

Research Article

Amplification of Methicillin Resistant Gene (*mecA*) gene from the MRSA strains

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Available Online: 11th April, 2015

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the vital causes for the hospital infections worldwide. High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2a. The *mecA* gene is a gene found in bacterial cells which allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics. The main objective of the present study was isolation, purification, and characterization of *Staphylococcus aureus* from the clinical bandages and swabs at the hospitals. Genomic DNA was extracted from the identified isolate, and analyzed using biochemical characterization. The MRSA was studied and confirmed at the molecular level by amplifying the gene *mecA* which in turn is responsible for antibiotic resistance. The selected colonies were sub cultured and confirmed of the strains on MSA assay. The isolated strains were morphologically characterized by Gram's Staining and were confirmed to be Gram positive and Cocci shape. The cultures which are *Staphylococcus* turned yellow in colour on MSA agar plate which further confirmed the strains as *Staphylococcus*. All the 10 isolates were confirmed as *Staphylococcus* strains. The *mecA* gene was amplified and the susceptibility and resistivity towards the antibiotics studies were confirmed by the antibiotic susceptibility disc diffusion method.

Keywords: *Staphylococcus aureus*, *mecA* gene, MRSA, PCR amplification

INTRODUCTION

Bacteria are the oldest living organisms that inhabit this planet. During evolution some of these prokaryotic cells have been working together to evolve into multicellular organisms¹. This has resulted in the multidiversity of organisms that have existed and exist to this day. Staphylococci are Gram-positive cocci, which often stick together in grape-like clusters. They belong to the family Micrococcaceae². *S. aureus* is commonly found in air, dust, water, and as normal flora on skin and in the respiratory tracts of humans. The most common mode of transmission is by skin-to-skin contact from an infected host^{3,4}. This common bacterium is the number one cause of nosocomial infections, meaning infections that are acquired while clients are receiving care in a hospital setting⁵⁻⁷. The limited number of morphological and cultural characters of *S. aureus*, and the lack of standardization of cultural conditions and virulence test among different researchers have led to confusion and uncertainty in the characterization of this pathogen⁸. The virulence mechanisms of *Staphylococcus aureus* are not fully known or understood. There is neither question about the present worldwide distribution of staphylococci nor that many of them have the capacity to cause disease. *Staphylococcus aureus* is one of the most important opportunistic pathogen among Staphylococci belonging to Micrococcaceae family causing significant infections under appropriate conditions^{9,10}. *S. aureus* is found as a commensal organism

on the squamous epithelium of the anterior nares up to 20% of the population at any one time, however, it has been estimated that *S. aureus* can transiently colonize up to 60% of the human population¹¹. Furthermore, *S. aureus* has been reported to frequently show multiple antimicrobial resistance patterns, particularly to methicillin and vancomycin^{12,13}. Many studies showed that there is a dramatic increase of *Staphylococcus aureus* that are resistant to multiple antibiotics which poses an increasingly serious problem in many hospitals¹, and it is responsible for numerous hospital outbreaks¹. Antibiotics have been successful in treating bacterial infections, but, due to overuse of antibiotics and incomplete drug courses taken by infected individuals, many clinically relevant bacteria have developed antibiotic resistance¹⁴. In recent years, many *S. aureus* strains have acquired resistance to commonly used antibiotics. Strains that are resistant to methicillin are common and are designated methicillin resistant *S. aureus* (MRSA)^{15,16}. Methicillin was first introduced in 1959 and was very effective in treating patients with penicillin-resistant *Staphylococcus aureus* infections¹⁷. Two years later, in 1961, the first case of MRSA was reported. The most commonly known carrier of the *mecA* gene is the bacterium known as MRSA. Apart from *Staphylococcus aureus* and other *Staphylococcus* species, it can also be found in *Streptococcus pneumoniae* strains resistant to penicillin-like antibiotics¹⁸. In *Staphylococcus* species, *mecA* is

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spread on the SCC *mec* genetic element. The *mecA* gene does not allow the ring like structure of penicillin-like antibiotics to attack the enzymes that help form the cell wall of the bacterium (transpeptidases), and hence the bacteria is allowed to replicate as normal^{19,20}. The gene encodes the protein PBP2A (Penicillin binding protein 2A). PBP2A has a low affinity for beta-lactam antibiotics such as methicillin and penicillin. This enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis^{21, 22}. Methicillin resistance is mediated by PBP-2a, a penicillin binding protein encoded by the *mecA* gene that permits the organism to grow and divide in the presence of methicillin and other β -lactam antibiotics²³. The *mecA* gene is located on a mobile genetic element called a staphylococcal chromosome cassette²⁴. The relative ease of transfer of this genetic element explains the growing resistance to β -lactam antibiotics such as penicillin and its chemical derivatives as well as the cephalosporin drug¹¹. The present study was undertaken to isolate the bacterial isolates especially *Staphylococcus aureus* strains. The isolates were screened and subcultured to isolate the genomic DNA and confirmed for the presence of *mecA* gene.

MATERIALS AND METHODS

Sample collection

Clinical samples like cotton bandages and swabs were collected in sterile test tubes from three different hospitals in Bangalore and are transported to the laboratory immediately under the temperature not less than 18 °C or more than 37 °C.

Isolation of *Staphylococcus*

The samples obtained were serially diluted and 10⁻⁹ and 10⁻¹⁰ dilutions were spread plated. The colonies obtained were screened for colony morphology, gram's staining and confirmatory tests specific for *Staphylococcus*.

MSA Test

In brief, streak a plate of mannitol salt agar with appropriate culture to obtain isolated colonies. The colonies that turn yellow in color after 24-48hrs of incubation with a yellow colored halo around them are MSA positive and the red or pink colonies are negative for MSA, indicating that they are not *Staphylococcus aureus*.

Biochemical Characterization

The different isolates obtained were screened for grams staining. Different mediums were used for the biochemical characterization of the isolated and selected bacteria for their identification according to *Bergey's Manual of Determinative Bacteriology*. Determination of the physiological properties of strains was performed according to the biochemical tests recommended. These tests included, among other assays, aerobic or anaerobic growth, pH range of growth, test for motility, methyl-red and Voges-Proskauer tests, Oxidase, Catalase, hydrolysis of casein, citrate utilization, nitrate reduction, Indole production, Urease test, gelatin hydrolysis, and hydrolysis of polysaccharides and fermentation of various sugars.

Antibiotic Sensitivity Testing: (Kirby Bauer-Disk Diffusion Method)

Antibiotic Sensitivity test was performed using commercially available antibiotic discs. Kirby-Bauer, recommended by the CLSI, was used for antimicrobial susceptibility testing. The identified 32 *P. aeruginosa* strains were tested against Ampicillin (AMP), Gentamicin (GEN), Tetracycline (TE), Methicillin (MET), Amoxicillin (AMX), Rifampicin (RIF) and Cefuroxime (CEF) on Muller Hinton Agar (MHA).

Molecular Characterization

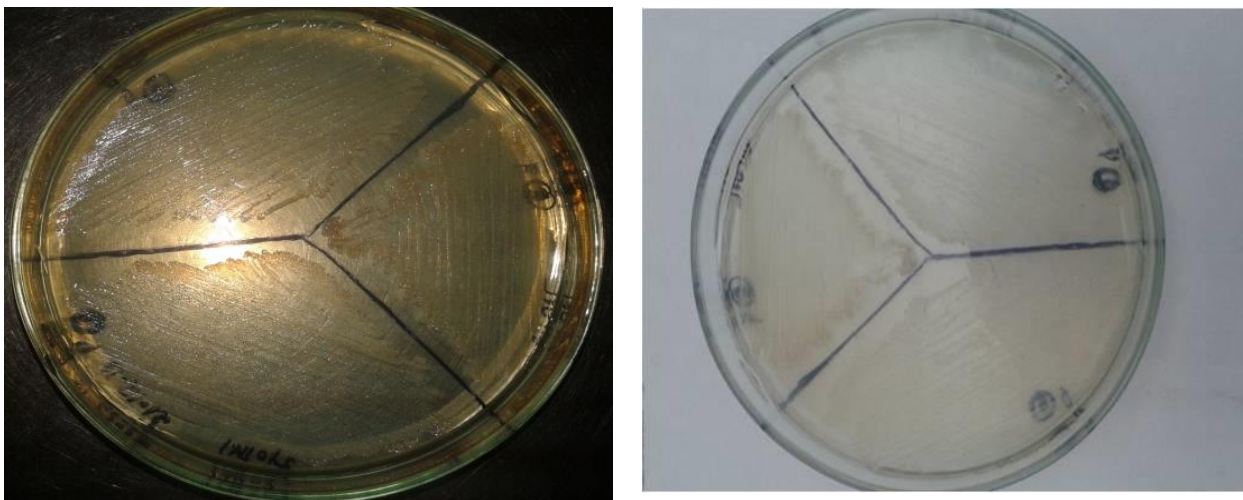
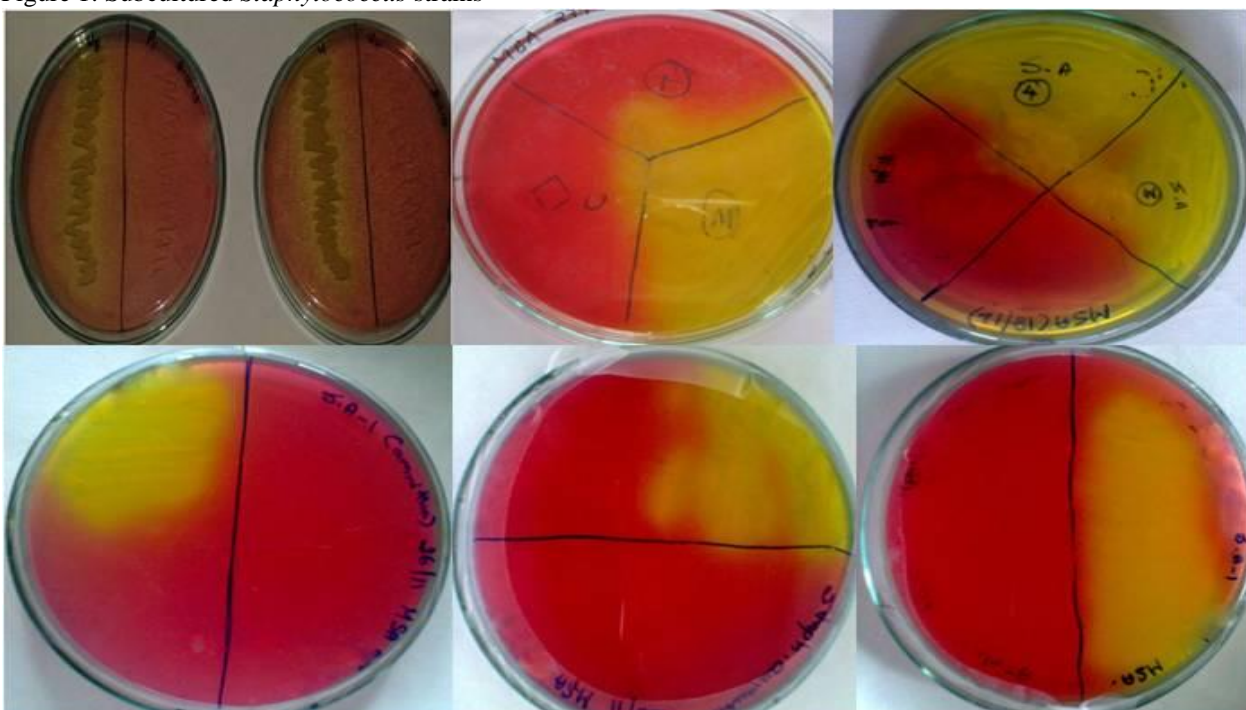
DNA isolation

The isolated colonies were then cultured in Luria-Bertani broth and incubated at 37°C for 48 hours. Following the incubation, 2ml bacterial culture was centrifuged at 6000 rpm for about 10 minutes. To the pellet 1ml of lysis buffer (10mM Tris HCl, pH 8; 0.5M EDTA; 0.5% SDS; 1M NaCl) was added and vortexed properly and incubated at 45 °C in boiling water bath for 10 minutes. Following incubation, 1ml of phenol: chloroform mixture (1:1) was added to the mixture and centrifuge at 10,000rpm for 10 minutes. The upper aqueous layer was transferred and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and then 1/10th volume of 3M sodium acetate was added. The contents are mixed properly and centrifuged at 10,000 rpm for 10 minutes. To the upper aqueous layer double the volume of chilled ethanol was added to precipitate the DNA and later centrifuged at 12,000 rpm for 10 minutes. The DNA pelleted was the stored in 20-50ml of TE buffer and stored at 4°C for further use. The extracted DNA was then quantified using the Nano drop spectrophotometer (ND-1000) to check for the purity. The pure DNA obtained thus obtained was run on 0.8% agarose gel to check for the DNA bands.

The samples were amplified with *mecA* primers namely Forward primer: *mec449F*, 5'-AAA CTA CGG TAA CAT TGA TCG CAA C-3', Reverse primer: *mec761R*, 5'-CTT GTA CCC AAT TTT GAT CCA TTT G-3'. The amplified product was run on a 1 % agarose gel and the result was viewed under UV transilluminator of the Gel Documentation system for further interpretation.

PCR amplification

The *mecA* gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of *mecA* gene. The forward primer for *ndvB*, 5' AAA CTA CGG TAA CAT TGA TCG CAA C 3' and the reverse primer, 5'- CTT GTA CCC AAT TTT GAT CCA TTT G 3', were purchased from Eurofins, Bangalore. These primers correspond to the gene *mecA* and thus the final PCR product was 350 – 400 bp. The PCR mixture consisted of 10x reaction buffer with MgCl₂ (1.5mM), 2 μ L of dNTP mix (2.5mM), 2 μ L each of forward and reverse primers (10picomoles/ μ l each primer), 0.3 μ L of Taq DNA polymerase (5 U/ μ L), and 50ng/ μ L of template DNA in a total volume of 20 μ L. The PCR was performed with the following cycling profile: initial denaturation at 94°C for 3 min, followed by 30 cycles of 30s denaturation at 94°C, annealing at 62°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 2 min. The PCR products amplified were then qualitatively analyzed on 1% agarose gel.

Figure 1: Subcultured *Staphylococcus* strainsFigure 2: Confirmatory test for *Staphylococcus*

RESULTS

Isolation of *Staphylococcus aureus*

6 *Staphylococcus aureus* colonies were isolated from various clinical samples. The strains were sub cultured and maintained on Agar plates. *Staphylococcus aureus* cultures appeared large white to golden colony on the Agar plates. The selected isolates were sub cultured and maintained on Nutrient Agar slants for further use.

Morphological Characterization

Gram Staining

The isolated strains were morphologically characterized by Gram's Staining and were confirmed to be Gram positive and Cocci shape. The cultures which are *Staphylococcus* turned yellow in colour on MSA agar plate which further confirmed the strains as *Staphylococcus*. All the 10 isolates were confirmed as *Staphylococcus* strains.

Biochemical Characterization

The isolated *Staphylococcus aureus* strains were biochemically characterized by IMViC. The test results were observed and tabulated. The results are as follows:

Antibiotic Susceptibility

The results obtained upon Antibiotic Susceptibility test is tabulated below. Most of the strains were found to be resistant to Ampicillin, Methicillin and Amoxycillin.

DNA Qualitative Analysis by Gel Electrophoresis

DNA isolation

The qualitative analysis of DNA by gel electrophoresis is shown in following pictures. The obtained DNA showed sharp single bands on 0.8% agarose gel without any degradation or RNA contamination.

PCR amplification of specific gene

The samples were amplified with *mecA* primers namely Forward primer: *mec449F*, 5'-AAA CTA CGG TAA CAT TGA TCG CAA C-3', Reverse primer: *mec761R*, 5'-CTT GTA CCC AAT TTT GAT CCA TTT G-3'. The amplified product was run on a 1 % agarose gel and the result was

Table 1: Biochemical test result.

Sample NO	Indole Test	Methyl Red test	VP test	Citrate Test	Catalase test
S1	-	+	-	-	+
S2	-	+	-	-	+
S3	-	+	-	-	+
S4	-	+	-	-	+
S5	-	+	-	-	+
S6	-	+	-	-	+
S7	-	+	-	-	+
S8	-	+	-	-	+
S9	-	+	-	-	+
S10	-	+	-	-	+

Table 2: Antibiotic Susceptibility assay. The zone of clearance is expressed in mm. All the values are average of triplicates.

Antibiotic	Zone of Clearance (mm)									
	SA1	SA2	SA3	SA4	SA5	SA6	SA7	SA8	SA9	SA10
Amp	-ve	3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Gen	18	17	1	8	8	1	1	8	10	1
Met	15	15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Tet	15	18	15	13	11	11	15	13	11	16
Amx	-ve	4	-ve	-ve	4	-ve	-ve	-ve	-ve	-ve
Cef	7	5	9		12	1	9	1	5	10
Rif	18	16	8	18	8	8	10	8	15	12

viewed under UV transilluminator of the Gel Documentation system for further interpretation.

DISCUSSION

The present study deals with the identification and amplification of *mecA* gene in *Staphylococcus* sp. isolated from clinical samples using PCR markers. The selected *Staphylococcus aureus* colonies were sub cultured and purified on Nutrient Agar plates and were further maintained on Nutrient Agar slants. *S. aureus* is a common species of this genus and is so named due to its characteristic golden colonies, the Latin word for gold being aurum which, literally translated means 'glowing dawn' after Aurora, the Goddess of dawn in Roman mythology⁷. For further confirmation the selected *Staphylococcus* strains were morphologically characterized by Gram's staining and was found to be gram positive, cocci. *S. aureus* is distinguished from other species on the basis of positive results of catalase, coagulase, mannitol-fermentation, and deoxyribonuclease tests. The bacteria can use galactose and lactose as energy sources, and as such is a notorious cause of bovine and bovine mastitis; and food poisoning via contaminated food, dairy related especially. *Staphylococci* together with Streptococci are members of a group of bacteria known as the invasive pyogenic cocci, since they can cause various suppurative or pus-forming diseases in humans and other animals. They were further biochemically characterized by tests such as Indole test, Methyl Red Test, Voges Proskauer test, Citrate test, Catalase test and Coagulase test. All the strains were negative for Indole test, positive for Methyl Red test and Voges Proskauer test and were negative for Citrate Utilization test and positive for Catalase test and Coagulase test. Hence from these tests it was further confirmed that the isolated strains belonged to the genus *Staphylococcus*.

The 10 selected strains were also checked for their antibiotic resistance against various antibiotics. *S. aureus* on the other hand, exhibits remarkable versatility in its behavior towards antibiotics and the capability of this bacterium to cause human diseases has not diminished even with the introduction of antibiotics²¹. For molecular characterization of the *Staphylococcus* strains, genomic DNA was isolated from 10 *Staphylococcus* sp. by chloroform: isoamylalcohol extraction method. The qualitative estimation of the DNA on 1% agarose gel gave single, sharp and distinct bands devoid of any smear for two samples. Hence the DNA isolation was repeated for those two samples which showed good sharp bands. Thus, genomic DNA of good quality without any degradation was successfully isolated from all the ten isolates.

The *mecA* gene is highly conserved among staphylococcal species. Selection of primers for the amplification of the *mecA* gene is significant for the accuracy of test results. The A+T content of *mecA* is high (c. 70%) and in order to minimize non-specific amplification of unrelated DNA regions, three primers were chosen to contain c. 50% G+C and were combined in two reactions in order to identify altered *mecA* DNA. The majority (89%) of this strain collection carried intact the *mecA* gene with the MRSE possessing *mecA* sequences that were more conserved (94.7%). In the present study the isolates were amplified using primers responsible for the detection of *mecA* gene. It was observed that 83.3% of the isolates showed the presence of *mecA* gene. The *mecA* gene is highly conserved among staphylococcal species^{11, 6}. Selection of primers for the amplification of the *mecA* gene is significant for the accuracy of test results. The A+T content of *mecA* is high (70%). The majority (83.3%) of the strain collection carried intact *mecA* gene possessing *mecA* sequences that were more conserved (94.7%). This study has helped in identifying the

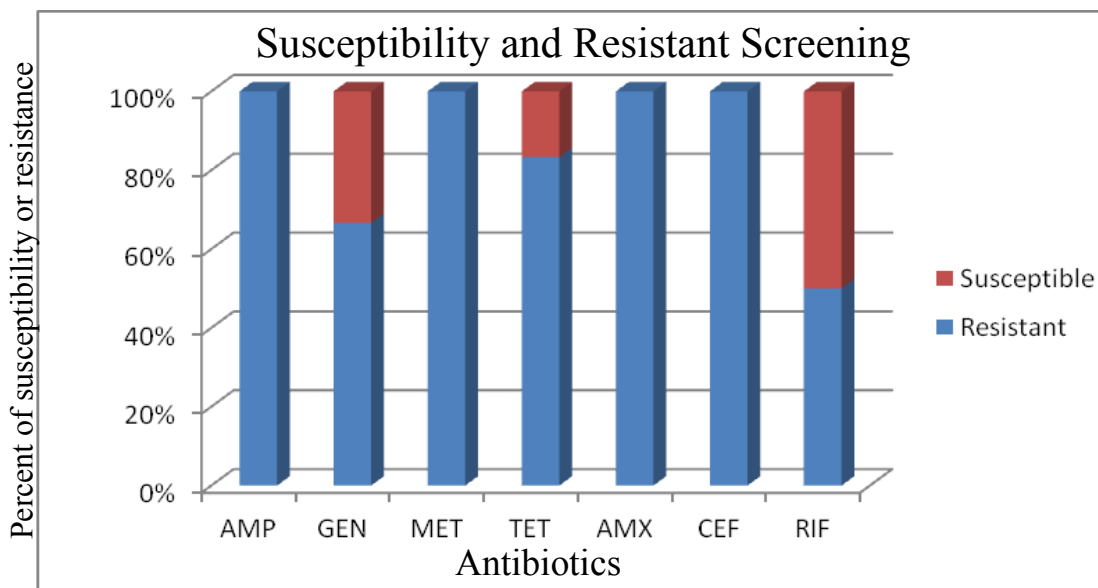


Fig 3: Graph representing the ratio of susceptibility and resistance to the antibiotics. All the values were average of triplicates.

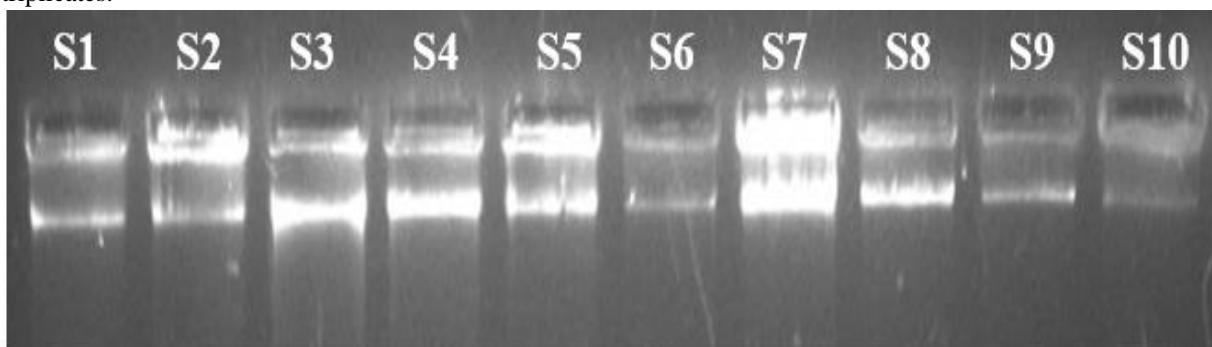


Figure 4: Isolated DNA run on Agarose gel to check purity

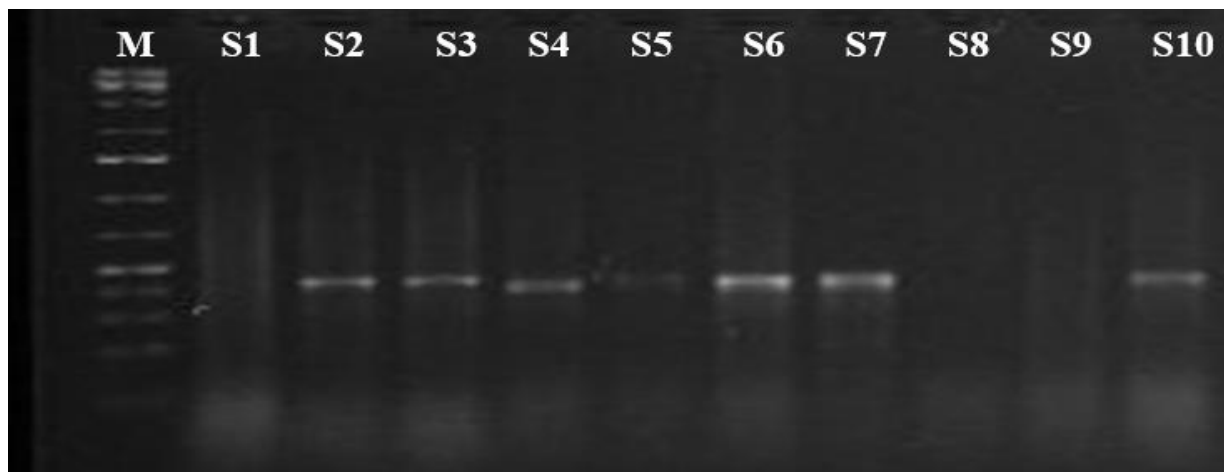


Figure 5: Amplification of the *mecA* gene viewed on 1% Agarose gel. M: Molecular marker, S1, .S10: isolates. The band was found to be near to 400bp. From the result obtained, presence of *mecA* gene was observed only in 7 of the 10 isolates. The presence of band indicates the presence of *mecA* gene in the selected isolates. The size of the gene obtained was found to be approximately 350 – 400bp.

methicillin resistant *Staphylococcus* species isolated from various clinical samples. The population of *mecA*-positive *S. aureus* strains seems to have emerged after acquisition of *mec* elements followed by genetic alterations.

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