Prevalence Study of MRSA with an Emphasis on Biofilm Production and Screening of mecA Gene

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ABSTRACT

Objective

The study aimed to address the growing prevalence of methicillin-resistant Staphylococcus aureus (MRSA) and its biofilm-forming capabilities in clinical settings. Specifically, the objectives were to isolate and identify MRSA from various clinical samples, assess the biofilm formation of MRSA, and detect the presence of the mecA gene using PCR. Additionally, the study sought to compare biofilm formation between MRSA and methicillin-sensitive Staphylococcus aureus (MSSA) strains.

Methods

A total of 120 clinical specimens were analyzed using standard microbiological methods. Antibiotic susceptibility was tested using the Kirby–Bauer disc-diffusion method, adhering to CLSI guidelines. Biofilm formation was evaluated using tube, plate, and tissue culture plate methods. MRSA was identified with cefoxitin (30 µg) discs and confirmed by PCR for the mecA gene.

Findings

Out of the 120 S. aureus isolates, 37.3% (45 isolates) were identified as MRSA. MRSA strains exhibited full resistance to penicillin and cefoxitin. All S. aureus isolates produced biofilms, with 48% showing weak biofilm production, 40% moderate, and 8.8% strong biofilm formation. Strong biofilm-forming MRSA strains were resistant to most antibiotics except vancomycin and clindamycin. The mecA gene was present in 75.5% of MRSA isolates, while MSSA strains did not carry this gene. No significant difference was observed between TM/CRA and TCP techniques for biofilm identification ($p = 1.0$). However, there was a significant correlation between mecA gene presence and biofilm formation with both TM/CRA ($p = 0.0013$) and TCP ($p = 0.0089$) techniques.

Novelty

This study highlights the strong correlation between mecA gene presence and biofilm formation in MRSA strains, emphasizing the association between antibiotic resistance and biofilm development. The findings underline the urgent need for enhanced biosafety measures to control MRSA spread in hospital settings.

Keywords: Methicillin-resistant Staphylococcus aureus (MRSA), mecA gene, biofilm formation, antibiotic resistance, PCR, clinical samples, biosafety measures.

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INTRODUCTION

Among the bacterial pathogens, *S. aureus*, is the prime factor that builds a broad range of clinical problems resulting in acquired infections both in hospitals as well as the community.[1]MRSA infections, being the key etiology for nosocomial infection, can result in serious disease, mortality, protracted hospital admissions, and expensive medical care. Hospital-associated (HA-MRSA) and community-associated (CA-MRSA) categories can be used to classify these infections.Their clinical manifestation, molecular traits, antibiotic resistance, and therapeutic modalities differ.[2] The fact that MRSA may grow biofilms on both non-living and living surfaces adds to the complexity of the problem. It has long been recognised that staphylococci are a major contributor to infections linked to biofilms. In spite of this, the majority of studies have concentrated on bacteria that are growing in free-floating (planktonic) cultures, even though antibiotics were first created to target specific bacteria cells. Nonetheless, it is clear that bacteria frequently choose growing in grouped populations.Nearly 80% of human infections are estimated to be caused by biofilms, and one of their most notable traits is their extreme resilience to antibiotics, immune system reactions, disinfectants, and environmental stress.[3] The staphylococcal cassette chromosome mec (SCCmec), a long DNA fragment ranging in size from 20 to 100 kb, was acquired by *S. aureus*, leading to the development of the MRSA strain. The penicillin-binding protein (PBP) is altered by this genetic element, changing from PBP2 to PBP2a. Because PBP2a, which is generated by the SCCmecA gene, has a poor affinity for beta-lactam antibiotics, MRSA can thrive in settings where these drugs are present in high concentrations. Even though PCR is frequently used to find the mecA gene and validate MRSA isolates, not all labs have the resources or capacity to apply this technique. Another way for identifying MRSA is to use the disc diffusion method with cefoxitin and oxacillin, then do further testing using oxacillin resistance screening agar base (ORSAB).[4]

MATERIALS AND METHOD

With ethical approval from the Institutional Review Committee of Dr.Moopen's Medical College, Wayanad, a prospective study was conducted from March to May of 2024. This study was qualitative and conducted in a lab. This research intended to identify the mecA gene in MRSA strains that were extracted from clinical specimens and to evaluate the development of biofilms in MRSA isolates from several age cohorts. Following the technician's first suspicion that the bacteria from several clinical specimens were *S. aureus*, the isolates were gathered and preserved for additional research. Biochemical techniques such as growth on MacConkey agar, Blood Agar, Chocolate Agar, catalase test, oxidase test, and coagulase test were used to confirm the isolates. Post 24-hour incubation period, at 37°C, the antibiotic sensitivity profile of the isolates was identified using the Kirby-Bauer disc diffusion method in Mueller Hinton Agar media. In accordance with CLSI recommendations, documentation of results was performed.[5] To distinguish between MRSA and MSSA, cefoxitin was also added to the antibiotic battery. Divided into two groups, MRSA and MSSA isolates were identified by having a zone of inhibition of 21 mm or less in each case. Three approaches were used to identify the development of biofilms: the Congo red agar method, which was developed by Freeman et al. Brain heart

infusion (BHI) broth, sucrose, Congo red dye, and agar (HiMedia, India) were combined with distilled water to create Congo red agar. The organisms underwent a 24-hour aerobic incubation period at 37°C after being inoculation. Colourless or smooth white colonies were regarded as negative biofilm observations, whereas brown or red colonies with a dry consistency were regarded as positive biofilm observations.[6] Using the test tube approach, 10 millilitres of BHI broth were used to inoculate a loopful of test organisms. The tubes were incubated at 37 °C for a duration of 24 hours. The tubes were dried, decanted, and washed with phosphate buffer saline (pH 7.3) after the incubation period. Following that, 0.1% crystal violet was used to dye the tubes. Any extra stain was removed with deionised water. The tubes' ends were facing up once they had dried.

The control strain's results were used to score the tube method, classifying the biofilm producers as strong, weak, and moderate. Biofilm production was deemed to be positive, when a notable film seemed to cover the tube's wall and bottom.[6] The MRSA isolates were added to 10 millilitres of brain heart infusion broth with sucrose using the tissue culture plate method (TCP), which was then incubated at 37°C for a whole day. After gently tapping each well to remove the contents, the cultures were diluted 1:100 with fresh media and cultured for 48 hours at 370C on a sterile 96-well polystyrene plate. The wells were then rinsed four times with 0.2 ml of phosphate buffer saline (pH 7.2). The wells were stained with 0.1% crystal violet and preserved with 2% sodium acetate. The plates were allowed to dry after deionised water was used to remove any remaining discolouration. The fixed dye was dissolved in each well with 0.2 ml of 33% glacial acetic acid, and the OD was measured in an ELISA plate reader (Thermofischer-MULTI SCAN FC) at 620 nm. In relation to the OD value of the control well (ODc), the isolates were classified as weak (ODc≤OD≤2×ODc), moderate (2×ODc≤OD≤4×ODc), strong (OD>4xODc), and non-biofilm producers (OD<ODc).

The MRSA isolates underwent standard polymerase chain reaction (PCR) genotypic identification of the mecA gene. The DNA was extracted using the thermal shock method described by Tarchouna et al, were the reaction set up involved were 3 minutes of initial denaturation at 95ºC; 30 seconds of additional denaturation cycles at 95ºC; 30 seconds of annealing at 57ºC; 1 minute of extension at 72ºC; and 5 minutes of final extension at 72ºC. The reaction mixture contained one microlitre of each forward and reverse primer.[8]DNA specimen solution 3 microlitre, 12 microlitre of PCR master mix (TaKaRa-Emalard Amp GT PCR master mix), and 20 microlitre of sterile distilled water to top it off. The thermal cycler machine's (HIMEDIA-PRIMA DUO) amplification cycle count was set to 34. Using agarose gel (1%) electrophoresis, the amplified mecA gene was identified, and a representative PCR product was sequenced for verification.[7]

Statistical Analysis

Data analysis was carried using SPSS (version 20.0) software. Chi-squared test helped evaluation of relationships between the biofilm formation techniques (TM, CRA and TCP) and the presence of mecA gene in MRSA isolates at $p < 0.05$, set significance.

RESULTS

All the 120 samples were identified as *S. aureus* by means of Gram staining, biochemical test including oxidase, catalase and coagulase, and characteristic growth pattern in the selective media used. (84.1%) of the isolates were resistant to penicillin followed by cefoxitin (55.8%). And most of the isolates were sensitive to Rifampicin and vancomycin. Based upon resistance to cefoxitin, 45 isolates (37.4%) were identified as MRSA and assigned the letter code S1 to S45 the statistical analysis indicates that the MRSA were more prevalent in adult (64.4%n=29) in age category, male (64.4%n=29) in gender category, (figure1a) and in pus (73%) in source category (figure 1 b). While detecting the resistance pattern of MRSA it was identified that all the isolates were resistant to multiple antibiotics (MDR-MRSA) (figure 1 c).

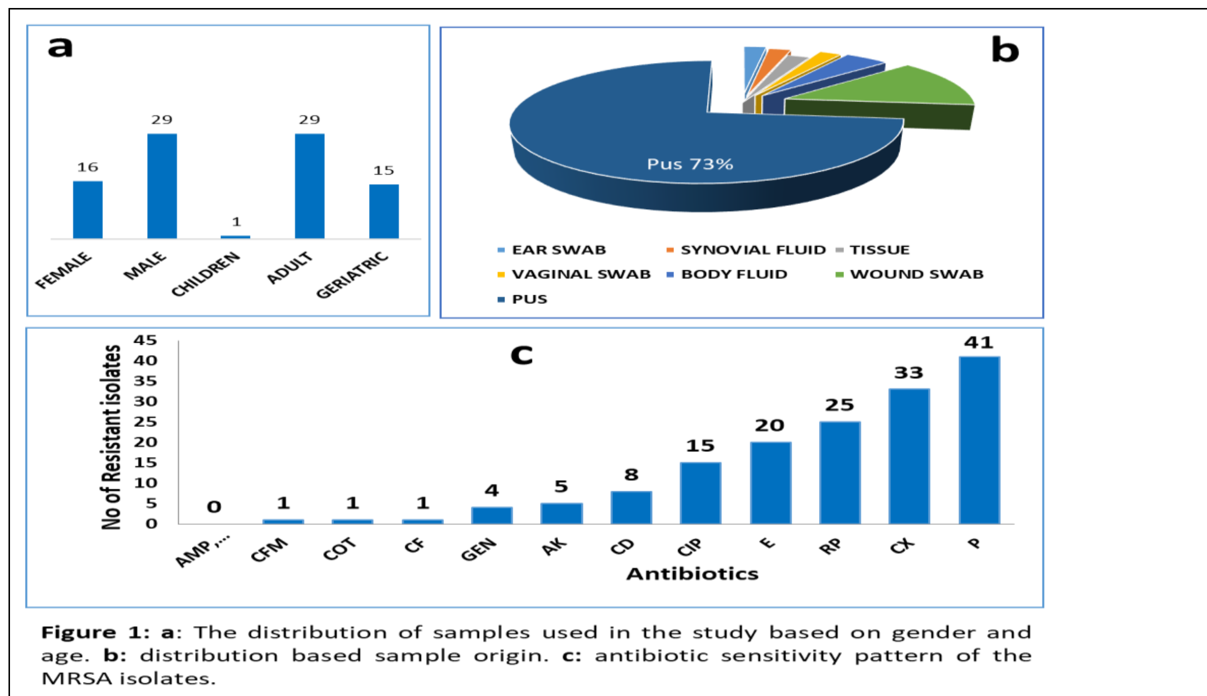

Figure 1: a: The distribution of samples used in the study based on gender and age. **b:** distribution based sample origin. **c:** antibiotic sensitivity pattern of the MRSA isolates.

Typical red dry and rough (RDAR) colored growth with a black coloration of the surrounding medium was observed for all the MRSA isolates on the Congo red agar medium indicating biofilm production (figure 2 a). Similarly, all of them were positive for biofilm production in the tube method as visualized by adhered growth on the wall of the test tube (figure 2 b). 58 % (n=26) were strong, 29% (n=13) were moderate producers of biofilm indicated by the visible intensity of crystal violet retention (figure 2 d). The average OD values obtained in the TCP method were compared with that of the control wells (0.2348) (figure 2 c) in order to categories the MRSA isolates as strong (9%,n=4),moderate (40%n=18)and weak (49% n=22)biofilm producers(figure 2 d) .

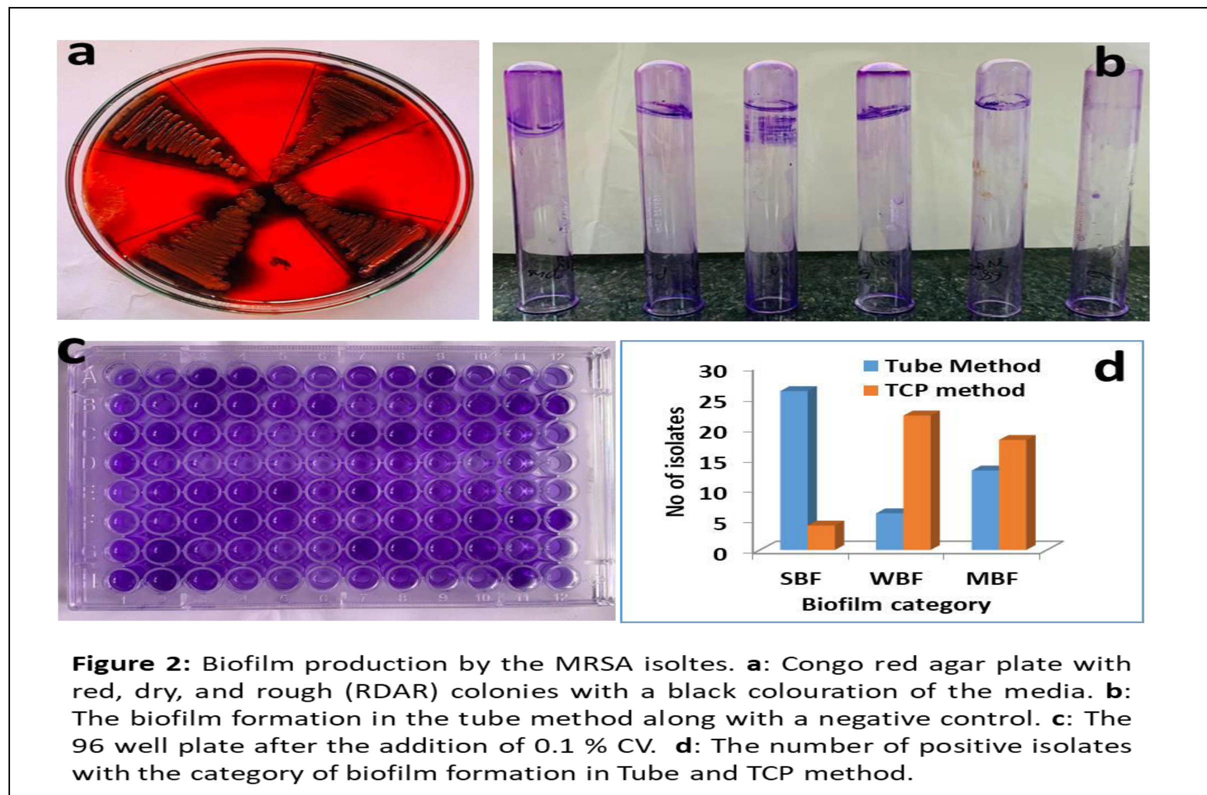

Figure 2: Biofilm production by the MRSA isoltes. **a**: Congo red agar plate with red, dry, and rough (RDAR) colonies with a black colouration of the media. **b**: The biofilm formation in the tube method along with a negative control. **c**: The 96 well plate after the addition of 0.1 % CV. **d**: The number of positive isolates with the category of biofilm formation in Tube and TCP method.

A clear visible band at the position of 533 base pairs were obtained after electrophoresis of the amplified PCR product in the agarose gel (figure 3). 76% of the MRSA isolates (n=34), were positive for the mecA gene. The similarity analysis (nBLAST) of the representative amplicon shows a 100% similarity, as a confirmation of the mecA gene in the MRSA isolates, to already reported mecA genes in the *S aureus* chromosome.

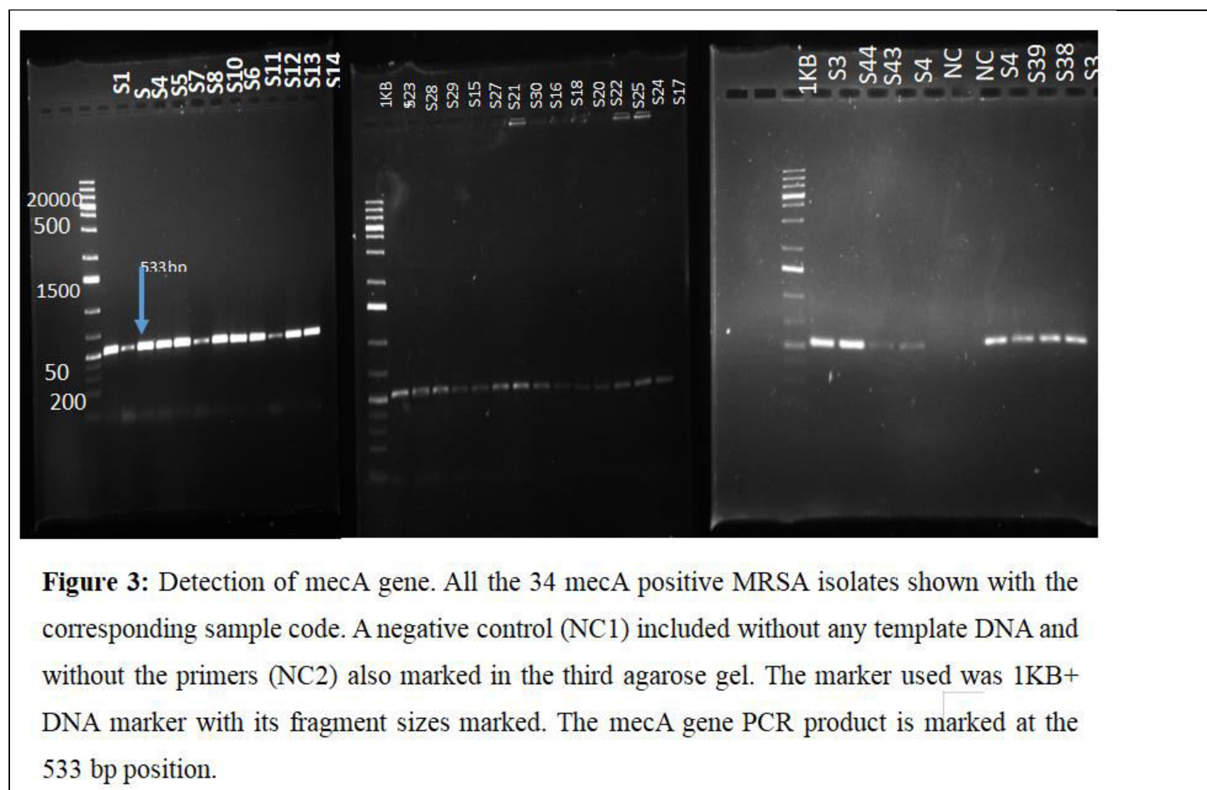

Figure 3: Detection of mecA gene. All the 34 mecA positive MRSA isolates shown with the corresponding sample code. A negative control (NC1) included without any template DNA and without the primers (NC2) also marked in the third agarose gel. The marker used was 1KB+ DNA marker with its fragment sizes marked. The mecA gene PCR product is marked at the 533 bp position.

Chi-square test employed towards correlation between the categorical variable revealed that there is no discernible difference in the ability to identify biofilm generation between the TM/CRA and TCP techniques (p = 1.0; □ = 1.0). MecA gene presence and biofilm development (TM/CRA technique) are significantly correlated (p = 0.0013). Similarly, the mecA gene was shown to be significantly correlated with biofilm development by TCP technique (p = 0.0089). These results imply that the presence of the mecA gene in MRSA isolates, apart from being connected with the development of resistance, is also strongly correlated with the development of biofilms.

DISCUSSION

The present investigation demonstrates that MRSA is an important pathogen in a range of clinical illnesses. MRSA develops biofilms, which adds to its increased drug resistance. Whereas no MSSA isolates in our investigation carried the mecA gene, all phenotypic MRSA isolates tested positive for it. These results are consistent with earlier studies carried out in India that examined the patterns of antibiotic resistance, biofilm formation, and clonal types of S. aureus from isolates obtained in the northeastern and southern regions of Mizoram and Tamil Nadu, respectively.[9]

In our investigation, only 37.5% (45/120) of the 120 samples that were thought to be contaminated with MRSA tested positive. The inclusion of almost all patient samples for testing may be the cause of this comparatively low detection rate. Some investigations, however, have found greater prevalence rates; among them, 46% (115 strains) were found to be methicillin-resistant. Furthermore, 73% of MRSA bacteria showed multidrug resistance; none of them had vancomycinresistance.[10]

Although 37.5% of *S. aureus* isolates in our investigation were methicillin-resistant, other studies have either indicated higher prevalence of MRSA or similar rates. Geographical location, the duration of the studies, infection control procedures, antibiotic prophylaxis and treatments utilised in various hospitals, and the epidemic nature of these diseases can all have an impact on variations in MRSA prevalence. According to research from tertiary care hospitals in Mangalore, India, a sizable percentage of MRSA isolates in the current research were resistant to penicillin.[11] Most *S. aureus* isolates were also resistant to cefoxitin and ampicillin after penicillin. Vancomycin was discovered to be the most effective antibiotic against MRSA isolates, which is consistent with findings from earlier Indian investigations.

Every MRSA isolate found in our investigation produced biofilm. These clinical isolates' increased frequency of biofilm formation could be a factor in their pathogenicity. Our findings concur with other studies conducted in Odisha, India, which found that 27.4% of the state produced biofilms.[12] While some MRSA strains in our study produced significant biofilms, the majority of them only produced weak ones. Furthermore, all MSSA isolates tested negative for mecA, however 75.5% of MRSA strains had the mecA gene, according to PCR analysis. These results are comparable to those of a study carried out in Nepal, where all MRSA isolates were mecA negative and 94.1% of MRSA strains were mecApositive.[13]

CONCLUSION

Given the high rate of methicillin resistance and biofilm formation observed in clinical *S. aureus*isolates, hospitals should reinforce infection control measures by enhancing hand hygiene, disinfection protocols, and isolation practices for MRSA-positive patients. Antibiotic stewardship is crucial, including optimizing prescribing practices, avoiding overuse, and monitoring resistance patterns. Addressing biofilm management through preventive measures, regular cleaning, and exploring antimicrobial coatings can help mitigate biofilm-related complications. Regular molecular screening for MRSA and mecA gene presence, along with tracking and tracing, will aid in timely identification and management of resistant strains. Investing in new treatment options, combination

therapies, and research into innovative diagnostics and preventive technologies is essential. Finally, ongoing education for healthcare staff and patient awareness campaigns are key to improving infection control and patient safety.

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