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Antibiogram Pattern and Biofilm forming Potential of Gram Positive Clinical Isolates from Karachi

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Abstract: Most microorganisms can live in both conditions, such as planktonic (as a single cell) or community in a matrix of exopolysaccharide (EPS) by forming biofilms (embedded in EPS as a community), adhering to a surface or rooting in a matrix. Exopolysaccharide is an extrapolymatrix substance that serves as a protective layer that surrounds the gathering of microorganisms (biofilm) from several environmental and antimicrobial effects. Biofilm production helps bacteria in multiple ways to survive, such as antibiotic resistance and persistent infections. A much-needed approach is required to evaluate methods for targeting biofilm formation and cellular progression by microorganisms within biofilm formation, as biofilm formation is responsible for several severe types of bacterial infections in the body. This study aimed to collect clinical bacterial isolates to determine their potential for producing biofilms by relating to antibiotic resistance. Memon Medical Institute (MMI) Hospital in Karachi, Pakistan, provided 150 gram-positive clinical samples, including 92 (61.33%) biofilm producers. An antibiogram assay was performed along with biofilm quantification tests to determine the relationship between antibiotic resistance and the biofilm formation potential. Cultures were obtained from humans (pus, blood, HVS, urine, and wound). Identified bacterial cultures were screened for biofilm production and non-production using the Congo Red Agar (CRA) method and quantified as no, weak, moderate, and strong biofilm formation by measuring the absorbance using the Trypticase Soya Broth (TSB) method in 96-well microtiter plates. Our findings revealed that *S. aureus* and CONS (coagulase-negative Staphylococci) were highly resistant to ciprofloxacin and moderately resistant against other antibiotics. A significant correlation was at the 0.01 level (2-tailed) with ($p < .001$), which indicates a strong positive relation between biofilm-forming potential and antibiotic resistance in prevailing isolates of CONS and *S. aureus*. PCR was performed for 05 strong biofilm-forming isolates of S. aureus for the icaA gene, and one isolate (20%) was positive for the presence of the icaA gene, which shows the presence of multiple genes responsible for biofilm formation in *S. aureus*. Results indicated a significant contribution in the increased biofilm production by the clinical pathogens resistant to two or more antibiotics, both practically and statistically.

Keywords: microorganisms, clinical bacterial isolates, biofilm, antibiotic resistance.

卡拉奇革蘭氏陽性臨床分離株潛力的抗菌譜模式和生物膜

摘要：大多數微生物可以在這兩種條件下生存，例如浮游（作為單細胞）或透過形成生物膜（作為群落嵌入胞外多醣）、黏附在表面或紮根於基質中的胞外多醣基質中的群落。胞外多醣是一種外多基質物質，可作為保護層圍繞微生物聚集（生物膜），免受多種環境和抗菌作用的影響。生物膜的產生以多種方式幫助細菌生存，例如抗生素抗藥性和持續感染。需要一種急需的方法來評估針對生物膜形成內的微生物的生物膜形成和細胞進展的方法，因為生物膜形成是體內幾種嚴重類型的細菌感染的原因。本研究旨在收集臨床細菌分離株，以確定其與抗生素抗藥性相關的產生生物膜的潛力。巴基斯坦卡拉奇梅蒙醫學研究所醫院提供了150份革蘭氏陽性臨床樣本，其中包括92份（61.33%）生物膜產生者。進行抗菌譜分析和生物膜定量測試，以確定抗生素抗藥性和生物膜形成潛力之間的關係。培養物取自人類（膿液、血液、高位陰道拭子、尿液和傷口）。使用剛果紅瓊脂方法篩選鑑定的細菌培養物的生物膜產生和不產生，並透過使用胰蛋白酶大豆肉湯法在96孔微量滴定板中測量吸光度來定量為無、弱、中度和強生物膜形成。我們的研究結果表明，金黃色葡萄球菌和凝固酶陰性葡萄球菌對環丙沙星高度抗藥性，對其他抗生素具有中度抗藥性。顯著相關性為0.01水平（2尾）（$p < .001$），這表明凝固酶陰性葡萄球菌和金黃色葡萄球菌的主要分離株中生物膜形成潛力與抗生素抗藥性之間存在很強的正相關關係。對05個強生物膜形成金黃色葡萄球菌分離株進行icaA基因聚合酶鏈反應，其中1個分離株(20%)的icaA基因呈陽性，這表明在金黃色葡萄球菌中存在負責生物膜形成的多重基因。金黃色葡萄球菌。結果表明，無論是實踐上還是統計學上對兩種或多種抗生素具有抗性的臨床病原體對生物膜產生的增加做出了重大貢獻。

关键词：微生物、臨床細菌分離株、生物膜、抗生素抗藥性。

1. Introduction

Under stressed conditions, bacteria develop survival strategies and attach themselves to available surfaces by forming biofilms. Microorganisms produce extracellular polymeric substances (EPS) as a matrix by fixing themselves to the surface, expressing a distorted phenotype in contrast to planktonic cells, particularly for their growth, gene transcription, protein production, and intercellular interaction within biofilms. This matrix facilitates the microbial community by providing a rigid structure, protecting them from unfavorable environmental conditions, gene regulation, and nutritional absorbance [10]. Microorganisms (bacteria, viruses, fungi, and others) can form biofilms on almost any organic or inorganic surface and are capable of surviving on dry surfaces for prolonged periods. These attached microbes are difficult to subside by conventional elimination methods as biofilm increases around 1000 folds in resistance to the same organism growing as planktonic [25]. With an increased rate of antibiotic resistance, evidence of a biofilm relationship with multidrug-resistant bacteria has been noticed. Microorganisms are capable of forming biofilms on both biological (human tissues and cells) and non-biological surfaces (medical devices).

Biofilm regulation is controlled using the process of the quorum sensing regulatory process, which is a complex mechanism that involves the regulation of several genes for the maintenance and maturation of biofilms. The high bacterial cell density activates QS regulation pathways. Apart from providing tolerance to different antimicrobial treatments, biofilms also contribute to the regulation and downregulation of virulence factors in some bacteria. For example, biofilm formation increases virulence factor expression in *Pseudomonas aeruginosa* while downregulating exotoxins and other virulence factors in the case of *Staphylococcus aureus* [13]. The release of signaling molecules or secretion of autoinducers controls QS.

These autoinducers are responsible for sensing the local concentration of bacteria to begin intensive inhabitant response such as biofilm formation. The chemical nature of the autoinducers is significantly different between gram-positive and gram-negative bacteria because QS is a highly conserved molecular mechanism [31]. For example, in gram-negative bacteria, autoinducers belong to the chemical class of acylhomoserine lactones, whereas in gram-positive bacteria, they are short peptides of approximately 5-50 amino acids, synthesized by ribosomes and often subjected to general posttranslational modification. Many researchers have investigated different approaches to control the process of bacterial quorum sensing, but this remains an area of continuous and rigorous research in the quest to control biofilm expansion [12, 18, 19, 23]. The shifting of bacteria from single living to biofilm formation occurs in a series of steps or phases. Generally, the stages of biofilm formation include i) conditioning film formation, ii) attachment of cells, iii) microcolony formation, which eventually merges to become mature biofilms, and iv) dispersion of biofilm and re-colonization [22]. In the clinical gram-positive pathogen S. aureus, organisms dispersed from a biofilm are then capable of producing biofilm in the future in the human body or other surfaces that can lead to the progression of severe infections [41].

2. Materials and Methods

2.1. Sample Collection

A total of 150 bacterial isolates from various clinical specimens such as pus, urine, blood, high vaginal swab (HVS), and wounds (from May to September 2019) were collected. Samples were grown on solid agar media plates, and colonial, morphological, and biochemical assays were performed for identification and differentiation.

2.2. Screening of Biofilm-Forming Bacteria

Screening of isolates capable of forming biofilms was done by Congo Red Agar (CRA) method, designed for identification and differentiation of bacterial colonies as biofilm and non-biofilm forming. CRA was prepared with the standard protocol as mentioned in [8]. Briefly, brain heart infusion broth (Oxoid) 37g/l, agar No 1 (Oxoid), sucrose 50 g/l, and Congo red stain 0.8 g/l in aqueous solution (autoclaved) were mixed separately. All bacterial isolates were cultured on CRA and incubated for 48 h at 37^{o}C. Organisms with grayish-to-black colonies were considered biofilm formers, whereas non-biofilm formers had pink colonies.

2.3. Microtiter-Plate Method

Quantitative analysis was performed using a microtiter-plate [6-15]. In brief, cultures were inoculated in 3-5 ml of trypticase soya broth (TSB) for 24 h and further diluted to 1:100 in fresh broth supplemented with 0.2% glucose. Culture was 200 µl of diluted and added to each well of a 96-well microtiter plate. Covered plates were incubated for 48 h. Free floating filling from each well was removed and washed three times with PBS. Staining for attached cells as biofilm was performed for 10 min with 125 µl of 0.2% crystal violet solution, followed by washing with clean tap water and leaving to air dry. 200 µl of 95% Ethanol was added to each well and incubated for 10 to 15 min at room temperature. The control was kept as blank TSB. The optical density (OD) of the wells measuring at 595 nm used a microplate reader (Diatek Dc-200Bc). Organisms were characterized as No, Weak, Moderate, and Strong biofilm formers by calculating the cutoff values below (Table 1) [27]. All tests were performed in triplicate.

Table 1 Values for NO, strong, weak, and moderate biofilm formation, ODc = optical density of control, OD test optical density

Cut-off value	Biofilm production
OD = ODc	No biofilm production
ODc < OD = 2 ODc	Weak biofilm production
2 x ODc < OD = 4 ODc	Moderate biofilm production
4 x ODc < OD	Strong biofilm production

2.4. Antibiogram Assay

The antibiotic susceptibility/resistance pattern of isolates was examined by the Kirby-Bauer method, following the Clinical Laboratory Standard Institute (CSLI) procedure [3, 32]. We used antibiotics:

- Amikacin (30 µg);
- Gentamicin (10 µg);
- Cefradin (30 µg);
- Amoxiclave (30 µg);
- Cefaclor (30 µg);
- Ciprofloxacin (5 µg);
- Erythromycin (15 µg);
- Cefuroxime (30 µg);
- Chloramphenicol (30 µg);
- Co-trimoxazole (10 µg);
- Nalidixic acid (30 µg);
- Fucidic acid (10 µg);
- Vancomycin (30 µg);
- Clindamycin (10 µg);
- Meropenem (10 µg);
- Tobramicin (10 µg);
- Ampicilin (10 µg);
- Ceftriaxone (30 µg);
- Levofloxacin (5 µg);
- Nitrofurantoin (50 µg);
- Teicoplanin (30 µg);
- Linezolid (30 µg) according to the organisms.

2.5. PCR for the icaA Gene

Primers icaA (F) ACACTTGCTGGCGCAGTCAA and icaA (R) TCTGGAACCAACATCCAACA were used for strong biofilm-forming isolates of *S. aureus, and* all 05 samples were tested for the icaA gene [42, 43]. In brief, monopole PCR for 25µl volume was performed containing 12 µl mastermix, DNA-free water, 1µl of reverse and forward primers, 3µl of DNA template. Amplification was performed under the following conditions: initial denaturation (94°C, 4 min). Denaturation (94°C, 30 s), primer annealing (56°C, 30 s), extension (72°C, 1 min), and final extension (72°C, 10 min) 35 cycles were performed and stored at -4°C. Analysis of the PCR product was performed using 2% agarose gel electrophoresis stained with ethidium bromide.

3. Results

Isolation of cultures made from human clinical specimen like pus, urine, blood, HVS (high vaginal swab), and wound gave a variety of bacterial growth in clinical laboratory like gram positive organism, gram negative organism and various pathogenic fungi species (data not shown).Gram positive bacterial cultures were identified as *S. aureus* (80) 53.33%, *CONS (coagulase negative Staphylococci)* (25) 16.66%, *Enterococcus Spp.* (15) 10.00%, *Streptococcus Spp.* (20) 13.33%,and *Micrococcus Spp.* (10) 6.66% (Table 2). Screening for the potential of biofilm formation with the help of Congo red agar (CRA) method qualitatively resulted in 92 organisms of 61.33% able to form biofilm. Quantitative testing and categorization of biofilm-forming isolates was done with the help of TSB by measuring optical density at 595nm suggested four categories of biofilm formation as No, Weak, Moderate, and Strong biofilm-forming bacteria by calculating of cutoff value formulation (Fig. 1, Tables 1 and 5). *S. aureus* and CONS were observed to be more biofilm formers with OD ranging 0.850 to 3.503. *S. aureus* and CONS were also the prevailing organisms reported in large number in all, associated with the highest biofilm-forming percentage at 81.25% and 56%, respectively (Table 2). *S. aureus* was observed as strong biofilm former, along with increased antibiotic resistance whereas *Enterococcus Spp* showed second highest biofilm activity as 73.33% in all but it was not observed to be a significant multidrug resistant except for Levofloxacin (10 isolates 90.90%), see Table 3. Antibiogram analysis results indicated that all organisms were resistant to two or more antibiotics. The highest resistance was seen by *S. aureus against ciprofloxacin* (isolates), cefuroxime and erythromycin (28 isolates), and meropenem (24 isolates). CONS were also resistant to clinically important antibiotics such as ciprofloxacin, cephradin, cefaclor, cefuroxime, and co-trimoxazole in a significant percentage (Tables 3 and 4). PCR was performed for 05 strong biofilm-forming isolates of *S. aureus for the icaA gene, and* one isolate was positive for the presence of the icaA gene (Fig. 2), which represents the presence of multiple genes responsible for biofilm formation in *S. aureus*. Overall statistical analysis of the relationship was done with Spearman's rho by measuring the correlation coefficient on SPSS 16.0. A significant correlation was observed at the 0.01 level (2-tailed) with ($p < .001$). Our results indicate a strong positive relationship between biofilm-forming potential and antibiotic resistance among the prevailing isolates of CONS and *S. aureus.* Results clarify the phenomena of Karachi, a metropolitan city and the center of business and trade in Pakistan is also among the cities of other developing countries, facing and fighting the crisis of antibiotic resistance. Among the others such as misuse, high mutation, and virulent factors of bacteria, biofilm has played an important role in antibiotic resistance. Biofilms have been responsible for the failure of antibiotic treatments, multidrug resistance (MDR), pan-drug resistance PDR (bacteria having no susceptibility to all available clinical drugs), and extensive drug resistance (XDR) globally, and our findings also reveal that phenomenon. A significant relationship has been observed from the results of our research as well.

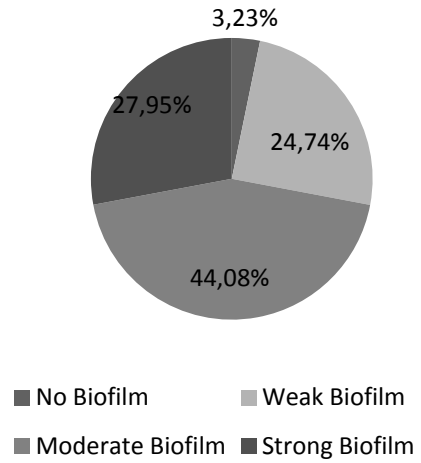

Fig. 1 Biofilm-forming categories of the isolates

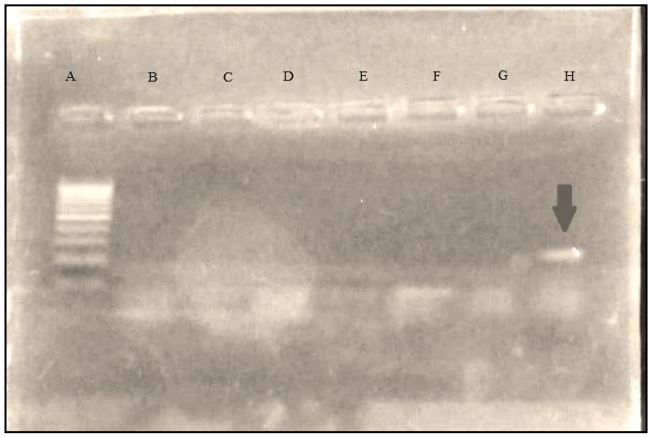

Fig. 2 PCR result on agarose gel for biofilm forming gene icaA. (A= DNA Ladder, B is negative control, C, D, E, F, G, H are tested bacterial isolates, arrow indicates the presence of gene)

Table 2 Sample collection

No.	Organisms	Number of isolates	Percentage	Biofilm forming
1	*S. aureus*	18	80.0%	64
2	*CONS (coagulase negative Staphylococci)*	25	56.0 %	14
3	*Enterococcus Spp*	15	73.0%	11
4	*Streptococcus Spp*	20	10.0%	2
5	*Micrococcus Spp*	10	10.0%	1
Total number of isolates		150	61.33%	92

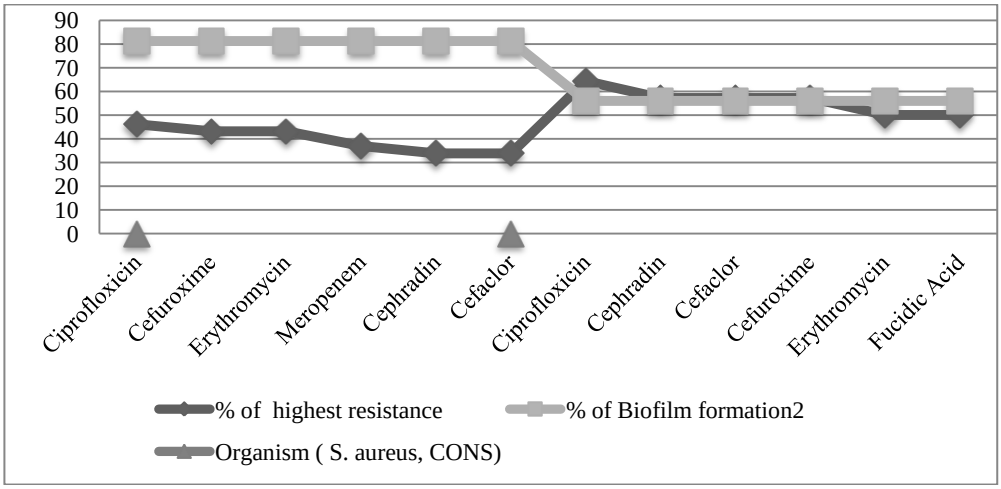

Fig. 3 % of biofilm formation and % of highest resistance

Table 3 Antibiogram analysis (R - resistance, S - sensitive, (-) - not determined, S.A - *S. aureus*, CONS - coagulase negative *Staphylococcus*, S.S - *Streptococcus Spp*, E.S - *Enterococcus Spp*, M.S - *Micrococcus Spp*)

Antibiotics	**S.A n = 64**		**CONS n = 14**		**S.S n = 2**		**E.S n = 11**		**M.S n=1**	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
Amikacin	8	57	3	11	1	1	-	-	1	0
Gentamicin	13	52	6	8	0	2	-	-	0	1
Amox + Clave	8	57	0	14	0	2	3	8	0	1
Cephradin	22	43	8	6	-	-	-	-	-	-
Cefaclor	22	43	8	6	-	-	-	-	-	-
Cefuroxime	28	37	8	6	-	-	-	-	-	-
Ciprofloxacin	30	35	9	5	-	-	-	-	-	-
Nalidixic acid	12	53	1	13	-	-	-	-	-	-
Erythromycin	28	37	7	7	1	1	-	-	-	-
Chloramphenicol	7	58	1	13	0	2	-	-	1	-
Co-trimoxazole	12	53	8	6	1	1	-	-	-	-
Fucidic Acid	20	45	7	7	-	-	-	-	-	-
Vancomycin	7	58	0	14	1	1	2	9	-	-
Clindamycin	9	56	1	13	1	1	-	-	0	1
Meropenem	24	41	1	13	0	2	2	9	-	-
Linezolid	2	63	0	14	1	1	0	11	-	-
Teicoplanin	-	-	-	-	-	-	2	9	-	-
Nitrofurantoin	-	-	-	-	-	-	1	10	-	-
Tobramycin	-	-	-	-	0	2	-	-	-	-
Ampicilin	-	-	-	-	2	0	4	7	-	-
Ceftriaxone	-	-	-	-	1	1	-	-	-	-
Levofloxacin	-	-	-	-	1	1	1	1	-	-

Table 4 Isolates with the highest resistance

Categories	**No. of organisms**	**%**	**Optical density range at 595 nm**
No biofilm	03	3.26	0.163 to 0.196
Weak biofilm	23	25.00	0.208 to 0.391
Moderate biofilm	40	43.47	0.395 to 0.764
Strong biofilm	26	28.26	0.850 to 3.503
Total	92	100	

Table 5 Biofilm-forming categories of isolates with optical density OD of 595 nm

Organisms	Antibiotics	No. of isolates resistant	% of resistance
S. aureus 65 isolates	Ciprofloxacin	30	46.15
	Cefuroxime	28	43.07
	Erythromycin	28	43.07
	Meropenem	24	36.92
	Cephradin	22	33.84
	Cefaclor	22	33.84
CONS 14 isolates	Ciprofloxacin	9	64.28
	Cephradin	8	57.14
	Cefaclor	8	57.14
	Cefuroxime	8	57.14
	Erythromycin	7	50.00
	Fucidic Acid	7	50.00

3.1. Result Statistics

Spearman's rho by measuring correlation coefficient on SPSS 16.0 was calculated at the 0.01 level (2-tailed) and used to evaluate the relationship linking biofilm formation and antimicrobial resistance. Optical densities of the biofilm were expressed as the mean of triplet observations.

4. Discussion

Microorganisms are responsible for infections and their spread in communities and healthcare settings. The rate of their spread has grown with the rate of antimicrobial resistance (AMR) [29]. The emergence of AMR is a rapidly growing problem in healthcare institutions worldwide [21]. Biofilm formation has now been adopted as a defensive mechanism by several pathogenic microorganisms against unfavorable conditions, such as in the presence of antibiotics, disinfectants, and other traditionally used antimicrobial agents. It is considered that biofilm-forming potential has a significant connection with AMR, which eventually results in therapeutic failure [23, 11]. The exploitation of antimicrobial drugs leads to changes in bacterial metabolism that support the attainment of resistance [30]. Biofilm is the most extensively disseminated and victorious mode of existence by organisms on earth [9]. These are composed of both single and different bacterial species with complex and active structures. Biofilms have a composite character that is extraordinarily essential to the microbial cells to live in intolerant situations, providing a gathering of strategy to avoid the effectiveness of antimicrobial agents [16]. Recently, effective approaches such as quorum sensing inhibition, biofilm denaturing, and biofilm interfering molecules are in research processes to fight this problem, but much analysis is still necessary to hinder, treat, and avoid biofilm-related infections on the molecular level [17]. In this study, the possible relationship between antimicrobial resistance and the ability to form biofilms from gram-positive clinical isolates was evaluated. With reference to past studies, the results of this study have demonstrated that there has been an interaction between biofilm formation and antibiotic resistance. The highest resistance was seen with strong biofilm formation by *S. aureus* against ciprofloxacin (46.15%), cefuroxime and erythromycin (43.07%), and meropenem 36.92%. CONS were also strong biofilm formers and resistant to clinically important antibiotics such as ciprofloxacin, cephradin, cefaclor, cefuroxime, and co-trimoxazole, ranging from 64% to 50% of isolates (table 4). This shows the emergence of antibiotic failure in Pakistan, similar to other countries in the Middle East. [37] investigated antibiotic resistance in Pakistan and highlighted the AMR representation in Pakistan for the last 10 years. Pakistan has an important ecological position as a bordering neighbor of the Middle East, bordering with China, Afghanistan, Iran, India, and Uzbekistan (central Asian state) [39]. Outcomes of study have demonstrated that most common clinical pathogens were able to fight against typically used antibiotics in Pakistan. This study reports significant incidences of antimicrobial resistance associated with biofilm formation from clinical isolates in Karachi hospital. Further studies can help in finding the overall increase of antimicrobial resistance to overcome this health hazard in the region. Karachi is the center of trade and business in the Sindh province, and increased AMR can therefore spread at a high level if proper measures are not taken. The molecular studies of antibiotic resistance are among the basic necessities to obtain a detailed understanding of antibiotic resistance, and this can further help to design novel or substituted treatments [38]. Our findings were also relatable to the results of [33] on biofilm-producing MDR and CONS strains; they showed resistance up to 100% to penicillins. In contrast, another study conducted in Pakistan [34] identified *S. aureus* as a common pathogen found in clinical medical devices forming biofilm (39%). [35] also concluded that 92 (95.8%) of the *S. aureus methicillin*-resistant *Staphylococcus aureus strains* collected clinically were able to form biofilms, and the four categories of isolates were (4.2%), 52 (54.2%), and 44 (35.4%) as strong, moderate, and weak, respectively. [22] studied 54 *S. aureus* isolates out of 2160 samples and their presence in meat products, dairy, fruits and vegetables, and desserts. Most strains forming biofilms (52/54) were resistant to at least one of the antibiotics, and 21

(38.9%) and three (5.6%) isolates were identified as MDR and MRSA, respectively. According to the conclusion of [26], biofilm analysis using Congo red agar and tube methods showed that 57% of the methicillin resistant isolates collected from different food commodities were biofilm producers. It was also observed that oxacillin induced a dogmatic influence on biofilm formation. Biofilm formation is one of the mechanisms used as a survival strategy to fight against tools and techniques used to kill them, and it also increases the virulent factors and targeting biofilm can help to reduce the severe infections and antimicrobial resistance globally. Biofilm formation in S. aureus is encoded by 12 genes such as; fibrinogen-binding proteins (*fib*) fibronectin-binding proteins (*fnbA* and *fnbB*) intercellular adhesion (*icaA, B, C* and *D*), clumping factor (clfA and B), elastin-binding protein (ebps), laminin-binding protein (*eno*), and collagen-binding protein (*cna*) genes [44, 45]. In these studies, biofilm formation in *S. aureus* was genetically tested by the presence of polysaccharide intercellular adhesion (PIA), which is coded by the ica operon. The presence of ABCD genes is responsible for biofilm production in *S.aureus* [42]. From the clinically isolated strains of *S. aureus, the presence of the icaABCD* gene indicates the potential biofilm formation from the organisms [43]. Biofilm production in *S. aureus* is connected to the presence of intracellular polysaccharide IPA locus and its product is icaABCD genes, which are responsible for the production of adhesion and multilayered biofilm. In this study, five *S. aureus* isolates with strong biofilm production were tested, from which one showed the icaA gene. Researchers should design strategies that focus on molecular mechanisms that relate to biofilm proliferation in bacteria [16, 14]. Pakistan is also among all other AMR-stricken countries, and researchers here are also trying to find ways to reduce AMR by targeting biofilm preregression in clinical isolates, and this study is one such approach.

5. Conclusion

This study has shown evidence of a relationship between biofilm production and antibiotic resistance among clinically isolated gram-positive bacteria. Biofilm production by bacteria facilitates antibiotic tolerance and makes it difficult to treat infections. Several studies (previously discussed in this paper) have demonstrated a critical role of biofilm in modulating antibiotic resistance. This study is also evidence of the relationship between antibiotic resistance and biofilm formation in clinical isolates from Karachi, Pakistan. This study is based on in vitro evaluations for general studies and can help future researchers plan antibiofilm approaches against MDR bacteria. Further evaluation of biofilm and antibiotic resistance kinetics at the genetic and molecular levels will help design antibiofilm drugs.

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