Biolife 2(4):1038-1044
AN INTERNATIONAL QUARTERLY JOURNAL OF BIOLOGY & LIFE SCIENCES
ISSN (online): 2320-4257
www.biolifejournal.com
BIOLIFE RESEARCH ARTICLE

STUDY OF ANTIBIOTIC RESISTANCE PATTERN AND DETECTION OF MECA GENE IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CLINICAL SAMPLES

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ABSTRACT

Staphylococcus sp. is an important opportunistic pathogen responsible for a variety of diseases, ranging from minor skin infections to life-threatening systemic infections. The objective of the study was to determine antibiotic resistance pattern of *Staphylococcus aureus* isolated from clinical samples and to detect the presence of mecA gene in the isolates. 10 different *Staphylococcus* strains were isolated from various clinical samples such as urine, pus, etc. Antibiotic resistance pattern of the isolated strains were studied using seven different antibiotics. Presence of mecA gene was analysed using PCR technique. mecA gene was observed in 70 % of the isolated *Staphylococcus* strains. Amplification of mecA gene gives an insight into pharmaceutical aspects of designing new effective drugs for the treatment of methicllin resistant *Staphylococcus sp.*

Keywords: *Staphylococcus sp.*, clinical samples, antibiotic resistance, mecA

INTRODUCTION

Staphylococci are Gram-positive cocci, which often stick together in grape-like clusters. They belong to the family Micrococcacea. There are 45 species and 24 subspecies of the genus Staphylococcus. The genus Staphylococcus, formerly classified in the family Micrococcaceae, is now recognized as belonging to the Bacillus-Lactobacillus-Streptococcus cluster of the low G+C content Gram-positive bacteria. Its closest relatives, with whom it forms the family Bacillaceae, are the genera Enterococcus, Bacillus, Salinicoccus, Planococcus, Brocothrix, and Listeria, but Micrococcus is no longer considered a closely related genus. *S. aureus* has been described as a variable bacterium with many pathogenic and antibiotic resistance variants. 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 pathogen (Kloos and Schleifer, 1981).

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. 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 resistance (Magilner et al., 2008). 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).

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 antibiotics. The mecA gene is located on a mobile genetic element called a staphylococcal chromosome cassette. 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 drug (Denton et al., 2008; Farzan and Hameed, 2006; Ito et al., 2004).
Rapid high-throughput screening tools are needed to reduce costs associated with maintaining strict MRSA control policies. Traditional screening cultures are still applied in most routine laboratories due to their technical simplicity and low costs per analysis. In low-prevalence settings, MRSA findings are commonly confirmed by the gold standard method: polymerase chain reaction (PCR) focusing on identification of the mecA gene. Moreover, complex real-time PCR assays with multiple target sequences, capable of identifying MRSA directly from clinical specimens in a few hours, have been introduced during the 2000s (Huletsky et al., 2004). A multiplex PCR assay for detection of genes for intrinsic methicillin resistance (*mecA*) was developed. Over the last few decades, there has been an enormous increase and emergence of *S. aureus* strains resistant to the antibiotic methicillin (MRSA strains), particularly in nosocomial settings. The intrinsic resistance to these antibiotics is attributed to the presence of *mecA*, whose product is a 78-kDa protein called penicillin binding protein (Malhotra et al., 2008).

MATERIALS AND METHODS

Sample Collection:
Clinical samples like blood, wound, pus, urine and nasal swab from infected patients were collected in sterile test tubes from three different hospitals namely Manipal hospital, Yashomathi hospital and Sparsha health care, Bangalore and transported to the laboratory immediately under the temperature not less than 18 °C or more than 37 °C, specially the blood samples.

Isolation:
100 µl of the collected clinical sample was transferred onto an agar plate. The sample was evenly spread over the surface by swabbing with sterile cotton swabs or by the help of L-shaped glass rod. The plates were incubated overnight at 37 ºC. For swab samples the swab was directly spread on the agar plate and incubated.

Isolation of Staphylococcus sp. on selective media:
The colonies which yielded gram positive cocci in clusters were further isolated and subcultured on to the media. Suspected *Staphylococcus aureus* colonies were streaked on a plate of Mannitol Salt Agar (MSA). The strains that turned yellow in colour after 24-48hrs of incubation with a yellow coloured halo around them are MSA positive suggestive of *Staphylococcus aureus* and the red or pink colonies are negative for MSA, indicating that they are not *Staphylococcus sp.*

Morphological and Biochemical Characterization:
The ten selected isolates were morphologically and biochemically characterized as per standard methods as per (Cappucino and Sherman, 1992).

Antibiotic Sensitivity Testing (Kirby Bauer-Disk Diffusion Method):
Antibiotic Sensitivity test was performed using commercially available antibiotic discs by Kirby-Bauer Disk Diffusion method recommended by the CLSI, 2009. The identified 10 *Staphylococcus* strains were tested against Ampicillin (AMP), Gentamicin (GEN), Tetracycline (TE), Methicillin (MET), Amoxycillin (AMX), Rifampicim (RIF) and Cefuroxime (CEF) on Muller Hinton Agar (MHA).

Amplification of mecA Gene

***Isolation of DNA*:**

DNA was isolated from all the samples by phenol-chloroform method. 2 mL of overnight culture was taken in an eppendrof tube and centrifuged for 10 minutes at 10000 rpm. The obtained pellet was suspended in 1 mL of lysis buffer and incubated at 45 C for 20 minutes. Then 600 µL of chloroform: isoamyl alcohol mixture in the ratio 1:1 was added to all the tubes, centrifuged at 10000 rpm for 10 minutes. The upper aqueous layer was transferred to another tube and was extracted with 500 µL of chloroform: isoamylalcohol in the ratio of 24:1 and centrifuged for 10 minutes at 10000 rpm. The supernatant was transferred to another tube and to that 1 mL of ice cold absolute ethanol was added and kept in deep freezer for 20 minutes then centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded and the obtained pellet was washed twice with 70% ethanol at 10000 rpm for 10 minutes. The obtained pellet was air dried and dissolved in 50-100 µL of nuclease free water and stored at 4 $^{\circ}$C until further use.

Estimation of DNA:

Qualitative estimation of DNA :

The quality of the DNA was estimated using Agarose gel electrophoresis. The obtained DNA was run on 0.8 % Agarose gel stained with Ethidium bromide at 80 – 90 V for 1 hour followed by viewing the gel under UV transilluminator.

Quantitative Estimation of DNA:

The quantity of the isolated DNA was further estimated by measuring the OD 260 and OD 280 or absorbance ratio. DNA yield and quality was calculated using a NanoDrop ND-1000 spectrophotometer (ThermoScientific) at 260 nm.

Analysis of mecA gene using PCR technique:

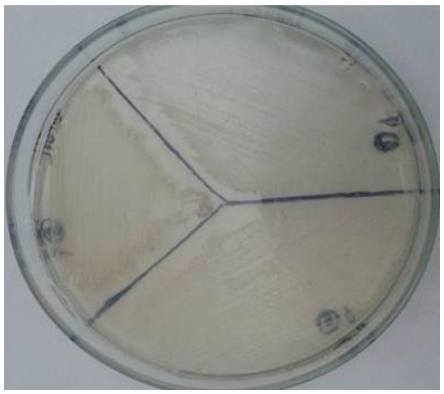
To amplify mecA gene both forward primer; **mec449F**, 5′-AAA CTA CGG TAA CAT TGA TCG CAA C-3′ and reverse primer **mec761R**, 5′-CTT GTA CCC AAT TTT GAT CCA TTT G-3′ were used. The total volume of the PCR reaction was 25 µL, which contained 1 µL of template DNA, 2.5 µL of 10x Taq buffer with $MgCl_2$ (10 mM), 1.5 µL of 2.5 mM dNTPs, 2 µL of Taq polymerase (Chromous), and 1 µL of both forward and reverse primer respectively. The PCR cycle was carried out with the initial denaturation at 94 °C for 3 minutes followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 2 minutes and a final extension of 72 °C for 10 minutes. The obtained PCR product was viewed in 1.5% agarose gel stained with ethidium bromide.

RESULTS

Isolation of Staphylococcus sps:

Suspected *Staphylococcus aureus* colonies were isolated from various clinical samples. The strains were sub cultured and maintained on Agar plates. *Staphylococcus aureus* cultures appeared large off white colour on the Agar plates (figure-1).

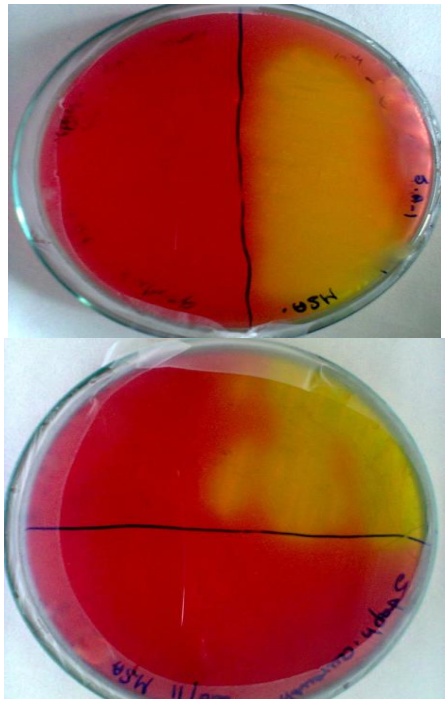
Fig 1: Subcultured *Staphylococcus* strains



Isolation of Staphylococcus sp. on selective media:

The suspected Staphylococcus strains which turned yellow in colour and showed a yellow coloured halo around them were suggestive of *Staphylococcus.* The other strains which did not turn yellow in colour were considered not to be *Staphylococcus.* Among the suspected colonies, 10 colonies turned yellow and showed the presence of yellow halo around them and hence were considered to be *Staphylococcus* positive (figure-2).

Fig 2: Isolation of *Staphylococcus* strains on selective media



Morphological and Biochemical Characterization:

The ten isolates were found to be Gram positive cocci in clusters by Gram's staining and were positive for catalase test.

Antibiotic Sensitivity Testing (Kirby Bauer-Disk Diffusion Method)

Among the 10 strains, 100% resistance was exhibited by the strains against Ampicillin, Methicillin, Amoxycillin and Ceftazidime. 20 % susceptibility was exhibited by the strains against Tetracycline while 35 % and 50 % of the strains were found to be susceptible to Gentamicin and Rifamycin respectively (Table-1) and (Figure-3 and 4).

Estimation of DNA:

Qualitative estimation of DNA

The qualitative analysis of DNA was estimated using Agarose gel electrophoresis as shown in (figure 5). The obtained DNA showed sharp single bands on 0.8% agarose gel without any degradation or RNA contamination.

Quantitative estimation of DNA:

Spectrophotometric analysis of the isolated DNA samples showed concentration in ng/µl ranging from 328.4 – 543.3 ng/µl. The 260/280 ratio of the samples obtained were found which indicates the presence of pure DNA (Figure 6).

Analysis of mecA gene using PCR technique:

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′. It was observed among the 10 strains, 7 *Staphylococcal* strains showed the presence of mecA gene. The obtained product length was found to be 300 bp (figure 7).

DISCUSSION

The present study deals with the determination of antibiotic resistance pattern, identification and amplification of mecA gene in *Staphylococcus* sp. isolated from various clinical samples. Suspected Staphylococcus colonies isolated from the clinical samples were further confirmed using selective media i.e. MSA media. Staphylococci can withstand the osmotic pressure created by 7.5% NaCl, while this concentration will inhibit the growth of most

Table 1: Observed zone of clearance in mm against various antibiotics

Serial No.	Antibiotic	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1	Amp	-ve	3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	Gen	18	17	1	8	8	1	1	8	10	1
3	Met	15	15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4	Tet	15	18	15	13	11	11	15	13	11	16
5	Amx	-ve	4	-ve	-ve	4	-ve	-ve	-ve	-ve	-ve
6	Cef	7	5	9	-ve	12	1	9	1	5	10
7	Rif	18	16	8	18	8	8	10	8	15	12

other gram-positive and gram-negative bacteria. Additionally, MSA contains mannitol and uses phenol red as a pH indicator (pK = 7.8) in the medium. When mannitol is fermented by a bacterium, acid is produced, which lowers the pH and results in the formation of a yellow area surrounding an isolated colony on MSA. A non-fermenting bacterium that withstands the high salt concentration would display a red to pink area due to peptone breakdown. In clinical samples, mannitol positive isolates that form yellow color colonies along with a yellow halo around them are suggestive of *Staphylococcus aureus.* 10 such *Staphylococcus* strains were selected for further study.

Bacterial resistance to antimicrobial agents is a growing problem. This is due to the alteration of resistance mechanisms, acquisition of resistance genetic element from other bacteria, and genetic changes in the bacteria (Hrabak et al., 2010). The phenotypic expression of antimicrobial resistance has been reported to be influenced by various factors (Baddour et al., 2007). In the present study, 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

Figure 3: Graph depicting the antibiotic sensitivity of the selected isolates against various antibiotics

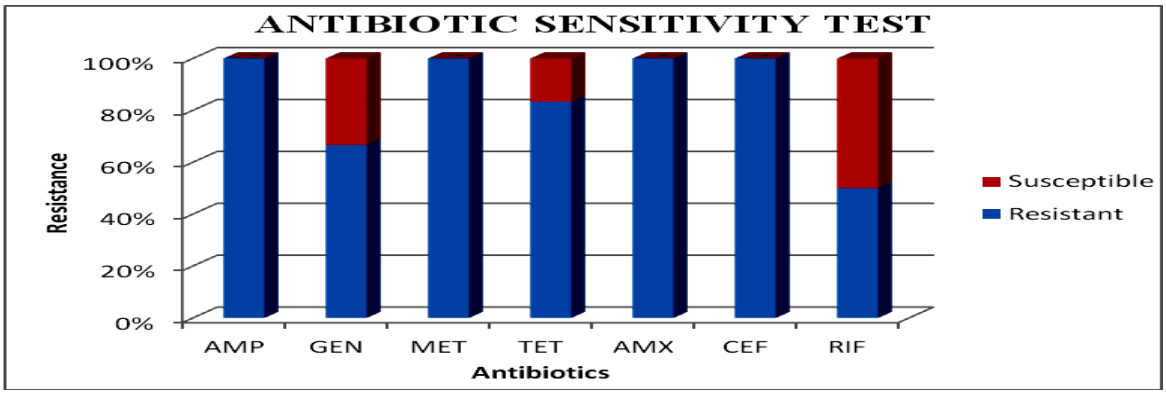


Fig 4: Zone of clearance observed in Antibiotic Sensitivity test

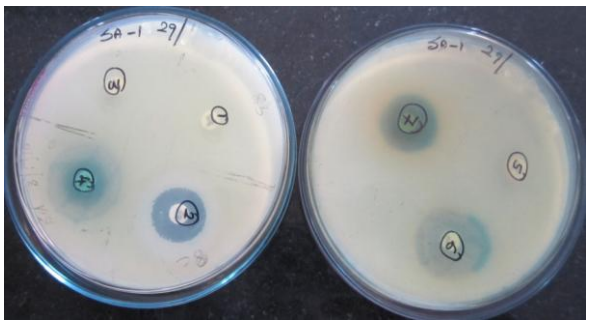


Fig 5: Isolated genomic DNA bands of various

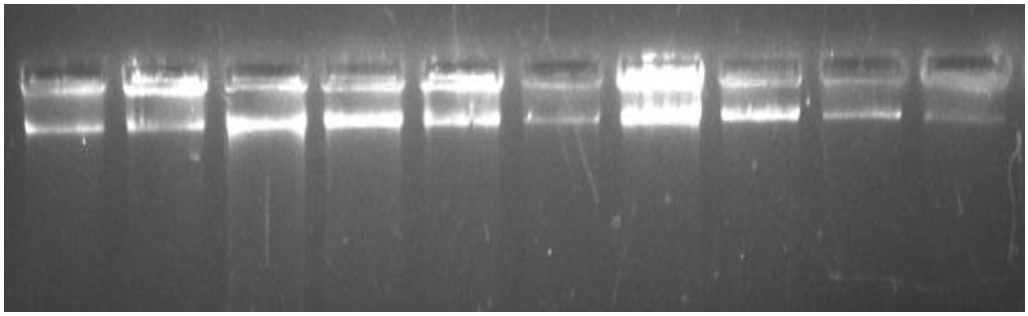


Fig 6: Spectra of genomic DNA obtained by Nanodrop Spectrophotometer for the isolated *Staphylococcus* strains

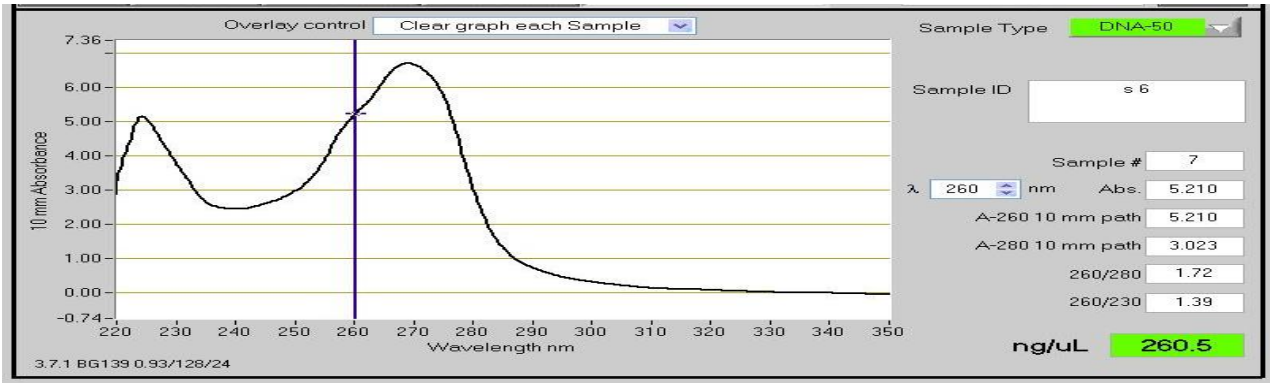
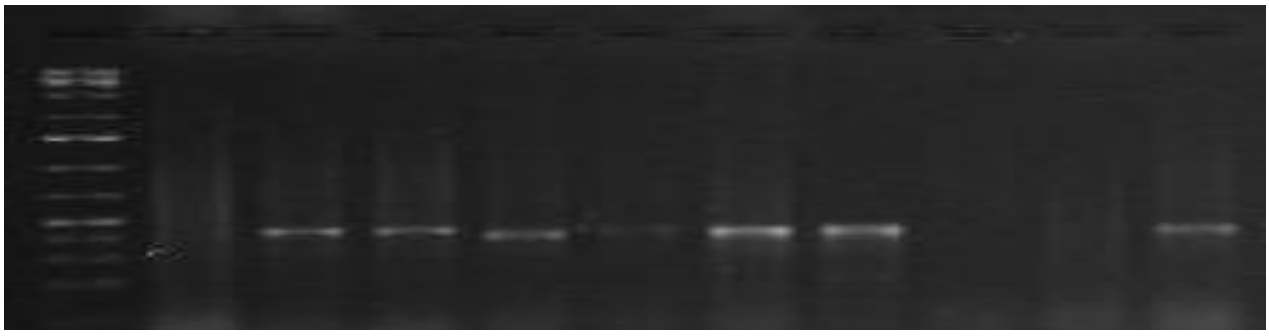


Fig 7: Amplification of mecA gene



antibiotics (Obiazi et al., 2007). 100% resistance was exhibited by the strains against Ampicillin, Methicillin, Amoxycillin and Ceftazidime.

The *mecA* gene is highly conserved among staphylococcal species (Suzuki et al., 1992; Archer et al., 1994). 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%). In the present study, majority (70%) of the strains carried intact *mecA* gene. This study has helped in identifying the 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. Rapid and reliable methods for antibiotic susceptibility are important to institute appropriate therapy. Multiplex PCR can be used for confirmation of the results obtained by conventional phenotypic methods when needed.

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