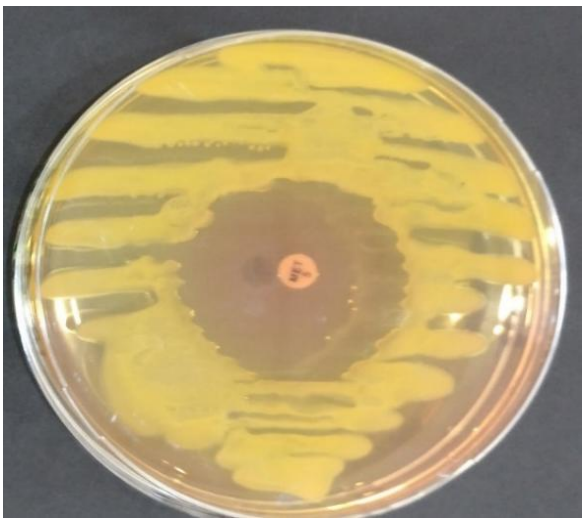

Fig 5 Methicillin Sensitive Strain with inhibition

Names Assigned and Zone of Inhibition of the Strains that were used for the rest of the Study.

Table 2. Strain Name Strains Used for the Study (Key; R= resistant, S= Sensitive)

Resistant	Zone of Inhibition diameter/mm	Sensitive	Zone of Inhibition diameter/mm
R1	13mm	S1	14 mm
R2	13 mm	S2	14 mm
R3	13 mm	S3	14 mm
R4	13 mm	S4	14 mm
R5	12 mm	S5	14 mm
R6	12 mm	S6	19 mm
R7	09 mm	S7	21 mm
R8	08 mm	S8	22 mm
R9	02 mm	S9	23 mm
R10	00 mm	S10	23 mm

10 Resistant and 10 Sensitive strains were selected and were sub cultured for preservation and further use in the study (For the rest of the study these 20 strains were used).

Fig. 6. Visible film lining the wall and bottom of the tube is indicative of biofilm formation

Table 3. Scores of biofilm production by tube method

Nature Resistant	Strains	Biofilm production score	Strains	Biofilm production score
	R10, R09	3	S01, S02	3
	R08, R07, R06	2	S03, S04, S05	2
	R05, R04, R03, R02	1	S06, S08, S10	1
	R01	0	S07, S09	0

3.5 To Determine the Relationship between the Nature of the Strains and Biofilm Production the following Protocols were Performed

3.5.1 Protocol 1: Tube method

Tube Method Results: Tubes were examined and amount of biofilm formation was scored as 0absent, 1-weak, 2-moderate, 3-strong [12].

3.5.2 Protocol 2: Congo Red Agar Method (CRA)

In sensitive, 50% strains had produced none-weak biofilms in all three protocols. The strains that had zone of inhibition of 14 millimeters, which are Sensitive(S) 01 to Sensitive(S), 05 produced weak-strong biofilms but they all produced weak biofilms in Congo red agar (CRA) method. This supports the argument that as these strains were almost near the antibiotic resistance which is less than or 13 millimeters therefore, they produced biofilms but as they were not completely resistant, they were unable to produce biofilms in all three protocols. strains Sensitive(S) 06 till Sensitive(S) 10 had no or weak biofilms in all protocols and as these strains had zone of inhibition greater than 18 millimeters which is far from resistance this supports the argument that the less the resistance to methicillin the weaker the biofilm is produced or perhaps the less chance of producing the biofilms The strains that produced strong biofilms formed black colonies and the ones that remained pink indicated weak biofilm production. [13].

Fig. 7. Black colonies indicating the production of strong biofilms

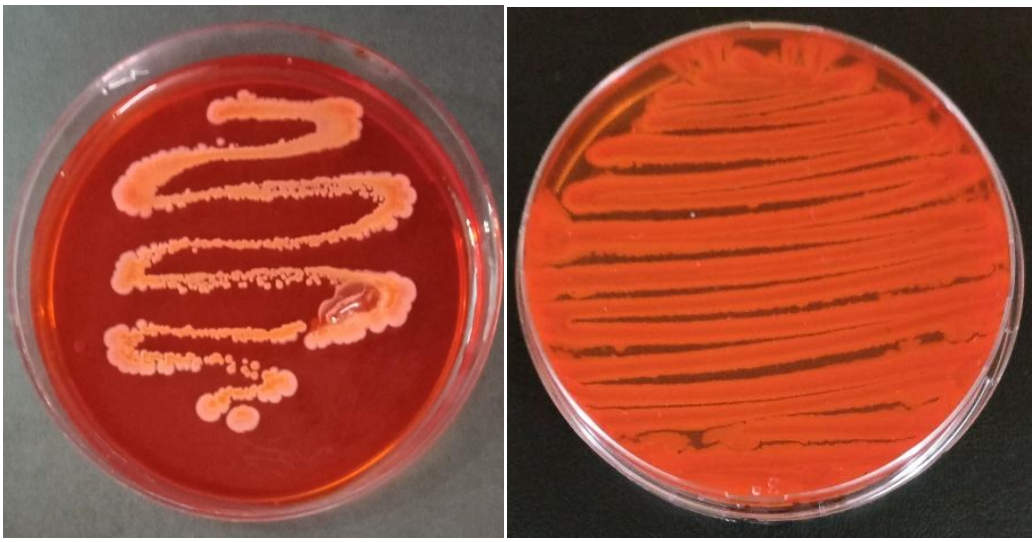

Fig. 8. Week biofilm producing stained remained pink

Table 4. Biofilm Production by CRA Method Results

Nature Resistant	Strain	Biofilm production
Sensitive	R10, R09, R08, R07, R06, R04	Strong
	R05, R03, R02, R01	Moderate-Strong
	S01, S02, S03, S04, S05	Weak
	S06, S07, S08, S09, S10	None

3.5.3 Protocol 3: Tissue Culture Plate Method (TCP)

According to reference value Resistance strain having strong biofilm [13,14].

Table 5. Tissue Culture Plate Optical Densities

Mean OD Values	Adherence	Biofilm Formation
< 0.120	None	None/weak
0.120 - 0.199	Weak	Weak
0.200 - 0.239	Moderate	Moderate
≥ 0.240	Strong	High

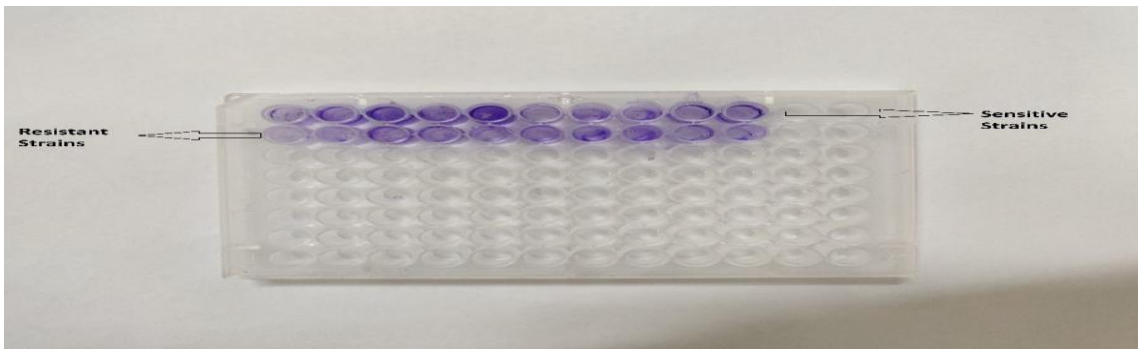

Fig. 9. Micro-titre plate stained with crystal violet.Classification of Bacterial Adherence by TCP Method [13,14]

Table 6. OD Values obtained by TCP Method Results

Resistant Strain	Mean OD Values	Sensitive Strain	Mean OD Values
R01	0.000	S01	0.483
R02	0.155	S02	0.304
R03	0.111	S03	0.234
R04	0.200	S04	0.270
R05	0.222	S05	0.179
R06	0.227	S06	0.130
R07	0.274	S07	0.119
R08	0.326	S08	0.130
R09	0.350	S09	0.145
R10	0.354	S10	0.172

Strain	Zone of Inhibition Diameter/mm	Biofilm Production By		
		Tube Method	CRA Method	TCP Method
Resistant				
R01	13	Absent	Moderate - Strong	None
R02	13	Weak	Moderate - Strong	Weak
R03	13	Weak	Moderate - Strong	None/Weak
R04	13	Weak	Strong	Moderate
R05	12	Weak	Moderate - Strong	Moderate
R06	12	Moderate	Strong	Moderate
R07	09	Moderate	Strong	Strong
R08	08	Moderate	Strong	Strong
R09	02	Strong	Strong	Strong
R10	00	Strong	Strong	Strong
Sensitive				
S01	14	Strong	Weak	Strong
S02	14	Strong	Weak	Strong
S03	14	Moderate	Weak	Moderate
S04	14	Moderate	Weak	Strong
S05	14	Moderate	Weak	Weak
S06	19	Weak	None	Weak
S07	21	None	None	None/Weak
S08	22	None	None	Weak
S09	23	None	None	Weak
S10	23	Weak	None	Weak

COMPARATIVE ANALYSIS OF THE ANTIBIOTIC SUSCEPTIBILITY AND BIOFILM FORMATION BY 3 DIFFERENT PROTOCOLS

Staphylococcus aureus has long been predictable as an imperative pathogen in human. Due to an expanding number of contaminations caused by methicillin-resistant *S. aureus* (MRSA) strains, treatment has become to be complicated[15] and develop multidrug resistant (MDR) strain with respect to antibiotic [16] multidrug resistant (MDR) strains having protective layer of Biofilm and that's is a basic reason of resistivity[17] The extracellular polymeric substance EPS is grow that is safety shield of microorganisms specially in case of *Staphylococcus aureus* [5].

Comparative analysis of the antibiotic susceptibility and biofilm formation by three different protocols shows that 70% strains that are resistant to antibiotic methicillin produced moderate-strong biofilms. 50% have produced the moderate-strong biofilms in all three protocols, which are Resistant (R) 10 till Resistant (R) 06. Resistant (R) 04 and Resistant

(R) 05 have produced moderate-strong biofilms in Congored Agar (CRA) and Tissue Culture Plate (TCP) method. Resistant (R) 09 and Resistant (R) 10 have produced strong biofilms in all three protocols and both of them had zone of inhibitions of 2 and 0 millimeters respectively, this therefore supports the argument that the more the resistance to methicillin the stronger the biofilm is produced and perhaps more chance of producing biofilms [13]

Furthermore, the strains of *Staphylococcus aureus* that have the ability to produce biofilms become methicillin resistant. This supports the argument that biofilms play major role in providing the antibiotic resistance to bacteria. Biofilm-producing strains of *Staphylococcus aureus* pose a serious threat in health sectors. These strains of bacteria are encased in a matrix that allows them to resist and exclude antibiotics and the host immune response. In addition to having structural barriers, the strains can rapidly undergo physiological changes such as slow growth rate and producing persistent cells. In these conditions, antibiotics fail to inhibit, kill, or eradicate these cells, which are found inside the biofilm matrix. Therefore, chronic infections caused by biofilms are often difficult to treat effectively in part due to the resistance of biofilms to antimicrobial therapy. In general, antimicrobial resistance along with biofilm formation becomes an escalating and intractable problem in the health sector.

4. CONCLUSION

The nature of biofilm structure and therefore the physiological attributes of biofilm organisms have inherent resistance to antimicrobial agents; no matter these antimicrobial agents are antibiotics or disinfectants. From the results obtained from the study it can be concluded that the greater the resistance to methicillin, the stronger biofilm is produced and less the resistance to methicillin the weaker, the biofilm is produced or perhaps the less chance of producing the biofilms.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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