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MOLECULAR DETECTION OF BIOFILM FORMING METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS IN NASAL CARRIAGE AMONG DENTAL HEALTHCARE PERSONNEL

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) presents a significant threat in healthcare settings due to its resistance to various antibiotics and its ability to form biofilms, which enhance its survival and persistence. Dental healthcare personnel (DHP) are at risk for MRSA colonization due to their frequent exposure to patients and contaminated surfaces. The study was planned to monitor the occurrence of MRSA nasal carriage among DHP, antibiogram analysis, the biofilm-forming capabilities of these isolates, and characterization of the nuc and mecA genes. Nasal swabs were collected from DHP, and 50 isolates were cultured for S. aureus using standard microbiological techniques. Methicillin resistance was confirmed using the cefoxitin disc diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines 2023. Of the 50 isolates, 9 (18%) were MRSA, and PCR was employed to detect nuc and mecA genes. The Kirby-Bauer (KB) disc diffusion method determined the antimicrobial susceptibility profile of 9 MRSA isolates. All were resistant to cefoxitin and penicillin while varying degrees of resistance were observed for other antibiotics. Vancomycin and linezolid show universal effectiveness, with all isolates being susceptible, confirming their role as a critical treatment option for MRSA infections. All MRSA isolates carried the nuc gene, confirming S. aureus, and 7 (77.7%) carried the mecA gene, confirming methicillin resistance; the remaining may carry other mec gene variants. Seven MRSA isolates carrying the mecA gene underwent SCCmec typing (I-V). Conventional PCR was used to amplify SCCmec types with the help of specific primers. Of the 7 isolates, 5 were positive for all five SCCmec types (I, II, III, IV, V). Two isolates carried different types n=1 (I, II, IV, V), and the remaining carried three SCCmec types n=1 (I, II, IV). Biofilm formation was quantified using a crystal violet assay. Among the MRSA

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isolates, 6 (56%) demonstrated moderate to strong biofilm formation. The study reveals potentially biofilm-forming MRSA strains in the nasal carriage of DHP. The high prevalence of biofilm-forming MRSA among DHP underscores the need for stringent infection control practices. The mixed susceptibility to other antibiotics like Piperacillin/Tazobactam, Gentamicin, and Ciprofloxacin indicates that antibiotic stewardship programs must be tailored to the specific resistance profiles of MRSA in different settings. In conclusion, these findings underscore the importance of infection control measures and ongoing surveillance within dental healthcare settings to moderate the risk of MRSA transmission and associated infections.

INTRODUCTION

Worldwide, about 2 billion healthy persons carry *Staphylococcus aureus* as a commensal and many of them, nearly 53 million (2.65%) are assumed to backpack with MRSA (Jayaweera et al., 2017). *S. aureus* is a cocci-shaped, facultatively anaerobic, non- motile, Gram-positive bacteria and tends to form "grape-like" clusters (Haag et al., 2019).

S. aureus is the second most familiar source of community-onset bacteremia, following *E. coli*. (Yarovoy et al., 2019). *S. aureus* is a normal flora of 25% to 30% of the general population and causes opportunistic contagions in community and hospital settings (Haag et al., 2019; Jaber et al., 2023).

S. aureus is classified based on its antibiotic susceptibility into MRSA and methicillin-sensitive *S. aureus* (MSSA) (Hasmukharay et al., 2023). In hospitals, HCWs and nasal carriers of *S. aureus* among patients are common reservoirs in these settings (Danelli et al., 2020).

In 1961, MRSA was first demarcated in England (Lee et al., 2018). In recent times, the significant increase in MRSA cases can be attributed to inadequate infection control practices and the lack of antibiotic stewardship programs (Hosseini et al., 2020).

Staphylococci is recognized as the foremost source of infections associated with biofilms, including dental plaque (Cihalova et al., 2015; Silva et al., 2021).

Biofilm production has three unique stages: early attachment, bacterial mat development, and dissemination (Kadkhoda et al., 2020; Silva et al., 2021).

Patients and dentists can share *S. aureus* via contaminated objects and surfaces used in medical settings can also spread the infection. Consequently, monitoring the occurrence of *S. aureus* more unambiguously, MRSA in the dental education environment is crucial for the efficient learning of infection and antibiotic resistance control techniques (Cavaco-Silva et al., 2021).

The primary emphasis of the proposed study is to isolate and genetically characterize MRSA associated with dental healthcare personnel. Furthermore, the biofilm-forming potential of MRSA will be explored in the second part of this study. Overall, this study will decipher the burden of biofilm-forming MRSA in dental healthcare personnel. The proposed study was planned to quantify the biofilm-forming MRSA burden among dental healthcare personnel.

The study aimed to achieve the following specific objectives:

1. Isolation of MRSA from dental healthcare personnel.
2. Biochemical and molecular identification of MRSA.
3. Molecular typing of MRSA based on the SCC*mec* gene.
4. Antibiogram analysis of MRSA against commonly used β-lactam drugs.
5. Determination of biofilm forming potential of MRSA isolates.

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MATERIALS AND METHODS

Isolation and identification of MRSA

Selection Criteria for Dental Healthcare Personnel

Inclusion criteria

- Dental healthcare personnel working in a dental clinic and hospital.
- Individuals who gave informed consent to take part in the research.

Exclusion criteria

- Individuals with recent antibiotic use (within the past month).
- Personnel with known chronic infections or underlying health conditions that may influence nasal carriage of MRSA.

Inoculation of nasal swabs

Morphological Identification of *S. aureus*

Growth of *S. aureus* on selective media

Mannitol salt agar (MSA) acts as a differential and discriminatory media for the growth of *S. aureus* as it is loaded with high sodium chloride concentration, which restricts other microbes from growing. Already prepared MSA plates were streaked with a loop full of overnight NB culture. Plates were incubated at 37°C for 24 hours. Deep yellow-golden coloured colonies were formed.

Growth of S. aureus on blood agar

S. aureus that formed golden yellow colonies on MSA were incubated on blood agar and incubated at 37°C for 24 hours. Staphylococci formed whitish-grey colored colonies exhibiting β-hemolysis. Colonies were picked up from blood agar and MSA and examined to determine their morphology microscopically with the help of Gram's staining.

The Gram staining protocol for S. aureus

For the Gram stain, freshly prepared bacterial suspension was placed on a clean glass slide and fixed the specimen by gentle heating. A few drops of crystal violet (primary stain) were added to the specimen covering the whole smear to colour the bacterial cell wall and allowing it to stand for 60 seconds. Washed with tap water thoroughly. A few drops of Gram iodine were added directly on the smear as a mordant for 60 seconds to fix crystal violet in the bacteria's cell wall. The iodine was poured on the slide and washed with tap water rapidly. The decolorizing agent 70% ethanol was flowed over the specimen for 5 seconds. The slide was again washed with tap water and safranin (counter stain) was added to the specimen for 60 seconds. The slide was washed with tap water and patted with filter paper to remove water. The slide was observed using bright field microscopy (Tripathi & Sapra, 2020).

Biochemical Characterization

All isolates were subjected to the following tests for biochemical characterization.

Catalase test for S. aureus

A 3% w/v hydrogen peroxide (H_2O_2) solution was added to 1 ml of the culture isolates, proving the isolates are catalase positive. Hydrogen peroxide is produced by bacteria living under aerobic conditions. Catalase neutralizes it to avoid its toxicity. The rapid growth of gas bubbles showed the breakdown of H_2O_2 to liberate O_2 and H_2O by catalase enzyme, indicating positive results.

$2H_2O_2 \rightarrow H_2O + O_2$

(Ekawati et al., 2020)

The test was conducted in a safety cabinet. 2-3 drops of hydrogen peroxide were poured on a clean and non-greasy slide. With the help of a sterile loop, a colony was taken from a fresh culture and mixed with hydrogen peroxide. Rapid formation of bubbles was observed by test clones indicating positive results. The test was repeated for all isolates.

Coagulase test for S. aureus

S. aureus secretes free plasma coagulase, which acts as a virulence element as well as differentiates it from coagulase-negative staphylococci (CoNS) (Tiwari et al., 2008). Coagulase is an enzyme that combines with prothrombin present in blood and forms a complex called Staphylothrombin. It allows

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protease enzymes to convert soluble fibrinogen of blood plasma into insoluble fibrin strands. It ends in the coagulation of blood plasma. It is used to differentiate between coagulase-positive and CoNS species. Two forms of coagulase enzyme are produced by the isolate (bound and free). The tube coagulase test is employed to identify free coagulase, while the slide coagulase test is utilized to detect bound coagulase.

Slide Coagulase for S. aureus

The cleaned, non-greasy glass slides were used for the slide coagulase test. In a single drop of saline, prepared smooth suspension of the test colony and mixed a small quantity of fresh human plasma in a circular motion for 10 seconds.

Tube Coagulase for S. aureus

The tube coagulase method was conducted as described by Fernandes Queiroga Moraes et al. (2021). A positive coagulase test differentiates *S. aureus* from the CoNS species. Freshly prepared human plasma was diluted by 1 in 10 with sterile saline. Aliquots of 1ml were pipetted into sterile test tubes and 0.1 ml of 24 hours MSA culture of the isolate was added, mixed and incubated at 37°C for 4 hours. A negative control containing only sterile MSA broth was also established. The incubation period was extended for 24 hours for delayed reactions because *S. aureus* is not susceptible to
impulsive clumping of plasma which is commonly followed by the use of citrate found in anticoagulants.

Mannitol fermentation for *S. aureus*

Many pathogenic staphylococci, such as *S. aureus*, ferment mannitol into pyruvate. Most of the non-pathogenic staphylococci will not ferment mannitol.
Mannitol →mannitol-1-phosphate→fructose-6-phosphate→pyruvic acid
(Lafta & Najem, 2020)

3.1.2 Phenotypic confirmation of MRSA using cefoxitin disc

CLSI anticipated *mecA* facilitated oxacillin resistance in staphylococci as the cefoxitin diffusion test is easy to read in reflected light for identification of resistant strains (Swenson et al). MRSA was phenotypically identified by using cefoxitin (30µg) disc diffusion test on MHA surface carpeted with bacterial colonies with the help of sterilized swab. After incubating at 37°C for 24 hrs, the inhibition zones were assessed. Results were interpreted according to CLSI guidelines 2023. An inhibition zone diameter of ≤ 21mm was considered methicillin-resistant and ≥ 22mm was reported as methicillin- sensitive (Gupta & Kumar, 2022).

3.2 Antibiogram analysis of MRSA isolates against commonly used drugs

KB method was employed to determine the AST profile of MRSA strains**.**

3.2.1 Preparation of 0.5 McFarland standard

To perform the disc diffusion test and to make sure that bacterial suspensions were within the required range, a 0.5 McFarland turbidity standard was prepared to ensure that bacterial suspension is not much concentrated or diluted and results are reliable. Barium chloride and sulfuric acid were mixed in a screw-capped test tube to make the McFarland standard, which resulted in the precipitation of barium sulfate. The chemical solution was prepared and stored at room temperature. The standard's optical density (OD) was dignified by a spectrophotometer at a 625nm wavelength, and the absorbance was between 0.08 and 0.1. By comparing the test suspensions to this
benchmark, the turbidity was regulated. Based on the 0.5 McFarland turbidity criterion, the prepared bacterial suspension had a cell density of roughly 1.5×10^8 CFU/ml.

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Table 3.1 Preparation of 0.5 McFarland turbidity standard.

Reagents	Quantity in ml
1.0% Sulphuric acid	9.95
1.18% Barium Chloride dehydrate	0.05

Preparation of Inoculum

2-3 colonies have been extracted from the overnight culture plates and diluted with 5 ml of 0.9% NaCl solution. The 0.5 McFarland standard was used to compare the suspension's turbidity. Twenty minutes after preparation, suspension was used.

3.2.2 List of antibiotics

Commonly used antibiotics from different groups were selected for AST and given in below Table 3.2.

Table 3.2 Antibiotics list used in Kirby-Bauer disc diffusion assay

Antibiotic Class	Antimicrobial Drug	Code	Potency (µg)	Manufacturer
Penicillins	Penicillin G	P	10	Oxoid
Penicillin+ β- lactamase inhibitor	Piperacillin/Tazobactam	TZP	100/10	Oxoid
Aminoglycosides	Gentamicin	CN	10	Oxoid
Fluoroquinolones	Ciprofloxacin Norfloxacin	CIP NOR	5 10	Oxoid
Folate Pathway Inhibitor	Trimethoprim- Sulfamethoxazole	SXT	25	Oxoid
Carbapenems	Imipenem	IPM	10	Oxoid
Cephalosporins	Cefoxitin	FOX	30	Oxoid
Glycopeptides antibiotics	Vancomycin	VA	30	Oxoid
Oxazolidinones	Linezolid	LZD	10	Oxoid
Lincosamide	Clindamycin	DA	10	Oxoid

Kirby Bauer Disc Diffusion Technique

This disc diffusion assay was accomplished consistent with the CLSI guidelines 2023. By obeying instructions stated by the manufacturer, MHA medium was prepared and then placed in the autoclave. The pH of the medium was maintained at 7.2–7.4.

Table 3.3 Muller Hinton Agar Composition

Ingredients	Amount
Starch	1.5 g
Acid hydrolysate casein	17.5 g

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Beef Extract	2 g
Agar	17 g
Distilled Water	1000 ml

The agar was then poured up to 4mm depth (nearly 25ml) in 90mm Petri dishes aseptically in a biosafety cabinet and allowed to solidify. Antibiotics were stored in a refrigerator and brought to room temperature before use. All the antibiotics were kept in sealed containers to maintain sterility. The antibiotics used in this test against MRSA are mentioned in Table 3.2.

3.2.3 Protocol for Disc Diffusion Assay

1. Sterile cotton swabs were rolled into the adjusted bacterial suspension.
2. The swabs were then rotated many times and blotted against the interior wall of the tube to get rid of extra fluid.
3. Already prepared MHA plates were inoculated via streaking of these swabs evenly in three directions and plates were continuously rotated at 60̊ to ensure a uniform distribution of the inoculum.
4. After that, the petridishes were desiccated at ambient temperature with the lid on for five minutes.
5. Antibiotic discs were placed to the inoculated agar plates using sterile forceps.
6. Discs were evenly spaced 15mm apart from the edges of plates and at least 24mm distance was between adjacent antibiotics to prevent overlapping of inhibition zones. Only 4-5 discs were placed in 90mm Petri plates.
7. To make sure every disc made contact with the agar surface, each disk was slightly pressed.
8. Next, petriplates were incubated at 35°C for 16 to 18 hrs after being covered with their lids and turned upside down.

Using a ruler, the diameter of each zone of inhibition (including the disc's diameter) was measured in millimeters following incubation.

The resistance to each antibiotic tested was determined by comparing measured zone diameters to CLSI guidelines 2023 interpretive criteria. Finally, classify as sensitive, intermediate, or resistant according to the CLSI breakpoints.

3.3 Crystal Violet Assay for Biofilm Formation of MRSA

The Crystal Violet Assay is frequently employed for MRSA biofilm development. A 96-well polystyrene microtiter plate was sterilized, and 200 µL of the diluted culture was drop in every well. For twenty-four hrs, plates were incubated at 37°C. Subsequent nurture, non-adherent cells were detached from the wells using three gentle washes with 200 µL of phosphate buffered saline (PBS) to get rid of any remaining planktonic bacteria. To fix the left over bacteria, 200 µL of 99% methanol was drop in every well for 15 minutes. After removing methanol, the wells were stained with 200 µL of 0.1% crystal violet solution for 5 minutes, as explained by Shukla and Rao (2017). Tap water was used to remove excess stain from plates. After air-drying the plate, 200 µL of 33% glacial acetic acid was drop in every well to dissolve the bound
crystal violet. A microplate reader was used to measure each well OD at 570nm. Biofilm formation was quantified by comparing the OD values to control wells containing TSB only. These steps, meticulously performed as enlightened by Kragh et al. (2019) and optimized for accuracy, allow for reliable and reproducible assessment of MRSA biofilm formation, essential for understanding the pathogenicity and virulence of antibiotic- resistant bacterium in healthcare settings.

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3.4 Molecular Analysis of S. aureus

3.4.1 DNA Extraction of S. aureus

DNA of 9 isolates that were resistant to cefoxitin were selected for DNA extraction. DNA was extracted for molecular characterization of *nuc* gene, *mecA* gene and SCC*mec* typing. BHI broth was used to culture isolates and was incubated at 37°C for twenty-four hrs in aerobic environment. Centrifugation at 10,000 rpm was performed to harvest the pellet and it was then washed with PBS. 200µl of PBS was added in washed cell pellets. DNA extraction of washed cell pellets was completed by following Favor Prep Blood/cultured cell Genomic DNA extraction mini kit. 200 µl lysozyme was added and samples were incubated at 37°C for 45 minutes. 20 µl of proteinase K and 200 µl of FABG buffer were added in tubes with cell pellets and vortexed thoroughly. The samples were then incubated at 60°C in the water bath for half an hour with shaking after every five minutes. Then vortexed tubes to drop all the liquid from inside of the lid. Mixed by pulse vortex for half minute after adding 200 µl of absolute ethanol. FABG columns were placed in collection tubes and samples were transferred in FABG columns with care. Centrifuged at 13,000 rpm for 60 seconds and discarded the flow through and transfer FABG column in new collection tube. FABG column was washed with 500 µl W1 buffer, centrifuged at 13,000 rpm and flow through was discarded. FABG column was again washed with 750 µl of Wash buffer and centrifuged for 60 minutes and flow-through was discarded. FABG columns were re-centrifuged for 180 seconds to dry the column. 200µl of elution buffer were added and centrifuged for 120 seconds to elute DNA. Eluted DNA was kept at 4°C.

3.4.2 Gel preparation

For the preparation of tris-acetic-EDTA buffer gel, we need agarose powder, TAE buffer and ethidium bromide (EtBr). The volume of TAE buffer and the amount of
agarose powder depends on the gene product and no. of wells. For 20 well gel preparation, 1 gm of agarose was added in 80 ml TAE buffer (1X) and heated in a microwave till it became transparent. After a few minutes, when the solution was cooled, 5µl of EtBr was dispended to stain DNA. The solution was shaken to mix and poured in gel mould with 20 well combs and allowed to harden for 30 minutes. 5 µl of eluted DNA were mixed with 1 µl of loading dye and run in 1.25%agarose gel at 220 volts for 30 minutes and visualized in gel doc. to confirm the presence of DNA in eluted solution.

3.4.3 Molecular Detection of *S. aureus* with *nuc*lease gene

Contents of a single PCR tube (25µl)

- Master mix 12.5 µl
- Forward primer 0.5 µl
- Reverse primer 0.5 µl
- PCR grade water 10.5 µl
- DNA template 1.0 µl

Except for the DNA template, all the above quantities were multiplied with no. of isolates and dispended in sterilized Eppendorf and vortexed. 24µl from the Eppendorf tube was poured into each sterilized PCR tube and then 1.0µl of DNA template was added in all PCR tubes. After the vortex, all PCR tubes were placed in a thermocycler under the following conditions to attain maximum yield.

Initial denaturation at 94°C for 3 minutes

For every next cycle, denaturation at 94°C for 1 minute Annealing at 55°C for 45 seconds

Amplification at 72°C for 1 minute Final extension at 72°C for 7 minutes Holding temperature 4°C for infinity Total 35 rounds of replication cycles

Amplicons were studied using a horizontal agarose gel electrophoresis system. For DNA staining, 8µl of undiluted PCR results were placed onto a 1.25% agarose gel
with 1 µg/ml EttBr (Fischer Scientific Ltd). Gels were run for 25 minutes at 220 volts in 1X TAE buffer. A gel documentation system (Slite 200W) was used to visualize amplicons. The size of the product was measured using 100 bp molecular markers.

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Table 3.4 Primer sequence for screening of *S. aureus* (*nuc* gene)

nuc gene	Sequence	Amplicon length	Reference
nuc forward primer	GCG-ATT-GAT-GGT-GATACG-GTT	278 bp	(Arshad et al., 2015)
nuc reverse primer	AGC-CAA-GCC-TTG-ACGAAC-TAA-AGC		

Molecular detection of *mecA* gene in phenotypically identified S. *aureus*

mecA gene detection is a golden marker for the pinpointing of MRSA.

3.4.4 Amplification of *mecA* gene of *S. aureus* using PCR

PCR mixture of 25µl containing 1µl of template DNA, 10.5µl PCR grade water, 0.5µl forward primer, 0.5µl reverser primer, and 12.5µl PCR master mix was prepared in sterilized PCR tubes for each DNA isolate. All isolates were centrifuged in a mini- centrifuge for 5 seconds and positioned in a thermal cycler. Amplification of the *mecA* gene was supported in the T100 Thermal Cycler under the following conditions.

The PCR procedure began with three minutes of initial denaturation at 95°C, followed by 60 seconds of denaturation at 94°C, 60 seconds of annealing at 55°C, and 60 seconds of extension at 72°C. A total of thirty-five cycles were completed, including a 7- minute final extension at 72°C. For (∞) infinity, the holding temperature was 4°C. Following gene amplification, the PCR products underwent electrophoresis and were seen under an illumination system. A 1.25 percent agarose gel was loaded with the amplicons. The last well was loaded with the ladder (50bp) plus ladder followed by the PCR products in order.

Table 3.5 Primer sequence for *mecA* gene of *S. aureus*

mec gene	Primer Sequence	Amplicon length	Reference
mec forward primer	5-GTA GAA ATG ACT GAA CGT CCG ATA A-3	310 bp	(Rana et al., 2018)
mec reverse primer	5-CCA ATT CCA CAT TGT TTC GGT CTA A-3		

3.4.5 Staphylococcal Cassette Chromosome (SCC*mec*) typing *S. aureus*

PCR was employed to recognize the *mecA* gene and ascertain the SCC*mec* types. It was accomplished as described already by Moosaviani et al., (2018). Primers were used for I-V SCC*mec* types as shown in table 3.6. The PCR mixture was prepared with SCC*mec* types primer according to the number of DNA templates. The PCR mixture was vortexed to homogenize and aliquated in volumes of 25 ul PCR tubes. After adding the DNA template, PCR tubes were centrifuged in a mini-centrifuge for 1-2 seconds to mix and then placed in the thermocycler. The optimization process was conducted as follows for primers I-V except for annealing temperatures that were 50°C for primers I & IV and 48°C for primers II and III.

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Preliminary denaturation at 95°C for 3 min
For every next round, denaturation at 95°C for 30 sec Annealing at (48°C/ 50°C) for 45 sec Amplification at 72°C for 45 sec Final extension at 72°C for 7 min Holding temperature 4°C for infinity Total 35 rounds of replication cycles

Table 3.6 Primers sequence for *SCCmec* typing *S. aureus*

SCC*mec* Typing	Primers Sequence	Amplicon length	Reference
*ccr*A2B- Forward *ccr*A2B- Reverse	ATTGCCTTGATAATAGCCYTCT TAAAGGCATCAATGCACAAACACT	937	(Moosavian et al., 2018)
*ccr*C- Forward *ccr*C- Reverse	CGTCTATTACAAGATGTTAAGGATA CCTTTATAGACTGGATTATTCAAAA	518	
IS 1272-Forward IS 1272-Reverse	GCCACTCATAACATATGGAA CATCCGAGTGAAACCCAAA	415	
*mec*AIS431-Forward *mec*AIS431-Reverse	TATACCAAACCCGACAACTAC CGGCTACAGTGATAACATCC	359	

The amplified DNA product was run on a gel in which 1 gram of agarose was warmed to mix in 100 ml of TAE buffer and 5 µl EtBr was added to stain the DNA. The PCR products were loaded on the gel with a 50bp ladder in the last well and run on an electrophoretic machine at 90 V for 30 minutes. Bands were visualized with the help of a gel doc. SCC*mec* types were determined based on the following data.

Table 3.7 *SCCmec* types based on specific primers of MRSA

SCC*mec* type	ccrA2B	ccrC	IS 1272	*mecA*-IS431
I			*	
II	*			
III		*		
IV	*		*	
V		*		*

(Moosavian et al., 2018)

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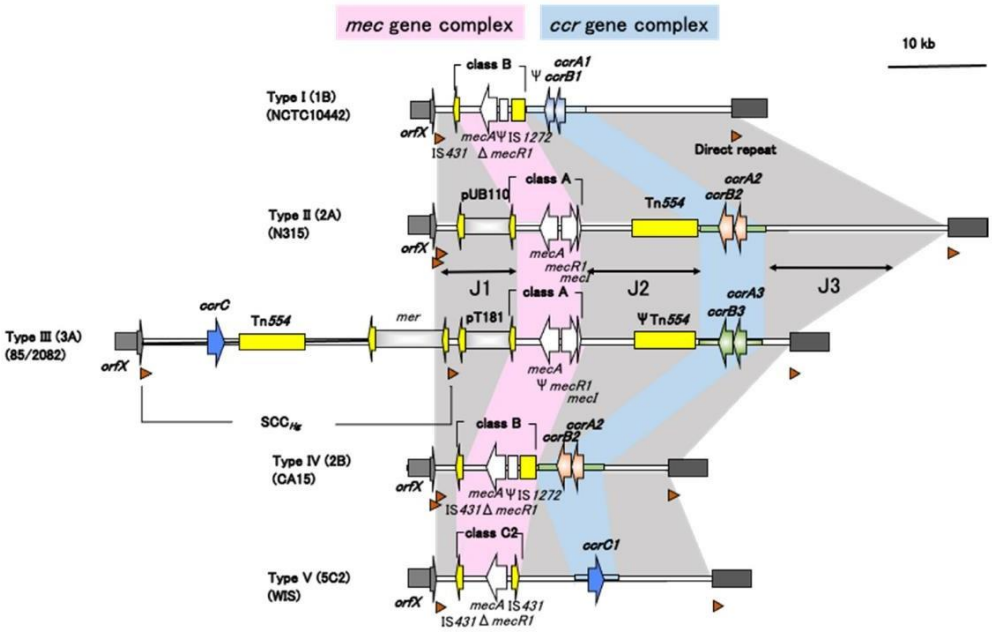
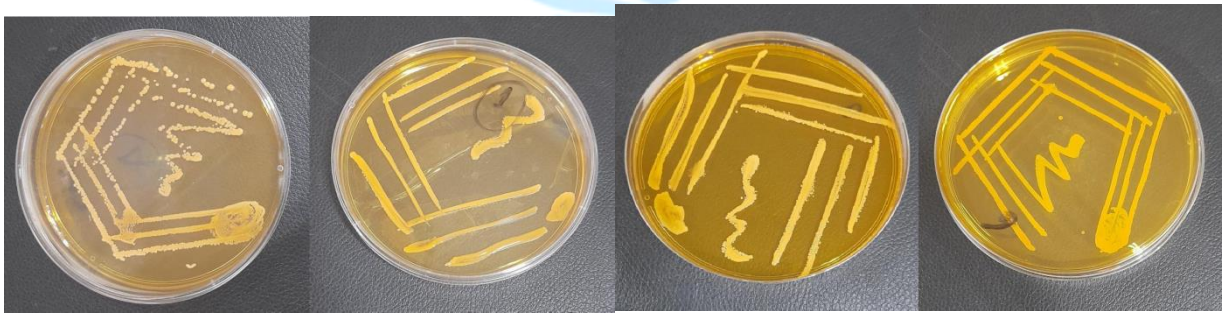


Figure 3.1 SCC*mec* types gene sequence of MRSA (Uehara, 2022) SCC*mec* types were determined with the help of Table 3.6 and Figure 3.1

RESULTS AND DISCUSSION



50 isolates of *S. aureus* were cultured on MSA (Fig.4.1). All 50 isolates grew on the media at 37°C for 24 hrs, forming golden yellow colonies. Bacteria used mannitol as a carbon source. The fermentation of mannitol yields pyruvic acid, which lowers the medium's pH. A color change indicates acid generation due to a pH reduction in the medium.

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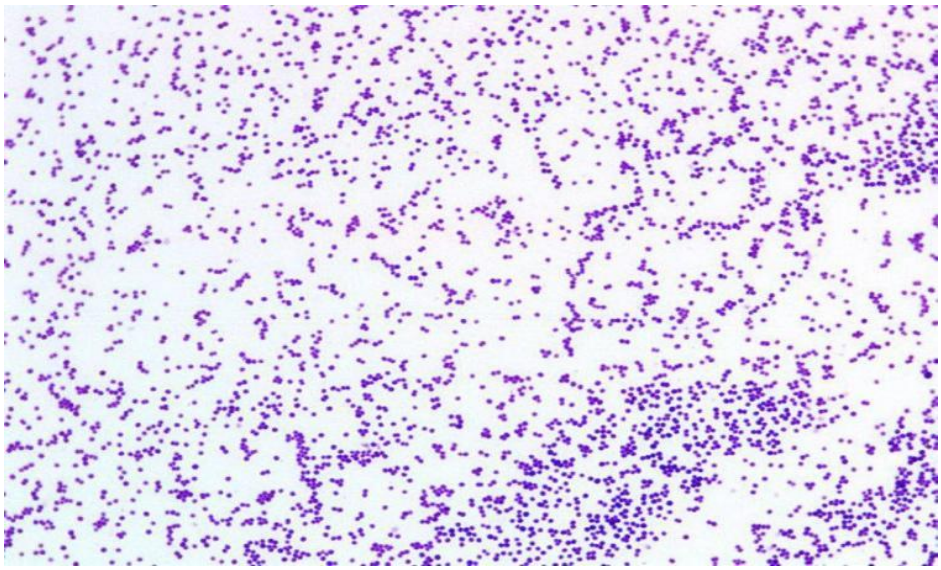

Figure 4.1 *S. aureus* colonies on MSA

Gram staining was performed for the determination of morphology. All isolates acquired a crystal violet color, proving themselves gram-positive. They have peptidoglycan and teichoic acid in their cell wall structure. They were observed as cocci, non-flagellated and non-spore forming bacteria. It was present as a single cell or in pairs or short chains but predominantly formed grape-like clusters when viewed through the microscope (Fig. 4. 2).

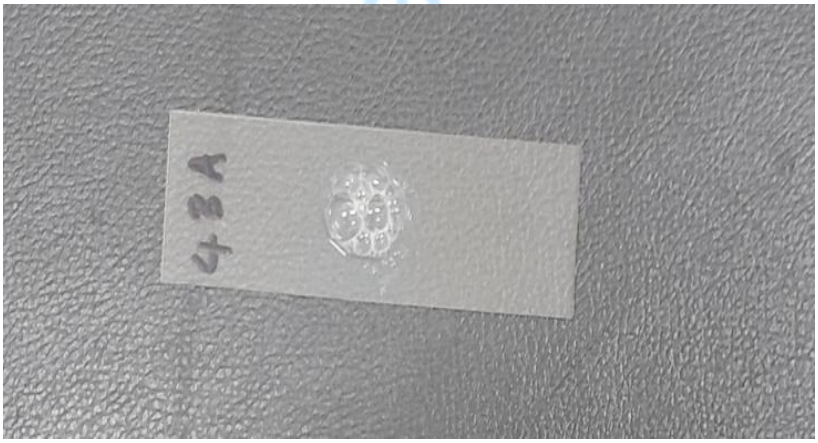

Figure 4.2 Gram staining reveals clusters of *S. aureus*

All 50 samples were catalase positive. During aerobic respiration, *S. aureus* produces hydrogen peroxide (H_2O_2) that is toxic for it. To neutralize hydrogen peroxide into water and oxygen, it produces catalase enzymes. Rapid bubbles (oxygen) development within 5-10 seconds was observed when hydrogen peroxide was poured on colonies of clinical isolates, ensuring clinical isolates as *S. aureus*. The procedure was repeated for all 50 isolates with controls.

Catalase positive (bubbles)

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Figure 4.3 Slide catalase test demonstrating bubble formation by *S. aureus*

Catalase negative (no bubbles)

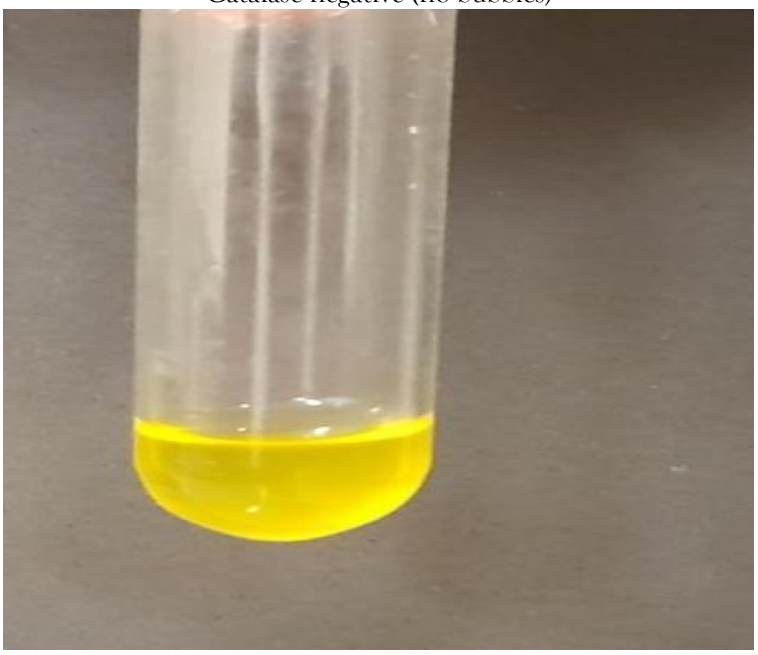

Figure 4.4 Slide catalase test showing no bubble formation

All 50 isolates were coagulase positive. It indicated that isolates could produce a coagulase enzyme that causes blood clotting. A tube coagulase test resulted in coagulation. It is accomplished to check the presence of free coagulase enzyme in the medium secreted by the bacteria. Free coagulase coagulated fibrinogen in blood plasma into fibrin and a gel like structure was observed.

Figure 4.5 Tube coagulase test exhibiting coagulation.

Hemolysis is the breakdown of red blood cells. It occurs due to the presence of toxins produced by infectious bacteria. *S. aureus* causes complete β-hemolysis due to the presence of hemolysin, leucocidin and toxic shock syndrome toxin-1. All 50 isolates showed β-hemolysis with whitish-grey colonies after 24 hours on blood agar.

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Figure 4.6 β-hemolysis exhibited by *S. aureus*

Susceptibility to cefoxitin by disc diffusion has been used to detect MRSA. Cefoxitin (30µg) disc was applied on the lawns. Of 50 isolates, 9 strains exhibited cefoxitin (30µg) resistance ≤ 21mm. This represented 18% phenotypically resistant MRSA (fig. 4.8 & pie chart).

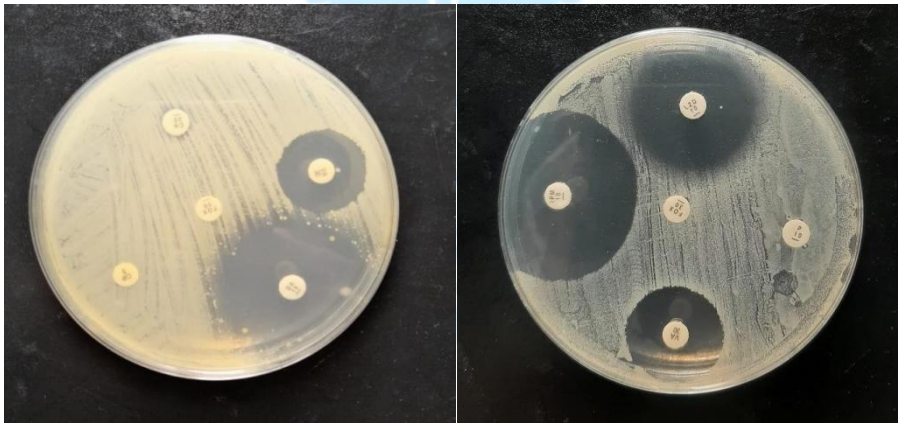

Figure 4.7 Demonstration of phenotypically resistant *S. aureus*

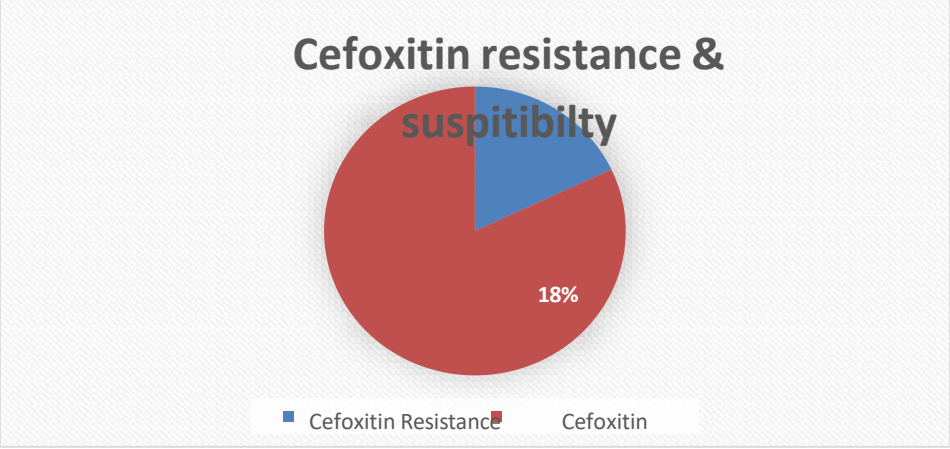

Figure 4.8: Cefoxintin susceptibility

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The KB method determined the AST profile of 9 MRSA isolates. The isolates classified as Susceptible(S), Intermediate(I), and Resistant(R), were interpreted according to the CLSI guidelines 2023. The antibiotics tested included Penicillin G, Piperacillin/Tazobactam, Gentamicin, Ciprofloxacin, Norfloxacin, Trimethoprim- Sulfamethoxazole, Imipenem, Cefoxitin, Vancomycin, Linezolid, and Clindamycin.

Breakpoint values for the KB disc diffusion test are given below in Table 4.1.

Table 4.1: Interpretation breakpoint values for the Kirby-Bauer disc diffusion test

Antibiotic	Disc Potency (µg)	Susceptible (S) Zone Diameter (mm)	Intermediate (I) Zone Diameter (mm)	Resistance (R)Zone Diameter (mm)
Penicillin G	10	≥ 29	-	≤ 28
Piperacillin/Tazobactam	100/10	≥ 21	15-20	≤ 14
Gentamicin	10	≥ 15	13-14	≤ 12
Ciprofloxacin	5	≥ 21	16-20	≤ 15
Norfloxacin	10	≥ 17	13-16	≤ 12
Trimethoprim-Sulfamethoxazole	25	≥ 16	11-15	≤ 10
Imipenem	10	≥ 16	14-15	≤ 13
Cefoxitin	30	≥ 22	19-21	≤ 18
Vancomycin	30	≥ 15	-	≤ 14
Linezolid	10	≥ 23	21-22	≤ 20
Clindamycin	10	≥ 21	15-20	≤ 14

Out of the 9 MRSA isolates tested, all were resistant to cefoxitin and penicillin, while varying resistance was observed for other antibiotics. Vancomycin and Linezolid exhibited universal effectiveness, with all isolates being susceptible, confirming their role as critical treatment options for MRSA infections. The details are presented in Table 4.2 and the chart below.

Table 4.2: Antimicrobial susceptibility profile for MRSA isolates

Antibiotic	Disc Potency (µg)	Susceptible Isolates	Intermediate Isolates	Resistance Isolates
Penicillin G	10	0	0	9
Piperacillin/Tazobactam	100/10	3	2	4

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Gentamicin	10	2	1	6
Ciprofloxacin	5	1	3	5
Norfloxacin	10	2	3	4
Trimethoprim-Sulfamethoxazole	25	7	0	2
Imipenem	10	6	2	1
Cefoxitin	30	0	0	9
Vancomycin	30	9	0	0
Linezolid	10	9	0	0
Clindamycin	10	7	1	1

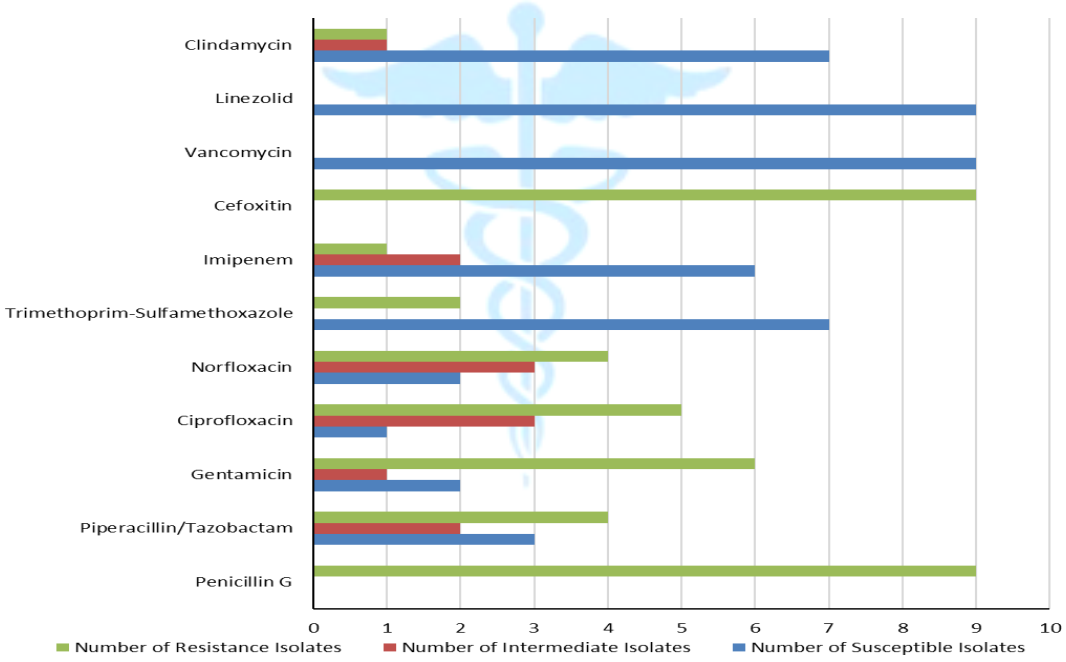

Figure 4.9: Antimicrobial susceptibility profile of MRSA isolates

The AST plates for the MRSA isolates demonstrate a diversity of susceptibility across the tested antibiotics. The plates reveal clear zones of inhibition for antibiotics such as vancomycin and linezolid, indicating high levels of susceptibility among the isolates. Conversely, there were no inhibition zones for antibiotics like penicillin G and cefoxitin, confirming universal resistance consistent with MRSA's known resistance mechanisms. Other antibiotics, such as piperacillin/tazobactam, gentamicin, ciprofloxacin, norfloxacin, trimethoprim-sulfamethoxazole, imipenem, and clindamycin, exhibit varying inhibition zone diameters, reflecting a mix of susceptible, intermediate, and resistant isolates.

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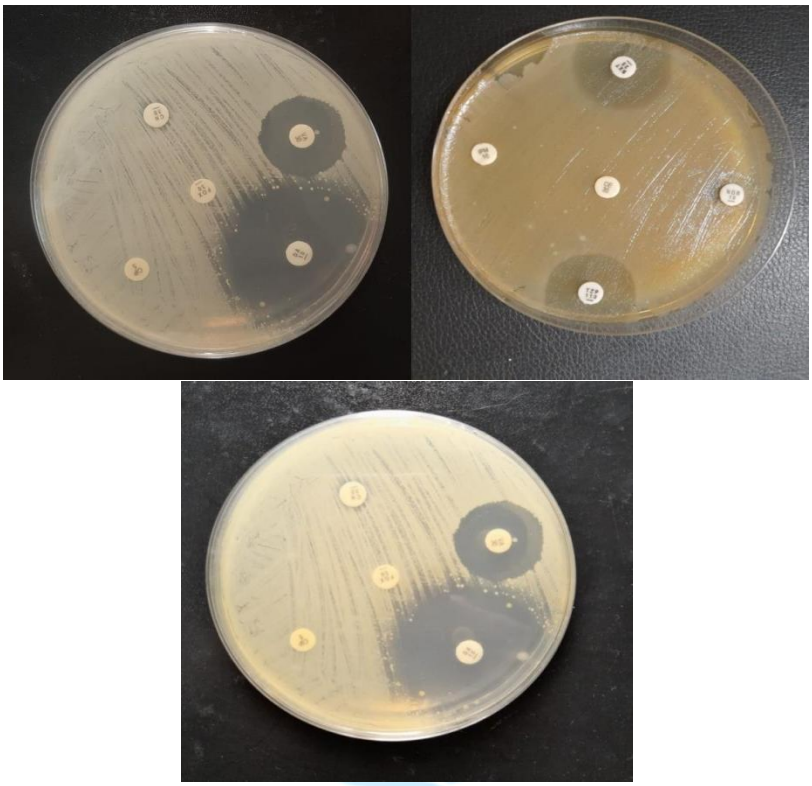

Fig 4.10: Antimicrobial Susceptibility testing plates

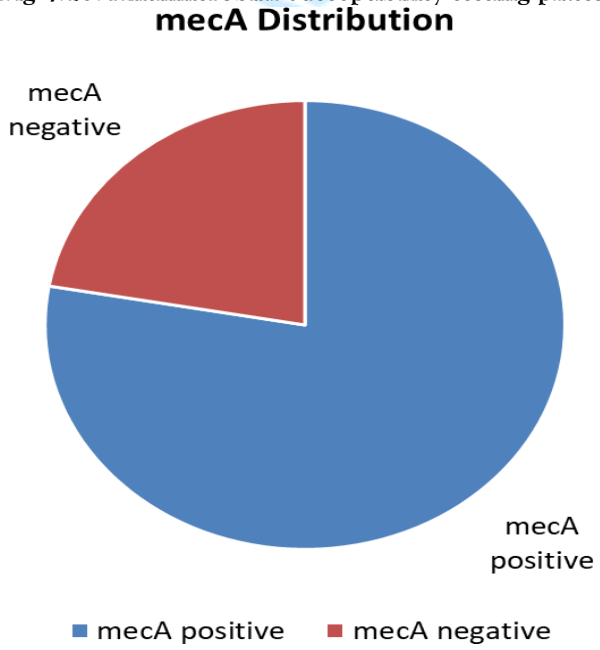

Figure 4.11:Graphical representation of *mecA* distribuation

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Table 4.3 *mecA* gene distribution among cefoxitin-resistant isolates

No. of Isolates	*mecA* positive	*mecA* negative
9	7	2

Thermonuclease (*nuc*) gene with 278bp was detected and amplified using specific primers, as mentioned in Table 3.4, and demonstrated on a 1.25% agarose gel. The existence of the *nuc* gene is a golden symbol for detecting *S. aureus*.

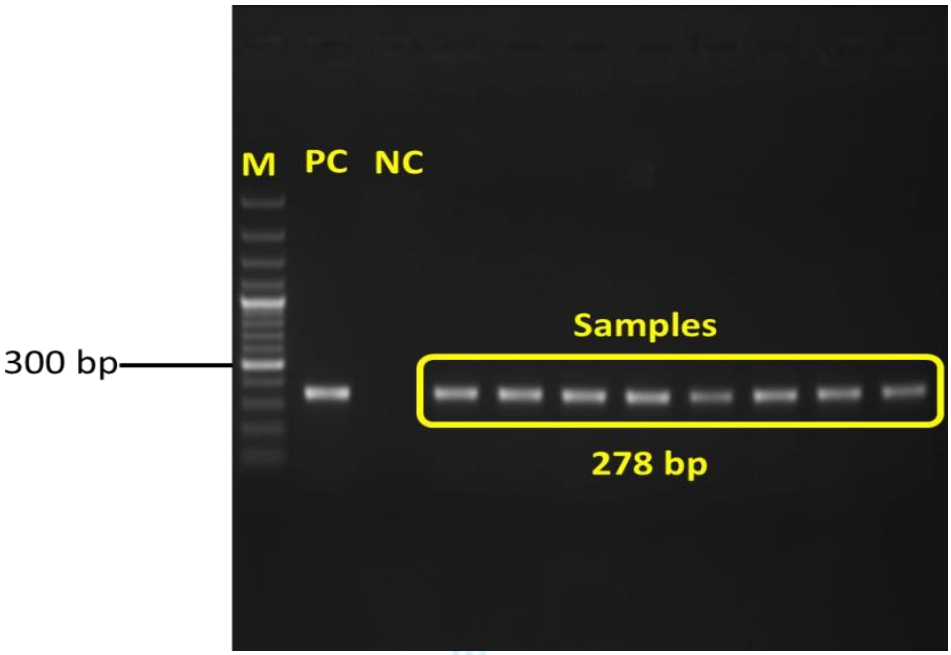

Fig 4.12: S. aureus nuc genes

Specific forward and reverse primers in Table 3.5 were used to amplify the *mecA* gene (310bp) in a PCR machine. The amplified gene was visualized through a UV illuminator in a 1.25% agarose gel compared to 50bp below the ladder. Seven (77.7%) isolates were flanked with the *mecA* gene (310bp).

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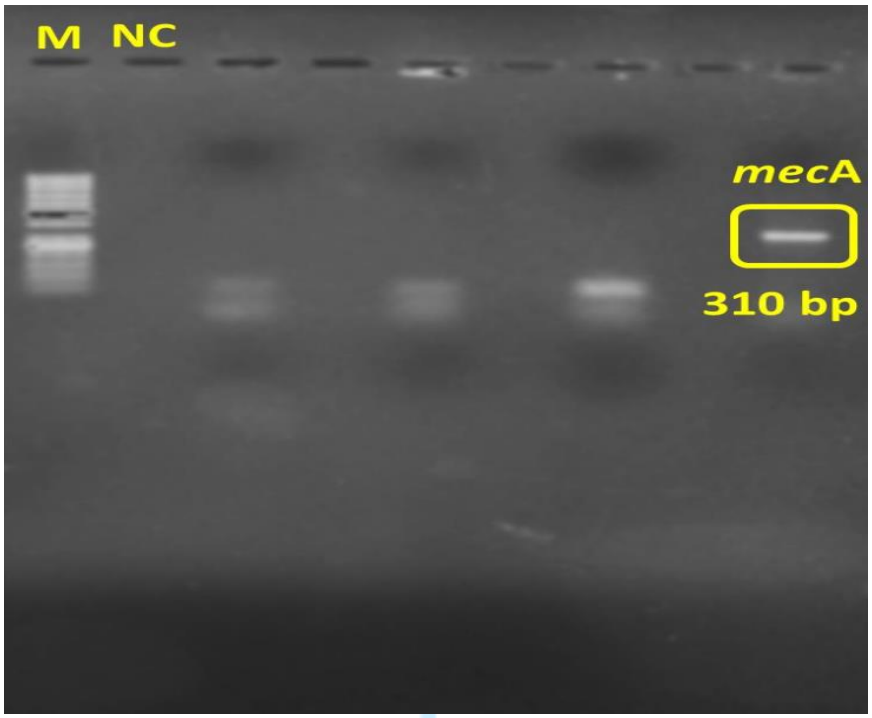

Fig 4.13: MRSA *mecA* gene

7 isolates positive for *mec*A were subjected to SCC*mec* typing using specific primers mentioned in Table 3.6.

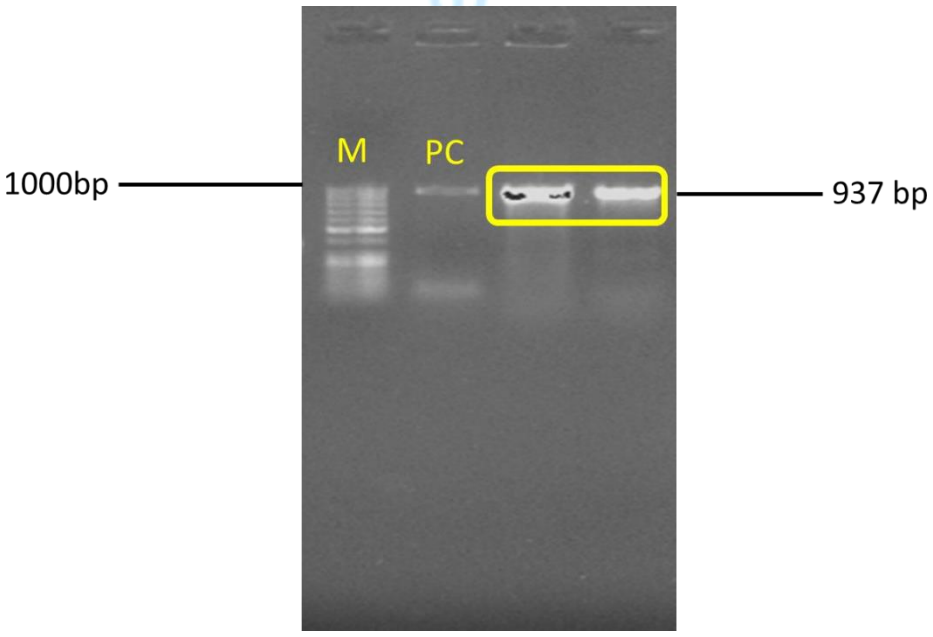

Fig 4.14: PCR product of *ccrA2-B*

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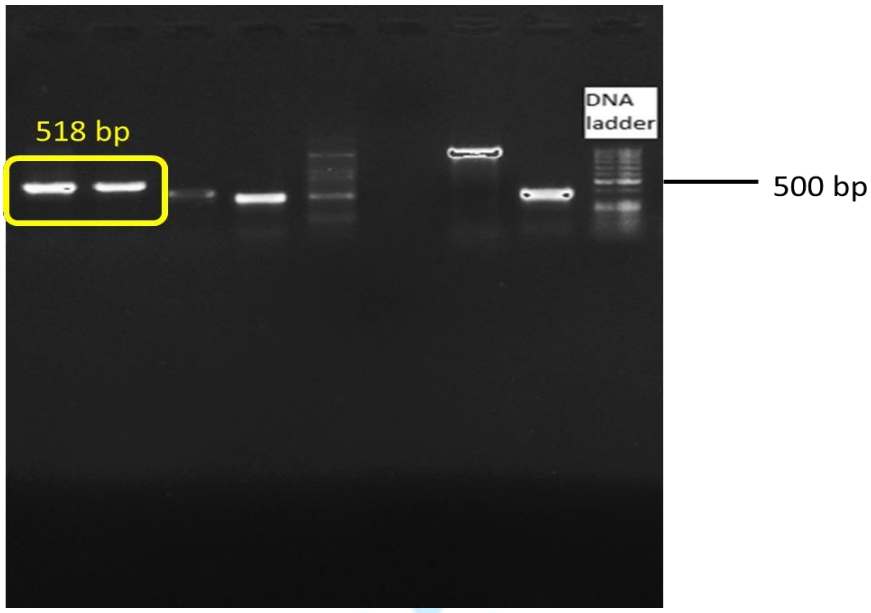

Fig 4.15: PCR product of *ccrC*

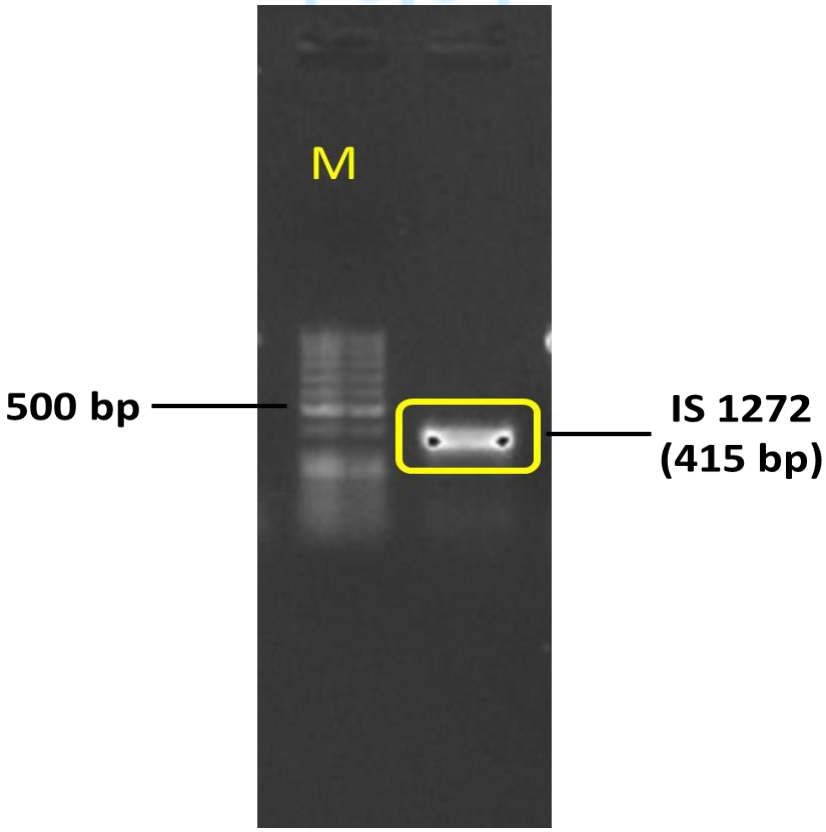

Fig 4.16: PCR product of IS 1272

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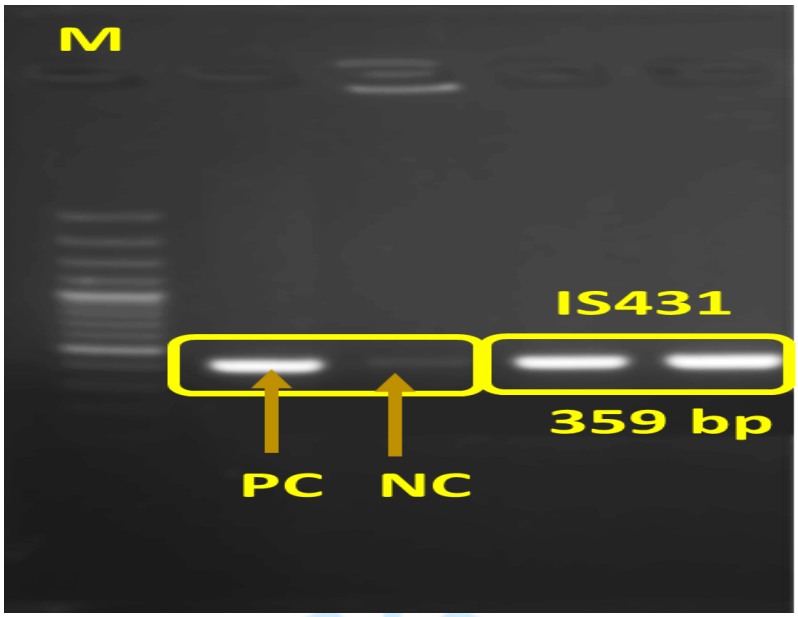

Fig 4.17: PCR product of IS 431

Table 4.4 SCC*mec* types in *mecA*-positive MRSA

Sr. No.	ccrA2-B	ccrC	IS 1272	SCC*mec*A-IS431	SCC*mec* types
1	*	*	*	*	I-V
2	*	*	*	*	I-V
3	*	*	*	*	I-V
4	*	*	*	*	I-V
5	*	*	*	*	I-V
6	*	*	*		I, II, IV, V
7	*		*		I, II,IV

Table 4.5 Various SCC*mec* Types in *mecA*-positive MRSA isolates

Isolates with 5 SCC*mec* Types	Isolates with 4 SCC*mec* types	Isolates with 3 SCC*mec* types
5	1	1

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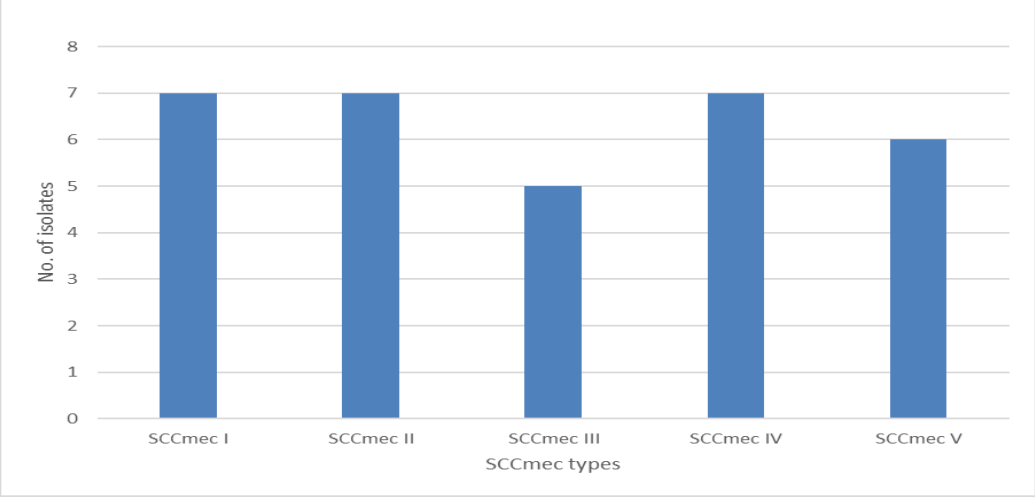

Figure 4.18: SCCmec types among SCCmec MRSA isolates

Table 4.6 Distribution of SCC*mec* types in MRSA isolates

SCC*mec* Types	SCC*mec* I	SCC*mec* II	SCC*mec* III	SCC*mec* IV	SCC*mec* V
No.	7	7	5	7	6

Nine MRSA variants were examined for their ability to generate bacterial mats. Using the crystal violet staining procedure, biofilm formation was assessed, and bacterial mat formation was quantified OD at 570 nm. The consequences indicated a variable capacity for bacterial mat formation among the MRSA isolates. Based on the OD values, the subtypes were categorized into the following four groups. The classification was conducted as stated by Stepanović et al. (2007), which defines cut-off values for each group.

- **Non-biofilm formers (OD ≤ 0.12):** 2 isolates (22%)
- **Weak biofilm formers (OD ≤ 0.24):** 2 isolates (22%)
- **Moderate biofilm formers (OD ≤ 0.48):** 4 isolates (44%)
- **Strong biofilm formers (OD > 0.48):** 1 isolate (12%)

Table 4.7 Biofilm formation capabilities among the isolates

Biofilm formation category	No. of isolates	Percentage
Non-biofilm former	2	22
Weak biofilm former	2	22
Moderate biofilm former	4	44
Strong biofilm former	1	12

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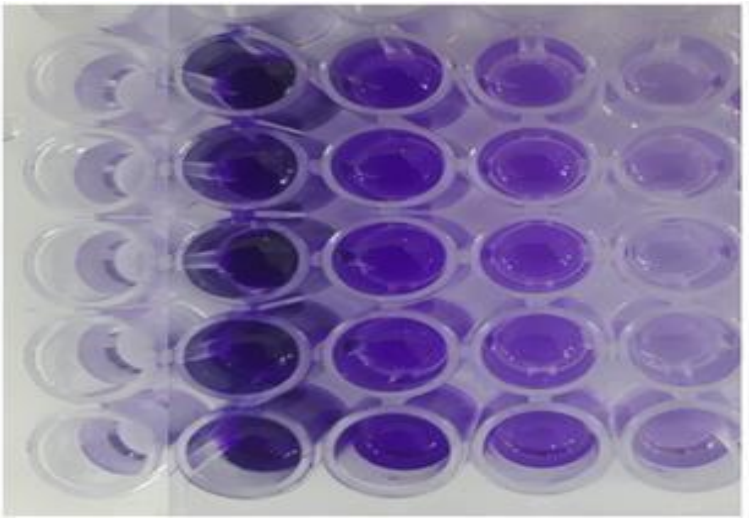

Figure 4.19: MRSA showing biofilm formation

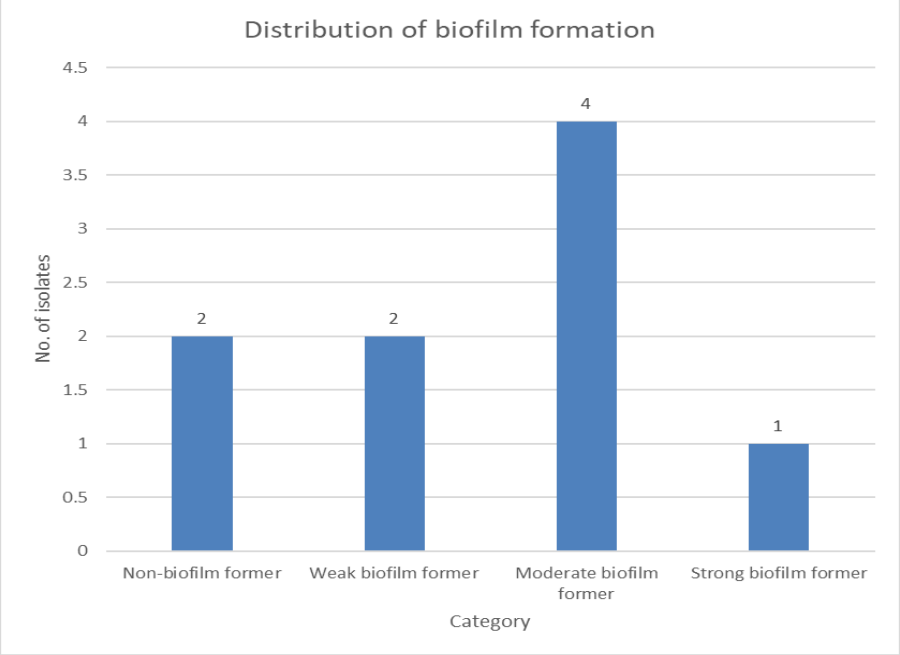

Figure 4.20: Distribution of biofilm formation

DISCUSSION

S. aureus is the most common pathogenic bacterium causing bacteremia and endocarditis, with high mortality rates if not treated on time with antibiotics. Its infection may result in small boils, abscesses and folliculitis. The dental environment, characterized by invasive procedures, exposure to body fluids, and the presence of medical devices, creates an ideal niche for the formation and dissemination of MRSA biofilms. Sepsis will be challenging to control if the *S. aureus* infection is because of the existence of *mec* genes on vector SCC in the bacterium that develops

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immunity against β-lactam antibiotics with production of PBP2a. Due to selection pressure induced by β-lactam antibiotics, different variants of *S. aureus* have exchanged genetic information through lateral gene transfer, resulting in the manifestation of different types of SCC*mec* elements. All isolates were incubated on MSA and blood agar as described by Haque et al. (2019). They collected 430 clinical isolates of *S. aureus* from wounds,

abscesses, aural swabs, burn exudates and diabetic ulcers and cultured them on MSA and blood agar. Morphologically, *S. aureus* was identified with microscopy through Gram's stain method as purple-stained cocci clustered like bunches of grapes, as performed by Al-Dmour et al. (2023). Al-Dmour collected 83 nasal swab samples from hemodialysis patients and confirmed their morphology with the help of Gram staining. A catalase test that resulted in bubble formation with the release of oxygen as described by Khairalla et al. (2017), they applied the catalase test on 1300 nasal swabs and identified it as *S. aureus* using routine microbiological techniques.

Agglutination and coagulation occurred in the case of slide and tube coagulation which reinforced specimens might be coagulase-positive *S. aureus* as Al-Dmour et al. (2023) identified *S. aureus* with the help of coagulase test

Molecular analysis for *S. aureus* was performed by PCR amplification method for the presence of a *nuc*lease gene that produced a thermostable *nuc*lease enzyme. The same test was performed by Khairalla et al. (2017) to confirm the *nuc*lease gene. Most MRSA harbor the SCC*mec*A gene, which is accountable for the synthesis of PBP2a that reduces the affinity of bacteria for β-lactam antibiotics. Ebadi and Khaliliazad (2018) used the *mec*A gene detection method to confirm MRSA molecularly. Genotypic confirmation of MRSA was performed for the *mec*A gene with the help of forward and reverse primers by Rana et al. (2018) with 310 bp depicting the presence of the *mec*A gene. 7 MRSA variants harbor the *mec*A gene and 2 may be bordered with *mec*C/*mec*B/*mec*D as the same results were monitored by Siddiqui et al. (2018). According to him, 36% of phenotypically identified MRSA isolates were flanked with the *mec*A gene and 64% were not bearing the *mec*A gene. Al-Dmour et al. (2023) also presented the same results. Makgotlho et al. (2009) also published that out of 97 isolates, 2 strains did not express the *mec*A gene. The absenteeism of the *mec*A gene was also observed by García-Álvarez et al. (2011) among livestock and humans in Great Britain and Denmark. Ruiz-Ripa et al. (2019) research work also coincides as out of four MRSA three specimens were flanked with the *mec*C gene while only one was harboring the *mec*A gene. The prevalence of MRSA with various SCC*mec* types in hospitals and communities is alarming for public health care authorities all over the world. Because different strains of MRSA may carry multiple genes against antibiotics (Milheiriço et al., 2017).

The occurrence of MRSA ranged from 44% to 57% in Pakistan (Bilal et al., 2021). In the current study, conventional PCR was used to amplify SCC*mec* types with the help of specific primers. 7 isolates harbouring the *mec*A gene were processed for the detection of types. All clinical isolates were loaded with multiple SCC*mec* types. Five isolates among 9 were harbouring five SCC*mec* types (I, II, III, IV, V). Two isolates carried different types n=1 (I, II,IV, V) and the remaining one carried three SCC*mec* types n=1 (I, II,IV). The existence of divergent SCC*mec* types in a single isolate is comparable to the formerly reported studies from Pakistan and all over the world. Zong et al. (2011) were among the first researchers to report the presence of a variety of SCC*mec*

elements in MRSA in 2011. They amplified 77 isolates to type SCC*mec*. About 30% of test clones were loaded with two different kinds of SCC*mec* types. Alharthi et al. (2016) detected eight test clones as MRSA. Six isolates among them carried composite genes of SCCmec type II & III and supported the results of this study.

The findings of this study underscore the heterogeneity in biofilm-forming potential among MRSA isolates from nasal carriage in dental healthcare personnel. With 56% classified as moderate to strong biofilm formers, the data highlight the necessity for routine screening and targeted interventions to mitigate the risk of biofilm-associated infections in clinical settings. The verdicts

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of this study are reliable with several aforementioned studies that have investigated the biofilm-forming potential of MRSA isolates. Moghadam et al. (2014) collected 135 pus/wound swabs from burn patients, and 40 were MRSA. MRSA biofilm formation was reported that 45% were moderate to strong biofilm formers, which aligns closely with our finding of 56%. Saud et al. (2023) collected 352 nasal swabs from healthcare workers, confirming 47.8% as MRSA. The data highlighted that 68.7% of isolates were biofilm-forming further corroborating our results.

MRSA can develop resistance and evolve new strains, so it emerged as a superbug that has become resistant to most antibiotics. The CDC and PHAC (Public Health Agency of Canada) take it as a serious matter of concern. It has established itself as a pathogen for humans and animals in both hospitals and communities. As it is stated, Prevention is better than cure, so physicians are trying to prevent MRSA infections rather than cure them. Clinicians are trying to develop decolonizing agents but are still striving. So, MRSA will be the point of research in the future

SUMMARY

S. aureus has emerged as a superbug causing nosocomial infections of the skin, mucous membranes of the gut and respiratory tract and endocarditis due to its resistance against most antibiotics, resulting in MRSA's evolution. MRSA is a critical threat in healthcare settings. DHP are particularly at risk for MRSA colonization because of their frequent exposure to patients and contaminated surfaces. MRSA represents a major pathogen responsible for numerous HAIs, with its biofilm-forming ability significantly enhancing its resistance to antibiotics and disinfectants. The nasal bearing of MRSA among healthcare workers carries a substantial risk for nosocomial infections, particularly within dental settings where close patient contact is prevalent. Utilizing PCR techniques, this research identified MRSA strains and assessed their biofilm-forming capabilities. High divergence in MRSA is because of different types of transposable elements like plasmids, bacteriophages, transposons, insertion sequences, genomic islands and SCC. Horizontal gene transfer among different species of staphylococci may be the cause of different emerging types of MRSA. SCC is considered an MGE that can acquire genes with different amalgamations of *ccr* and *mec* gene complexes.

To determine the occurrence of MRSA in nasal carriage among DHP, this study intended to isolate, identify, and characterize MRSA genotypically and phenotypically. Additionally, antibiogram analysis, the aptitude of these isolates to form bacterial mats, and the presence of the *nuc* and *mecA* genes as well as SCCmec typing were evaluated. 50 isolates were initially cultured on MSA and blood agar. Methicillin resistance among clinical isolates was phenotypically confirmed through cefoxitin (30µg) disc diffusion methodology according to CLSI guidelines 2023. Out of the 50 isolates, 9 were confirmed as MRSA. All were positive for the nuc gene, confirming them as *S. aureus*, and 7 had the *mecA* gene, indicating methicillin resistance. These 7 MRSA isolates underwent SCC*mec* typing using conventional PCR with specific primers, revealing multiple SCCmec types among the isolates.

Cefoxitin and penicillin were resistant to all MRSA isolates. Vancomycin and linezolid showed universal effectiveness, with all isolates being susceptible. The detailed susceptibility profile disclosed that 33.3% were susceptible to piperacillin/tazobactam, 77.8% to gentamicin, 66.7% to ciprofloxacin, 44.4% to norfloxacin, 55.6% to trimethoprim-sulfamethoxazole, 77.8% to imipenem, and 44.4% to clindamycin. Cefoxitin resistant isolates then underwent molecular analysis for the *mecA* gene through specific amplification by PCR. A set of primers was used to type SCC*mec* gene through single-plex PCR. In the present study, conventional PCR was used to amplify SCC*mec* types with the help of specific primers. 7 isolates harboring the *mecA* gene were processed for detection of types. All isolates were loaded with multiple SCC*mec* types. 5 isolates among 9 were harboring all five SCC*mec* types (I, II, III, IV, V). Two variants carried different types n=1 (I, II, IV, V) and the remaining one carried three SCC*mec* types n=1 (I, II, IV). Bacterial mat development was enumerated using a crystal violet assay, with 6(56%) of the MRSA isolates demonstrating moderate to strong biofilm formation. This biofilm-forming capability is a

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significant virulence factor that complicates infection control efforts, as biofilms enhance bacterial survival on surfaces and medical devices.

The study's limitations include a relatively small sample size and the exclusion of other *mec* genes like *mec*C, which could contribute to methicillin resistance. Future research should involve larger sample sizes and comprehensive molecular analyses to understand MRSA resistance mechanisms fully. In conclusion, this study underscores the significant presence of biofilm-forming MRSA in the nasal carriage of DHP. The findings emphasize the importance of severe infection control measures and continuous surveillance to alleviate the risk of MRSA transmission and associated infections in dental healthcare settings.

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