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Detection of MRSA and MSSA biofilms in clinical specimens from a tertiary care hospital located in Hyderabad

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Abstract
Background: MRSA, or methicillin-resistant *Staphylococcus aureus*, is a common pathogen that causes a wide range of infections, ranging from minor skin infections to severe conditions such as endocarditis and osteoarticular infections, which are associated with significant morbidity and mortality rates. Biofilm formation is crucial in MRSA's capacity to infiltrate, propagate, and withstand antimicrobial therapies.
Methods: This study was performed at the microbiology division of a tertiary healthcare facility in Hyderabad. The identification of MRSA was carried out using the cefoxitin disk diffusion method. At the same time, the detection of biofilm was performed through the utilization of microtiter plate and Congo red agar techniques.
Results: In this study, 235 samples of *S. aureus* were analyzed. Of these, 104 samples were identified as MRSA, and the remaining 131 were classified as MSSA (Methicillin-Susceptible *Staphylococcus aureus)*). Seventy of the 104 MRSA (67%) samples and sixty-two of the 131 MSSA (47%) samples were found to be multidrug-resistant, and these samples were checked for biofilm production in this study. According to the microtiter plate method, MRSA showed 26% of the biofilm production, while only 4.3% was found on Congo Red Agar. Comparatively, the Congo red agar method demonstrated just 2% and the microtiter plate method demonstrated 10% of biofilm formation in MSSA.
Conclusion: This study shows that, when compared to Congo red agar, the Microtiter plate method is more accurate for detecting biofilm-producing *staphylococci*. Compared to MSSA strains, MRSA strains typically produced more biofilm. Biofilm producer exhibits broad antibiotic resistance. Before administering treatment, clinicians, along with the microbiologists, should routinely monitor biofilm formation in hospitals. To enhance biofilm management in healthcare settings, educating stakeholders about biofilms and ad hoc efficacy tests is crucial, which are frequently academic.

Keywords: *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, methicillin sensitive *Staphylococcus aureus*, biofilm, Congo red agar

Introduction
Staphylococcus aureus is a resilient pathogen that is resistant to drugs. It has the potential to induce infections in the skin and soft tissues, which can subsequently progress to serious conditions such as endocarditis, osteomyelitis, pneumonia, and various other invasive diseases [1]. The word "biofilm" was initially coined in 1975 following the observation of biofilms in a trickling wastewater filter, referring to the complex microbial community that attaches to various surfaces, including both abiotic and biotic substrates [2]. The development of biofilm required an appropriate substrate or surface as well as environmental conditions. Specifically, the process of colonization and subsequent biofilm formation is more common on rough surfaces due to their increased surface area and advantageous physicochemical properties [3, 4, 5]. Implanted medical device materials or biomaterials can serve as susceptible targets for biofilm formation due to their inherent characteristics. The adherence of biofilms can differ in terms of both rate and extent, influenced by the specific chemical composition that coats the biofilm [6, 7, 8]. *Staphylococci,* such as MRSA, are recognized as the primary source of infections associated with biofilms [9]. The composition of *S. aureus* biofilms primarily comprises water and organic components. Within the biofilm structure, bacterial micro colonies and extracellular polymeric substance (EPS) play a significant role [10]. EPS is a complex mixture of various polymeric compounds, such as polysaccharides, extracellular DNA (eDNA), and proteins.

Biofilm formation occurs through a series of distinct stages, encompassing attachment, formation/maturation, and dispersal [11].The primary adhesion of a bacterium to living (such as endovascular, bone, or joint) or non-living (like prosthetic device/catheter) surfaces requires the interaction of various proteins referred to as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) [12].The key proteins responsible for *S. aureus* adhesion are clumping factor A (ClfA) and clumping factor B (ClfB), which are part of the Microbial Surface Component Recognizing Adhesive Matrix Molecule [13]. By binding to fibrinogen, these clumping factor proteins direct the initial attachment process [9]. Additional microbial surface components that recognize adhesive matrix molecules in the arsenal of *S. aureus* encompass bone sialo-binding protein (Bbp), responsible for attaching to the extracellular matrix, and collagen adhesion (Can), which specifically binds to collagen [13]. Another similar protein that is involved in initial attachment that is expressed by the eno gene is alphaenolase. By binding plasminogen, it plays a role in *S. aureus*'s adhesion to the extracellular matrix [14]. Due to this ability to bind fibrinogen and plasminogen makes it easier for MRSA to colonize injured areas [14]. After the attachment and initial growth of the micro colonies, the biofilm commences its maturation process through the secretion of the extracellular polymeric substance (EPS).

Biofilm-related MRSA has the potential to induce a wide array of infections, spanning from skin and soft tissue infections to more severe cases such as bloodstream infections (BSIs), osteomyelitis, and infective endocarditis (IE). The prevalence of MRSA infections poses a major challenge in intensive care units, as numerous strains have acquired resistance to multiple antibiotics. The gravity of these infections is exacerbated by their connection to healthcare facilities and the significant morbidity and mortality they entail [15, 16].

Biofilm-associated microorganisms possess the capacity to undergo dormancy for prolonged durations, spanning from weeks to years. This dormant state can endure until favorable conditions emerge, resulting in the development of localized or systemic signs and symptoms of infection. The recurrence of infections following multiple antibiotic treatments is frequently linked to the existence of biofilms [17, 18]. Due to their composition and polymeric matrix, biofilms offer a distinct protective mechanism that allows bacteria to resist antibiotics by inhibiting antibiotic diffusion, resulting in the development of multidrug-resistant bacterial populations. Bacteria within biofilms demonstrate antibiotic resistance levels that can be up to 1000 times higher than those found in their planktonic counterparts [19] Multidrug-resistant infections present a significant obstacle for antibiotic treatment and elimination of infections. The primary risk factor linked to MRSA as the underlying cause of certain healthcare-associated infections is the utilization of intravenous antibiotics within the preceding 90 days. Apart from antibiotic resistance, biofilms are crucial in different infections like skin and soft tissue infections, medical-device-related infections, and catheter-associated intravascular or urinary tract infections by providing protection against environmental stressors (such as sheer forces, drying) and phagocytosis [20, 21].

Material and Methods

The study was conducted within the microbiology division of the tertiary healthcare facility located in Hyderabad from 2021 to 2023, spanning duration of two years. A variety of clinical specimens were employed in this study, such as blood, pus, sputum, urine, and other bodily fluids. Blood agar, MacConkey agar, and Nutrient agar were utilized for the cultivation of all samples, except for urine. Cysteine lactose electrolyte deficient agar (CLED Agar) was specifically used for the analysis of urine samples [22].

The antimicrobial susceptibility of the isolates was assessed using the Kirby-Bauer method, also known as the disk diffusion method, on Muller-Hinton agar plates. Fourteen antibiotics were evaluated against *Staphylococcus aureus* in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI). To determine the presence of methicillin-resistant *Staphylococcus aureus* (MRSA), a cefoxitin (30mg) disk was used as a surrogate marker [23].

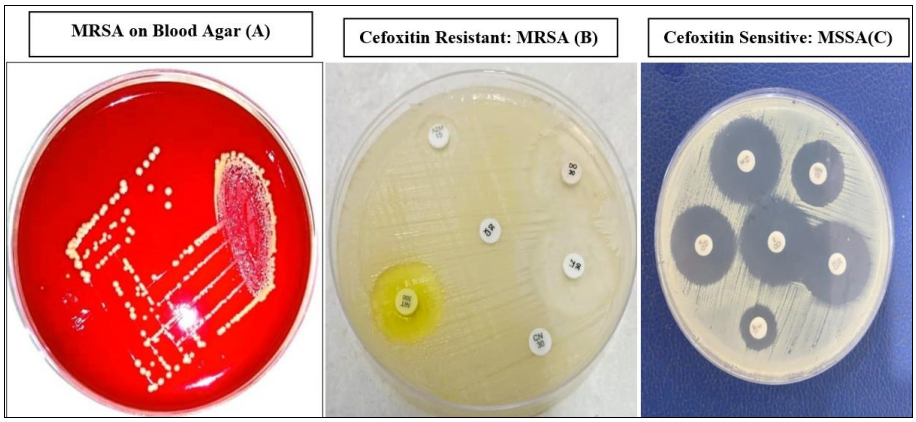

Fig 1: In picture (A), colonies grown on blood agar were golden yellow in color; in picture (B), colonies were cefoxitin-resistant, indicating MRSA; and in picture (C), colonies were cefoxitin-sensitive, indicating MSSA

Biofilm-Production Identification Using the Tissue Plate Method

The isolates were revived by incubating them on 5% sheep blood agar at 37°C for 18 to 24 hours. Following this, the bacterial cells were transferred to brain heart infusion broth (BHIB) to create a cell suspension with approximately 108 CFU/mL. Next, 200mL of this BHIB suspension was inoculated into the wells of a tissue culture polystyrene 96-well plate in duplicate. The biofilms were allowed to develop for 48 hours at 37°C. Once this time had elapsed,

the supernatant was removed and any no adherent bacterial cells were eliminated by washing the biofilms three times with 250mL of sterile phosphate-buffered saline (pH 7.4). The biofilm was then fixed with 200mL of methanol per well for 15 minutes and stained with 200mL of 1% crystal violet per well for 5 minutes. After rinsing with distilled water, the plates were left to air dry. Subsequently, the colorant was dissolved in 95% ethanol and the absorbance at 490 nm was measured using a micro plate reader. Four wells were dedicated to positive and negative controls each time. A cutoff value was established, and any absorbance value above this cutoff was considered positive. The biofilm was then categorized as moderate, or severe based on the absorbance readings [24]. An OD value below 0.120 indicates that the organism is not capable of producing biofilm. On the other hand, an OD value ranging from 0.120 to 0.240 suggests moderate biofilm production. Finally, an OD value exceeding 0.240 signifies a strong ability to produce biofilm [25].

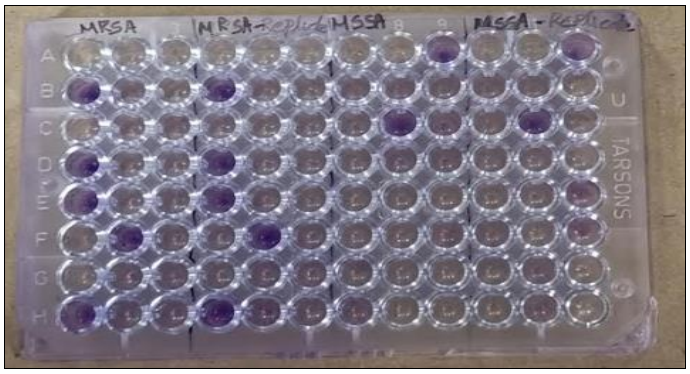

Fig 2: Biofilm formation in a microtitre plate

Congo Red Agar Method for Biofilm Production Detection

Briefly, CRA plates were prepared using trypticase soy agar supplemented with 5% sucrose and 40 µg/mL Congo red dye (Sigma-Aldrich; Budapest, Hungary). Congo red is a secondary diazo dye, which can be used as a pH indicator (with a detectable color change at pH 3.0–5.2). Strains were cultured on trypticase soy agar plates at 37 °C for 16–18 h; cells were resuspended in trypticase soy broth at a density of OD600=2; 10 µl of the suspension was spotted on CRA plates. The inoculated CRA plates were incubated at 37 °C in aerobic conditions for 24 h, followed by incubation at room temperature before the reading of the plates for an additional 24 h. The isolates were assessed for their colony morphologies: black colonies with a dry consistency and rough surface edges were considered as biofilm-producers in this assay, while red colonies with smooth, round and shiny surface were read as negative for biofilm production [26].

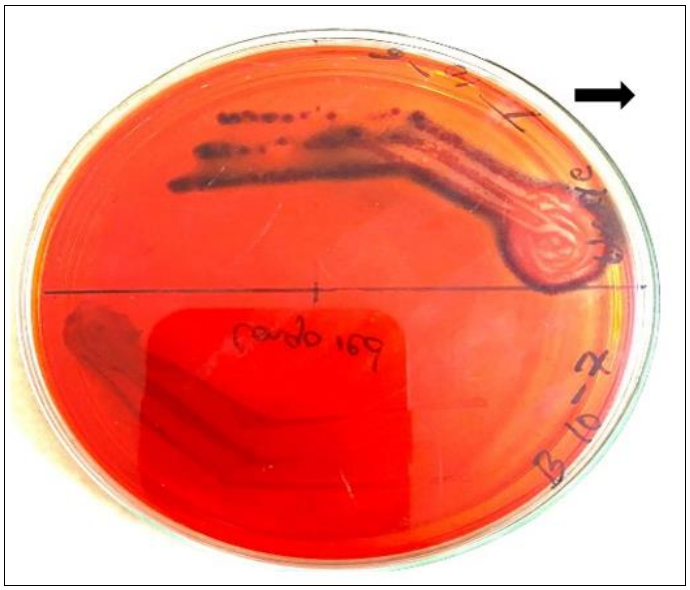

Fig 3: The upper section colonies displays the black color pigmentation on Congo red agar and lower section colonies are negative for biofilm production

Extraction of DNA and polymerase chain reaction

DNA was isolated from colonies of *S. aureus* utilizing the Qiagen Mini kit (cat no51304). Primers were utilized to amplify a 310 bp fragment of the mecA gene through PCR amplification

Given primers were utilized for the amplification of 310 bp fragment of mec Agene. *S. aureus* ATCC 43300 was used as a positive control and negative control also included [27].

mecA1: 5[1]GTA GAA ATG ACT GAA CGT CCG ATA A

3^1

mecA2: 5^1CCA ATT CCA CAT TGT TTC GGT CTA A 3^1

Amplification

The PCR solution was prepared by combining 12.5 µL of mastermix (Fermentas), 1 µL of mecA 1, 1 µL of mecA 2, 5.5 µL of water, and 5 µL of extracted DNA, resulting in a final volume of 25 µL. The PCR was set up using the BIO-RAD T-100 Thermal cycler, following these steps, begin by denaturing at 94°C for 1 minute, followed by annealing at 60°C for 1 minute, extension for72°Cat 40 seconds, and final extension at 72 °C for 5 minutes. PCR products were analyzed using 2% agarose gel electrophoresis [27].

Results

In this study, a total of 70 samples of MDR (Multi drug resistance) MRSA and 62 samples of MDR MSSA were incorporated to assess their capacity for biofilm production. In the analysis of 70 MRSA samples, it was found that 18 (26%) samples demonstrated biofilm production when utilizing the tissue culture plate method. Furthermore, only 3 (4.3%) samples were identified as having the ability to produce biofilms when Congo red agar was employed. In contrast, out of 62 MSSA samples, only 6 (10%) were found to be biofilm producers using the micro titer plate technique, while just one sample (2%) exhibited biofilm production according to the Congo red agar method.

The table 1 below illustrates the antibiotic sensitivity pattern of MRSA towards various antibiotics. Azithromycin exhibits a resistance rate of 72%, while Cefotaxime demonstrates a resistance rate of 91%. On the other hand, cephalexin exhibits a complete resistance of 100%, and clindamycin shows a resistance rate of 49%. However, a resistant pattern of 14% was observed for Teicoplanin, while Vancomycin and Linezolid exhibited resistance rates of 2% and 6% respectively.

Table 2 displays the antibiotic sensitivity pattern of MSSA to various groups of antibiotics. Levofloxacin exhibited a resistance rate of 73%, while ofloxacin showed a resistance rate of 69%. Clarithromycin demonstrated a resistance rate of 57%, whereas Erythromycin had a resistance rate of only 51%. Cefotaxime exhibited a resistance rate of 84%, while cephalexin showed complete resistance at 100%. On the other hand, Teicoplanin, vancomycin, and linezolid were more effective against MSSA.

Table 1: The antibiotic susceptibility pattern of various group of drugs to MRSA(n=104)

Antibiotic class	Antibiotics	Sensitive		Resistant	
		No	%	No	%
Aminoglycosides	Amikacin	66	63	38	36
	Gentamicin	70	67	34	33
Quinolones	Ofloxacin	54	52	50	48
	Levofloxacin	55	53	49	47
Cephalosporin's	Cefotaxime	09	9	95	91
	Cephalexin	0	0	104	100
Macrolides	Clarithromycin	48	46	56	54
	Erythromycin	37	35	67	64
	Azithromycin	29	28	75	72
	Clindamycin	53	51	51	49
Sulfonamides	Co-trimoxazole	64	61	40	38
Glycopeptides	Teicoplanin	89	85	15	14
	Vancomycin	102	98	02	2
Oxazolidinones	Linezolid	98	94	06	06

*No: Number

Table 2: The susceptibility pattern of different groups of drugs to MSSA (n=131)

Antibiotic class	Antibiotics	Sensitive		Resistant	
		No	%	No	%
Aminoglycosides	Amikacin	70	53	61	46
	Gentamicin	80	61	51	39
Quinolones	Ofloxacin	40	30	91	69
	Levofloxacin	35	28	96	73
Cephalosporin's	Cefotaxime	21	16	110	84
	Cephalexin	0	0	131	100
Macrolides	Clarithromycin	56	43	75	57
	Erythromycin	64	49	67	51
	Azithromycin	72	55	59	45
	Clindamycin	91	69	40	30
Sulfonamides	Co-trimoxazole	87	66	44	33
Glycopeptides	Teicoplanin	119	91	12	9
	Vancomycin	130	99	01	1
Oxazolidinones	Linezolid	131	100	0	0

Table 3: This table displays the total number of MDR MRSA and MSSA along with their biofilm production, using two distinct methods

Total MRSA isolates	Total MDR MRSA isolates	Biofilm production by			Total MSSA isolates	Total MDR MSSA isolates	Biofilm production by		
		Microtiter plate method		Congo red agar method			Microtiter plate method		Congo red agar method
104	70	18		03	131	62	06		01
		11(S)	07(M)				02(S)	04(W)	

S: Strong, M: Moderate& W: Week

*****MDR is characterized by a resistant pattern that encompasses more than three classes of antibiotics

The data presented in the table indicates that among the 70 MDR MRSA strains, a mere 4% (3 isolates) were observed to exhibit biofilm production when assessed using the Congo red agar method. However, when the microtitre plate method was employed, 26% (18 isolates) were classified as biofilm producers. Among these 18 biofilm producers, only 11 samples demonstrated strong biofilm production, while the remaining 07 samples displayed moderate biofilm production. In contrast, out of the 62 MDR MSSA isolates, only a single isolate (2%) was found to be biofilm-positive through the Congo red agar method. Conversely, when the microtitre plate method was utilized, six isolates (10%) were identified as biofilm producers. Among these six biofilm producers, only two samples exhibited strong biofilm production, while the remaining four samples displayed weak biofilm production.

In the microtitre plate method, MRSA biofilm-producing strains exhibited higher resistance to nearly all antibiotic classes. Levofloxacin displayed a resistance rate of 58%, while Clarithromycin demonstrated a resistance rate of 61%. Clindamycin exhibited a resistance rate of 67%, Erythromycin displayed a resistance rate of 70%, and Azithromycin exhibited the highest resistance rate of 88%. In contrast, MSSA biofilm-producing strains exhibited

varying levels of resistance to different antibiotics when tested using the microtiter plate method. Erythromycin demonstrated a resistance rate of 33%, while Clarithromycin showed a resistance rate of 50%. Levofloxacin resistance was observed in 67% of the strains, and Ofloxacin displayed the highest antibiotic resistance at 83%.

Table 4: This table presents the overall count of mecA gene detection in MRSA and its correlation with biofilm production

Total Number of MRSA Isolates	mecAgene Detected	mecA gene not detected	Total no.of biofilm producers by microtitre plate method	No.of isolates positive for mecA gene	No.of isolates Negative for mecA gene
70	62(88%)	08 (11%)	18	14 (78%)	4(22%)

Above table displays PCR method was employed to analyze 70 MRSA clinical isolates, revealing the presence of the mecA gene in 62 isolates, while 08 isolates tested negative for the mecA gene.When correlating biofilm production with the presence of the mecA gene among 18 biofilm producers, 14 isolates were found to test positive for the mecA gene (78%), while 4 isolates tested negative (22%).The presence of the mecA gene was linked to an elevation in biofilm production.

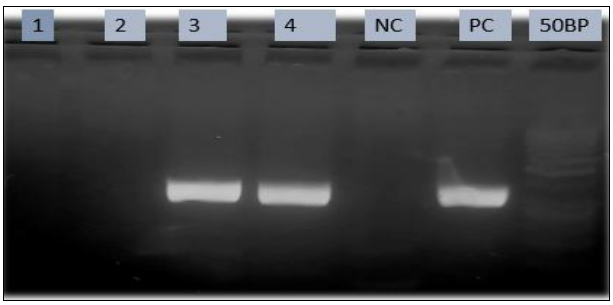

Fig 4: Amplification of mecA gene. The isolates in lanes 1 and 2 were found to be negative for the mecA gene, while the isolates in lanes 3 and 4 showed a distinct band at 310 bp, indicating a positive result for the mecA gene. Lane 5 is negative control and lane 6 is positive control (ATCC43300)

MRSA biofilm production was predominantly detected in pus samples (61%) using the microtitre plate method, with blood samples following at 39%. In contrast, MSSA exhibited the highest biofilm production in pus samples (10%) only when analyzed by the microtitre plate method.

According to the Congo red agar method, the MRSA strain exhibited a biofilm production rate of only 4.2% exclusively in pus specimens. Conversely, the MSSA strain displayed biofilm production in just one sample (2%), also limited to pus specimen.

The prevalence of MRSA biofilm producers was found to be higher in the ICU ward, while the orthopedic ward exhibited the second-highest prevalence.

Discussion

Biofilm is a crucial factor in the development of staphylococcal infections. Under stressful circumstances, microorganisms trigger the gene expression of biofilm as a response to stress. This biofilm enables bacteria to endure the harsh conditions, facilitating their attachment and colonization on both living and non-living surfaces like prosthetic devices, ultimately contributing to their prolonged presence on these devices [28, 29]. mecA is a component of the *Staphylococcus* cassette chromosome mec (SCCmec), commonly present in MRSA strains, providing resistance against β-lactam antibiotics [30].

In this study, a total of 62 MSSA and 70 MRSA samples obtained from various clinical sources were subjected to phenotypic characterization in terms of biofilm formation using both the microtitre plate and Congo red agar methods. We employed two distinct approaches to assess the biofilm-forming ability of MSSA and MRSA isolates. The results from both methods indicated a greater and more robust biofilm production in MRSA strains as compared to MSSA strains. Lee *et al*. (2016) [31], Askhan *et al*. (2021) [32], and Piechota *et al*. (2018) [33] have also reported similar findings in their respective studies. Whereas Abdel Halim *et al* (2018) [34] study showed biofilm production was high in MSSA as compare to MRSA.

In MRSA, our analysis of microtiter plate results revealed a greater occurrence of the mecA gene in isolates that produce biofilm (78%) compared to isolates that do not form biofilm (22%). By using the Congo red method, only three (4%) samples produced biofilm, and all three of those samples positive for the mecA gene. The results obtained align with the findings presented by Pozzi *et al*. (2012) [30], who emphasized the correlation between the existence of mecA and the formation of biofilms in MRSA strains. Whereas Tamar Leshem (2022) [35] shown more biofilm producers by Congo red agar method.

The Congo red agar technique proved to be more efficient and quicker compared to the microtiter plate method. However, it only identified 4.2% of MRSA and 1.4% of MSSA in our research. Similar results were reported by Abdel Halim *et al* (2018) [34], where 1.3% of biofilm producers were detected using the Congo red agar method

This study illustrates that blood samples exhibit a greater potential for biofilm formation. Furthermore, Agarwal and Jain (2013) [36] have reported similar results. In contrast, Piechota *et al*. (2018) showed that isolates derived from nasal passages or wounds displayed superior biofilm-producing abilities in comparison to those acquired from blood samples.

Biofilm producing strains in our work were resistant to almost all groups of antibiotics. Among our isolates, Levofloxacin (67%), Gentamicin (61%), Clarithromycin (57%), Clindamycin (52%), Erythromycin (50%) in MRSA. This is concordant with Sharvari and Chitra, 2012 [37], Ramakrishna *et al*., 2014 [38] and Singh *et al*., 2017 [39] who found that staphylococci biofilm producers were more resistant to commonly used antibiotics.

In our study, all the strains were sensitive to vancomycin (100%) while (6%) were resistant to linezolid. The findings align with Sharvari and Chitra, 2012 [37], where they found that all their isolates were susceptible to vancomycin, while a small percentage (4.1%) showed resistance to linezolid.

Conclusion

The findings of this research indicate that the Microtiter plate method shownhigher biofilm-producing staphylococci compared to Congo red agar. It was observed that MRSA strains produced a higher amount of biofilm in comparison to MSSA strains. Additionally, biofilm-producing

staphylococci displayed extensive resistance to high end antibiotics. Our study revealed that the majority of biofilm producers in MRSA exhibited the mecA gene.Enhanced infection control measures and the validation of combination therapies through additional *in vitro* research on biofilm development are essential for controlling of biofilms in healthcare settings.

Conflict of Interest: None.

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