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MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF MRSA ISOLATES IN VIDARBHA REGION OF CENTRAL INDIA

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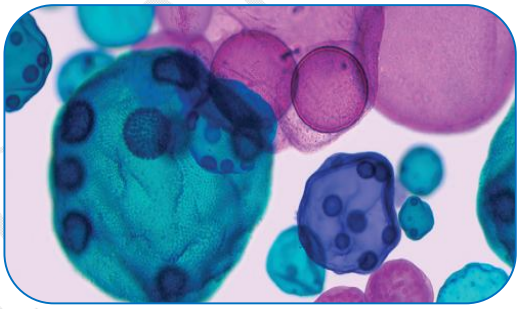
ABSTRACT :



Introduction: *The emergence of hospital acquired methicillin resistant Staphylococcus aureus in patients population is a major public health concern. The coagulase positive species S. aureus is well documented as a human opportunistic pathogen and responsible for more serious infection. The purpose of present study was to evaluate molecular identification and characterization MRSA in Vidarbha region.* ***Materials and Methods:*** *Total 329 MRSA isolates were obtained from three tertiary care clinical centres of Vidarbha region by disc diffusion and MIC method. The presence of mecA gene was determined by PCR among MRSA isolates. The PCR products of 16S rRNA and methicillin resistance gene were Sequences and verified by BLAST search using the NCBI website.* ***Result and Discussion:*** *The prevalence of MRSA in Vidarbha region was found 60.92%. The novel finding of this study is all the tested MRSA strains were found positive to mecA gene. The blast analysis results were showed the 100% matching of both sequences of mecA of B26 isolates with USA 300 strain.* ***Conclusion****: The diverse genetic group of clinical MRSA isolates found globally, however four distinct genotypes of MRSA strains were identified among hospitalized patients in ICU. Our study revealed that ST8/SCCmec/IV/PVL clone of MRSA with multidrug resistance was found in hospitals of central region of India. Study proposed the continuous and nationwide MRSA surveillance studies are essential to find clonal distribution of MRSA in community to hospitals.*

KEYWORDS : *Methicillin resistant S. aureus, PCR, mecA gene.*

1. INTRODUCTION

Staphylococci grow readily on most bacteriologic media under aerobic or microaerophilic conditions. They grow most rapidly at 37 °C but form pigment best at room temperature (20–25 °C). Colonies on solid media are round, smooth, raised, and glistening. *S. aureus* usually forms grey to deep golden yellow colonies. Pathogenic *Staphylococci* are commonly identified by their ability to produce coagulase, and thus clot blood[1].*S. aureus* is well documented as a human opportunistic pathogen. Some of the more serious infections produce by *S. aureus* are bacteremia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, cerebritis, meningitis, chorioamnionitis and scaldead skin syndrome[2].*Staphylococcus aureus* is a major pathogen of increasing importance due to the rise in antibiotic resistance[3]. To date the *S. aureus* genome databases have been completed for 7 strains; 8325, COL, MRSA, MSSA, N315, Mu50, and MW2. The

average size of the *S. aureus* genome is 2.8Mb[4]. The *S. aureus* genome consists of a singular circular chromosome of about 2.7 to 2.9 Mbp containing about2600 genes composed of core and auxiliary (accessory) genes. The majority of genes comprising the core genome are those associated with central metabolism and other housekeeping functions. Supplementing these are genes that are associated with common species functions but that are not essential for growth and survival, including virulence genes. Also included are surface binding proteins, toxins, exoenzymes and the capsule biosynthetic cluster[5,6]. Accessory genes typically have a different G + C content than those in the core genome, often because they are obtained from other species of bacteria. Many of these genetic elements are known to carry genes associated with virulence, drug and metal resistance, to substrate utilization and miscellaneous metabolism[7].

Antimicrobial resistance is natural phenomenon & its effects, amplified by continuing and unnecessarily increase exposure to antimicrobials. *Staphylococcus aureus* have a distinct property to develop resistance against antimicrobials for their survival. Methicillin is most important drug used for the treatment of severe cases of *S.aureus* infection. Resistance to this β-lactam drug in *S. aureus* is of great concern to medical and scientific personnel. The genes for methicillin resistance are located on the chromosome. The genes in the signaling pathway for methicillin resistance are *mecA, mecRl, mecR2* and *mecl. mecA*codes for a penicillin-binding protein, PBP2a (also called PBP2) which has a lower binding affinity for β-lactam drugs than regular PBPs. PBPs are transpeptidases involved in the construction of the bacterial cell wall.

Vidarbha region of Maharashtra state is a central part of our country including tribal district Gadchiroli and Chandrapur, which have various tertiary care clinical centers. Limited reports were available on development of Methicillin resistant *Staphylococcus aureus* form this part of India. Vancomycin from glycopeptides category is last resort of drug. Hence there is need of continuous surveillance of antibiotic resistance patterns of *Staphylococcus aureus* and there genotypic variation to controlled antibiotic resistance problem so that we may not fall back into pre-antibiotic era.The purpose of present study was to evaluate molecular identification and characterization MRSA in Vidarbha region.

2. MATERIALS AND METHOD

The practical work was done in the Department of Microbiology, Sardar Patel College, Chandrapur. The specimens were collected from government hospital of Chandrapur, Gadchiroli and Indira Gandhi Government Medical college and Hospital, Nagpur. These hospitals provide tertiary health care to people of respective district and referrals from other hospitals of province.

2.1 Isolation and Confirmation of bacterial isolates

The specimen (pus, burn wound and sputum) were collected during the study period from all these three hospitals by using sterile cotton swabs and sterile containers (from HiMedia) from different age groups of individual. Specimens were processed within 2 hours of collection by the standard Microbiology technique. The quality control and rejection criterion of the specimen was followed[8].The *S. aureus* isolates were identified on the basis of morphological characteristics, microscopic examination of stained preparation and various biochemical tests such as catalase reaction, coagulase production (slide test and tube test), voges-proskauer (VP). Coagulase positive *S. aureus* are only selected for further study.

2.2 Antimicrobial susceptibility test

In vitro antimicrobial susceptibility tests were performed on Mueller-Hinton agar by two methods: The disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) standards. The Minimum Inhibitory Concentration (MIC) as described by the Etest method in accordance with CLSI standards[9].

2.3 Molecular Characterization

Four highly resistant MRSA isolates were select for molecular study. DNA extraction 1ml of microbial suspension culture was heat inactivated by incubating at 80°C for 15 min. The culture was then centrifuged

at 12000 rpm for 10 min. The cells were isolated and taken in an eppendorf. To these 1X PBS was added. These tubes were centrifuged at 2000 rpm for 10 min. The supernatant was discarded except 0.1 ml. To this, PBS was added up to 0.5 ml, and later 15 µl SDS and 5 µl Proteinase K were added and vortexed. The tubes were incubated in water bath at 55°C for half an hour, after which the tubes were vortexed and incubated for another half hour. Then 5 µl Proteinase K was added and the tubes were incubated for further half an hour.

Following incubation, 100µlNaCl and 80 µl CTAB were added to these tubes and incubated again for 10 min at 65°C. After 10 min, the tubes were brought down to room temperature and equal volume of phenol and chloroform isoamyl alcohol mix (350µl phenol and 350µl chloroform isoamyl alcohol mix) was added. The contents were invert mixed and centrifuged at 12000 rpm for 10 min. The aqueous layer was transferred to new tubes and equal volume of chloroform isoamyl alcohol mix was added. The tubes were gently inverted and centrifuged at 12000 rpm for 10 min. The aqueous layer was again transferred to fresh tubes. To these 30 µl Sodium acetate and 0.6 volumes Isopropanol were added. The tubes were incubated at -20°C for half an hour. The tubes were then centrifuged at 12000 rpm for 10 min. The supernatant was removed except less than 0.1 ml. To this 1 ml of chilled 70% Ethanol was added. The tubes were again centrifuged at 12000 rpm for 10 min. the supernatant was discarded and the pellets were air dried.The pellets were re-suspended in 30 µl of 1X TE buffer. Conventional PCR were performed for the detection of 16S rRNA and *mec*A gene. The visualization of isolated DNA was confirmed by Agarose gel electrophoresis. The PCR products were separated on a 2 % agarose gel, purified and sequenced by Sanger sequencing method at the SciGenom Labs, Cochin India. 16S rRNA and methicillin resistance gene sequences were analysing by BLAST search using the NCBI website for significance.

3. RESULT AND DISCUSSION

3.1 Processing of clinical samples

The research processed a total number of 1162 clinical samples. These were collected from three different districts tertiary care centres of Vidarbha region. This study included 619 clinical samples from IGMC Nagpur, 309 samples from Government District Hospital Chandrapur and 234 from Government District Hospital Gadchiroli. Pus, sputum and burn wound swab were the source of clinical samples in this study. Total 540 coagulase positive *S. aureus* clinical samples, 318 (58.88%) and 222 (41.11%) were isolated from males and females, respectively. The age wise distribution of total patients with Coagulase positive S. *aureus* infection were as follows; the age group 0-10 years included 92(17.03%), 11-20 years 29(45.37%); 21-30 years 40(7.4%); 31-40 years 82(15.18%); 41-50 years 107(19.81%); 51-60 years 93(17.22%); 61-70 years 78(12.3%); and 71-80 years 19(3.5%). Of the 916 *S. aureus* positive clinical samples, 62.6% (573) were of male patients and 37.4% (343) of female patients.

3.2 Antimicrobial Resistance determination

The prevalence rate of methicillin resistance among 540 *S. aureus* isolates on disc diffusion and MIC and was 67.49% and 60.92% respectively. Antimicrobial susceptibility test by MIC is considered as gold standard; therefore the prevalence rate of MRSA in Vidarbha region was 60.92%. Out of three districts the prevalence rate of MRSA is found higher in Chandrapur district (68.00%) as compare to Nagpur (61.60%) and Gadchiroli (48.71%). Prevalence of MRSA has increased rapidly since 1993 at tertiary care centre from 12% in 1992 to 80.89% in 1999. The MRSA isolates showed resistance to several other therapeutic drugs. Incidence of MRSA was as low as 6.9% in 1988 in India and reached to 24% & 32.8% in Vellore &Lucknow reported in 1994[10,11]. Overall prevalence remained in the same range in Mumbai, Delhi & Bangalore in 1996 and Rohtak[11] & Manglore[12] in 1999. However, the situation appeared to be more alarming in Tata hospital in Mumbai[13]where, it reached to 87% in 1995 and tapered to 64% in 1996. In 2001 Vidhaniet. al. found 51.6% MRSA prevalence in New Delhi[14]. The INSAR group[15], India reported MRSA prevalence was 42% in 2008 and 40 % in 2009. According to Khan, *et. al*., study prevalence of MRSA 32% in 2011[16].Tambekar, *et. al,*.were reported high level of HA-MRSA in Vidarbha region[17].

3.3 Molecular characterization of MRSA in Vidarbha region

The primer for 16S rRNA of MRSA was obtained from CIIMS Hospital and Research centre, Nagpur. The obtained primers were tested by aligning them to the reference sequences using BLAST and found to be 100% similar. The amplification reactions were carried out for few MRSA strains. All the strains were positive to 16S rRNA and band size of 1100bp was visualized. The gel photograph is given in **figure 1**

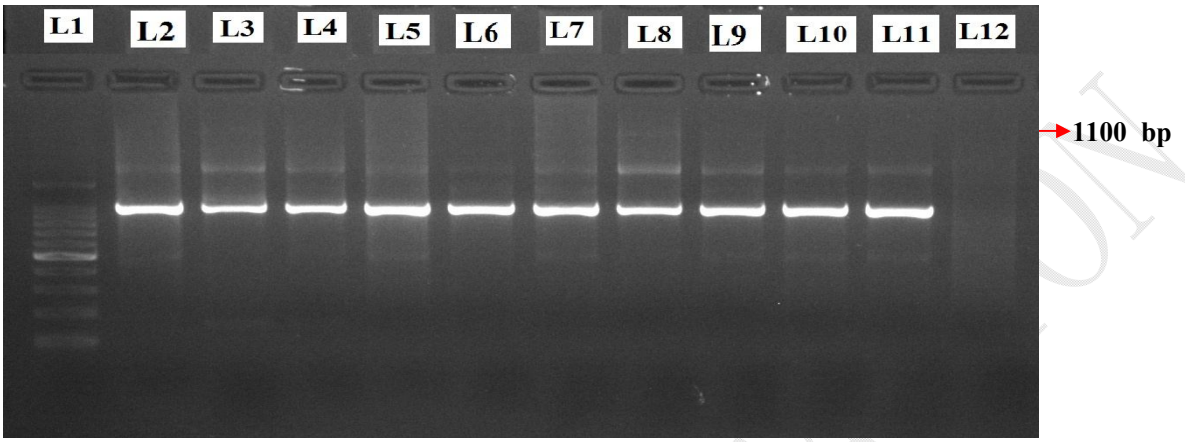


Fig. 1: PCR products of 16S rRNA gene PCR in DNA extracted from *S. aureus* cultures when electrophoresced on 2% agarose gel. L1: 100 bp molecular ladder, L2: 16S positive control, L3-L4: A12 *S. aureus* culture isolate, L5: A63 *S. aureus* culture isolate, L6-L7: A97 *S. aureus* culture isolate, L8-L9: B-26 *S.aureus* culture isolate, L10: 29213 *S. aureus* culture isolate, L11: 25913 *S. aureus* culture isolate, L12: Negative control.

PCR amplification of 16S rDNA of the clinical isolates A12, A63, A97 and B26 using primers specific for *Staphylococcal* 16S rDNA produced an identical 1.1 kb amplicon of 16S rDNA in all the strains. All the tested *S. aureus* isolates were 16S positive

3.4 Phylogenetic analysis

16S rRNA gene sequences were aligned and a phylogenetic tree was constructed. The output was visualized in CLCViewer as a cladogram. Phylogenetic relationship was established with 16S rRNA sequence of other organism available in database. (**Figure 2**)

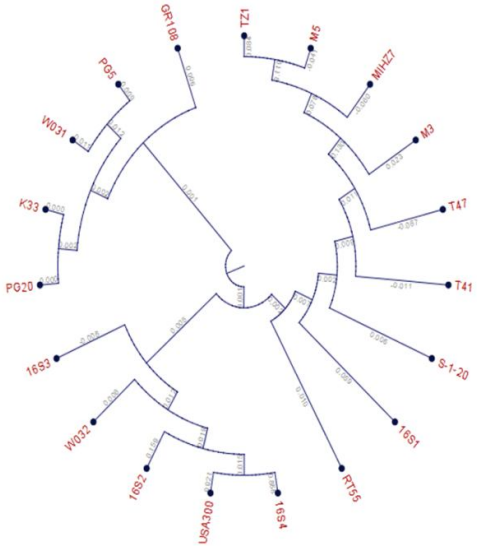


Figure 2: Cladogram depicting comparison of highly conserved 16S rRNA gene sequence of four clinical isolates included in this study with 15 strains that have been reported worldwide. The horizontal bar at the base of the figure represents 0.350 substitutions per nucleotide site.

The output was visualized in CLC Viewer as a cladogram. Isolate B26 was found to be closely related with highly reported strain USA300. The community-associated MRSA strain USA300, which nearly always carries genes for the Panton-Valentine leukocidin (PVL) and the *Staphylococcal* cassette chromosome *mec* (SCC*mec*) type IV, has been reported to be the predominant strain type of MRSA circulating in the United States[18].

3.3 Evaluation of *mec*A genes

The 16S rDNA confirmed MRSA isolates were further used for detection of methicillin (*mec*A) resistant genes. Detection of the *mecA* genes was considered the gold standard for MRSA confirmation[19,20].The obtained primers were tested by aligning them to the reference sequences using BLAST as given below and found to be 100% similar. The amplification reactions were carried out for few MRSA strains. All the strains were positive to *mec*A gene and band size of 310bp. The gel photograph is given in **Figure 3.**

3.4 BLAST analysis results for *mec*A gene primers:

Oligonucleotide primers for mecA gene

Products on target templates

>KM505042.1 *Staphylococcus aureus* strain TN/CNH/6/14 MecA (*mec*A) gene, complete cds
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 178 202
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 487 463
>KM505041.1 *Staphylococcus aureus* strain TN/CNH/5/14 MecA (*mec*A) gene, complete cds
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 178 202
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 487 463
>KM505040.1 *Staphylococcus aureus* strain TN/CNH/4/14 MecA (*mec*A) gene, complete cds
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 178 202
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 487 463
>KM505039.1 *Staphylococcus aureus* strain TN/CNH/3/14 MecA (*mec*A) gene, complete cds
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 178 202
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 487 463
>KM505038.1 *Staphylococcus aureus* strain TN/CNH/2/14 MecA (*mec*A) gene, complete cds
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 178 202
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 487 463
>AB781444.1 *Staphylococcus aureus* subsp. *aureus* 120 DNA, SCC*mec* type V *staphylococcal* cassette chromosome *mec*region
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 1897 1921
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 2206 2182

>AB777516.1 *Staphylococcus aureus* subsp. *aureus* 118 DNA, SCC*mec* type V *staphylococcal* cassette chromosome *mec* region
product length = 310
Forward primer 1 GTAGAAATGACTGAACGTCCGATAA 25
Template 1897 1921
Reverse primer 1 CCAATTCCACATTGTTTCGGTCTAA 25
Template 2206 2182

The Primer-BLAST search was performed by entering the forward and reverse primers without entering any template. The results indicated that all variants from the *mec*A resistance gene have the same amplicons. The above table shows the details only for 7 variants due to space limitation. The current search generated 20,038 BLAST hits. This primer pair indeed showed perfect matches to the *mec*A resistance gene as well as other variants from the same gene and would generate a 310 base amplicon. No non-specific amplifications were found

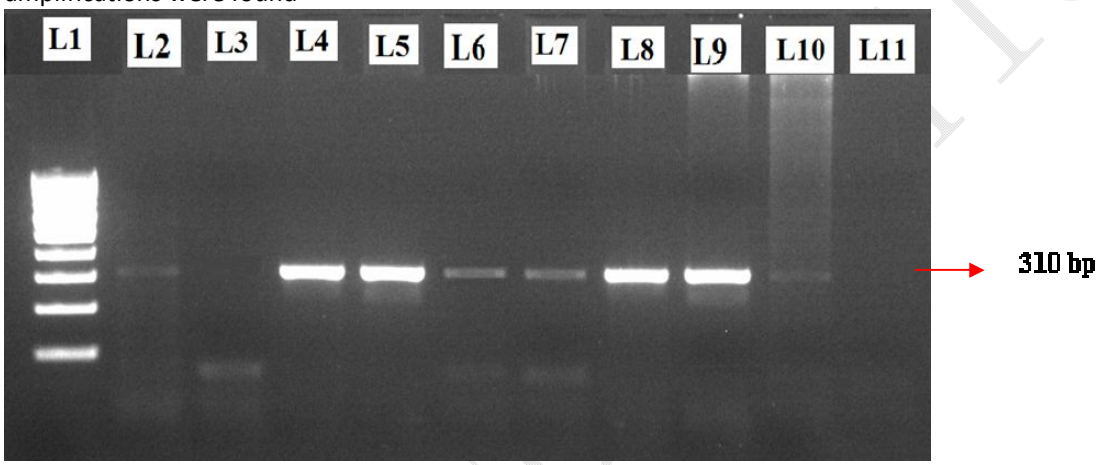


Fig. 3: PCR products of mecA resistance gene PCR in DNA extracted from *S. aureus* cultures when electrophoresced on 2% agarose gel. L1: 100 bp molecular ladder, L2: 29213 *S. aureus* culture isolate, L3: 25913 *S. aureus* culture isolate, L4-L5: A12 *S. aureus* culture isolate, L6-L7: B26 *S. aureus* culture isolate, L8-L9: A97 *S. aureus* culture isolate, L10: A63 *S. aureus* culture isolate, L11: Negative control.

Current study used *mecA* gene detection by PCR as gold standard PCR by using oligonucliotide primer suggested by Tiwari and Sen[21]. In the present study *mecA* gene analysis was positive to all the tested oxacillin resistant isolates on disc diffusion and on MIC. (Table 1)

Table 1: Methicillin Resistance pattern of *Staphylococcal* isolates as determined by PCR

Strain	*mecA* gene
29213 (600 ng/µl) Positive control	*mecA*$^{+}$
25913 (412 ng/µl) Negative control	*mecA*$^{-}$
16S1: A12 (94.2 ng/µl)	*mecA*$^{+}$
16S2: A63 (600 ng/µl)	*mecA*$^{+}$
16S3: A97 (79.8 ng/µl)	*mecA*$^{+}$
16S4: B26 (73.7 ng/µl)	*mecA*$^{+}$

3.5 Sequencing and BLAST analysis of *mec*A gene

The PCR product was then sequenced by Sanger sequencing method at the SciGenom Labs, Cochin India

Sr. No.	Gene	Isolate	Sequence	No. of BLAST hits	Specificity
1	***mec*A**	**A12**	ATAAACATTCAGGATCGTAAAATAAAAAAAGTATC TAAAAATAAAAAACGAGTAGATGCTCAATATAAAA TTAAAACAAACTACGGTAACATTGATCGCAACGTTC AATTTAATTTTGTTAAAGAAGATGGTATGTGGAAG TTAGATTGGGATCATAGCGTCATTATTCCAGGAATG CAGAAAGACCAAAGCATACATATTGAAAATTTAAA ATCAGAACGTGGTAAAATTTTAGACCGAAACA	100	No non-specific hits were seen.

Alignments

No definition line found
Sequence ID: lcl|Query_16965 Length: 2007 Number of Matches: 1
Range 1: 1533 to 1776

Score	Expect	Identities	Gaps	Strand	Frame
451 bits(244)	5e-131()	244/244(100%)	0/244(0%)	Plus/Minus	

Features:

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Query  1     ATAAACATTCAGGATCGtaaaataaaaaaagtatctaaaaataaaaaacgagtagatgct  60
Sbjct  1776  ATAAACATTCAGGATCGTAAAATAAAAAAAGTATCTAAAAATAAAAAACGAGTAGATGCT  1717
Query  61    caatataaaattaaaacaaaCTACGGTAACATTGATCGCAACGTTCAATTTAATTTTGTT  120
Sbjct  1716  CAATATAAAATTAAAACAAACTACGGTAACATTGATCGCAACGTTCAATTTAATTTTGTT  1657
Query  121   AAAGAAGATGGTATGTGGAAGTTAGATTGGGATCATAGCGTCATTATTCCAGGAATGCAG  180
Sbjct  1656  AAAGAAGATGGTATGTGGAAGTTAGATTGGGATCATAGCGTCATTATTCCAGGAATGCAG  1597
Query  181   AAAGACCAAAGCATACATATTGAAAATTTAAAATCAGAACGTGGTAAAATTTTAGACCGA  240
Sbjct  1596  AAAGACCAAAGCATACATATTGAAAATTTAAAATCAGAACGTGGTAAAATTTTAGACCGA  1537
Query  241   AACA  244
Sbjct  1536  AACA  1533
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Methicillin resistant *S. aureus* strains infections have emerged globally as an significant clinical pathogen, including in India. HA-MRSA are emerge as major cause of morbidity and mortality in Intensive care units worldwide[22,23].The novel finding of this study are all the tested MRSA strains were found positive to *mec*A gene. The blast analysis results were showed the 100% matching of both sequences of *mec*A of B26 isolates with USA 300 strain.ST8 MRSA is one of the main causes of human infections worldwide and has progressively obtained SCCmec types I, II, and IV[24]. ST8 MRSA has been found as a distinctive clone and they are main causes for S. aureus infection in Japan and Bangladesh[25,26]. However, ST8/SCCmec/IV/PVL-positive, known as USA 300, is a major cause of soft tissue infection in USA[27]. The emergence of this type in central part of India is an alarming situation for all tertiary care hospitals. There is need of continuous monitoring of this infection and management of *Staphylococcal* infection. Improved professional practices and higher infection-control precautions are essential for controlling and preventing the healthcare-associated infections caused by MRSA in a hospital setting.

4. CONCLUSION

The diverse genetic group of clinical MRSA isolates found globally, however four distinct genotypes of MRSA strains were identified among hospitalized patients in ICU. Our study revealed that ST8/SCCmec/IV/PVL clone of MRSA with multidrug resistance was found in hospitals of central region of India. The present study also concluded that the phenotypic test for antimicrobial susceptibility study could be produce false positive result. This problem might be overcome by using *mec*A gene based detection system by using PCR. As its presence was final confirmation of MRSA. Therefore, it is most sturdy and accurate method for prediction of resistance to oxacillin. 16S rDNA based phylogenetic study indicated that one of the clinical isolate (B26) was closely related to the highly reported community-associated MRSA strain USA300. It also suggested that the three *Staphylococcal* isolates A12, A63 and B26 have originated from a common ancestor. 16S rDNA nucleotide sequence had showed 99% similarity with USA 300 strain. Blast analysis of *mec*A gene nucleotide sequence was given 100% gene sequence similarity with *mec*A gene of sequence USA 300 strain.Continuous and nationwide MRSA surveillance studies are essential to find clonal distribution of MRSA in community to hospitals.

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