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ORIGINAL ARTICLE

Molecular characterization of typing and subtyping of Staphylococcal cassette chromosome SCC*mec* types I to V in methicillin-resistant *Staphylococcus aureus* from clinical isolates from COVID-19 patients

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ABSTRACT

Background and Objectives: Methicillin resistance is acquired by the bacterium due to *mecA* gene which codes for penicillin-binding protein (PBP2a) having low affinity for β-lactam antibiotics. *mecA* gene is located on a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* genomic island comprises two site-specific recombinase genes namely *ccrA* and *ccrB* [cassette chromosome recombinase] accountable for mobility. Currently, SCC*mec* elements are classified into types I, II, III, IV and V based on the nature of the *mec* and *ccr* gene complexes and are further classified into subtypes according to variances in their J region DNA. SSC*mec* type IV has been found in community-acquired isolates with various genetic backgrounds. The present study was undertaken to categorize the types of SCC*mec* types and subtypes I, II, III, IVa, b, c, d, and V and PVL genes among clinical MRSA isolates from COVID-19 confirmed cases.
Materials and Methods: Based on the Microbiological and Molecular (*mecA* gene PCR amplification) confirmation of MRSA isolated from 500 MRSA SCC*mec* clinical samples, 144 cultures were selected for multiplex analysis. The multiplex PCR method developed by Zhang et al. was adapted with some experimental alterations to determine the specific type of these isolates.
Results: Of the total 500 MRSA, 144 MRSA (60 were CA-MRSA and 84 were HA-MRSA) were selected for characterization of novel multiplex PCR assay for SSC*mec* Types I to V in MRSA. Molecular characterization of multiplex PCR analysis revealed results compare to the phenotypic results. Of the 60 CA-MRSA; in 56 MRSA strains type IVa was found and significantly defined as CA-MRSA while 4 strains showed mixed gens subtypes. Type II, III, IA, and V were present in overall 84 HA-MRSA. Molecular subtyping was significantly correlated to define molecularly as CA-MRSA and HA-MRSA however 15 (10%) strains showed mixed genes which indicates the alarming finding of changing epidemiology of CA-MRSA and HA-MRSA as well.

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Conclusion: We have all witnessed of COVID-19 pandemic, and its mortality was mostly associated with co-morbid conditions and secondary infections of MDR pathogens. Rapid detections of causative agents of these superbugs with their changing epidemiology by investing in typing and subtyping clones are obligatory. We have described an assay designed for targeting SSC*mec* types and subtypes I, II, III, IVa,V according to the current updated SCC*mec* typing system. Changing patterns of molecular epidemiology has been observed by this newly described assay.

Keywords: Methicillin-resistant *Staphylococcus aureus*; SSC*mec* types; Staphylococcal cassette chromosome *mec* (SCC*mec*)

INTRODUCTION

Staphylococcal pneumonia is caused by *Staphylococcus aureus (S. aureus)* with a high case fatality rate. The infection is generally endogenous, the bacteria frequently being derived from the patient's skin or nose and the infection is air-borne. However, staphylococcal pneumonia or lung abscess sometimes follows bacteraemia or septicaemia, particularly in individuals with weakened immune systems, low birth weight premature neonates and drug addicts with right-sided bacterial endocarditis (1-3).

MRSA (Methicillin Resistant *Staphylococcus aureus*) was identified in 1960 soon after the introduction of antibiotic methicillin for the treatment of penicillin-resistant *Staphylococcus aureus* due to beta-lactamases penicillinase production (4). Since then, it is the most common pathogens causing various types of skin and soft tissue infections, bacteraemia, infective endocarditis and osteomyelitis, pneumonia, meningitis, septic arthritis, brain abscess, subdural empyema, spinal epidural abscess, neonatal pustulosis, neonatal MRSA sepsis (2, 3).

Nevertheless, a common community pathogen, it is originated twofold as persistently in cases of pneumonia in hospitalized patients. It often attacks the geriatric, neonatal age groups, and patients with Cystic Fibrosis (CF) and arises as a co-infection with influenza viral pneumonia. The clinical course is characterized by high fevers, chills, a cough with purulent bloody sputum, and speedily developing dyspnea. It may evolve into a necrotizing cavity with congested red/purple lungs and airways that contain bloody fluid and thick mucoid secretions. The histologic pattern is characterized by bronchopneumonia that spreads distally from the small airways into the alveolar spaces to form abscesses that connect with the pleural surface and may result in empyemas. The treatment of this organism has become gradually challenging due to multi-drug-resistant (MDR) strains, especially methicillin-resistant *S. aureus* (MRSA). It often complicates influenza with a death toll significantly surpassing those of many past pandemics. *S. aureus* owes its pathogenicity to a series of necrotizing exotoxins that induce a marked neutrophil response resulting in suppurative tissue necrosis (4-7). Particularly potent is a strain of MRSA that secretes an exotoxin known as Panton–Valentine leucocidin (PVL) as well as complicating influenza, this strain has resulted in fatal septic shock in previously healthy persons (8, 9).

Concomitant pandemic COVID-19; a public health emergency of international concern viral respiratory tract infections possibly play a role in promoting the dissemination of MRSA amongst hospitalized patients and in acclimatizing colonization to the disease. MRSA co-infection with COVID-19 viral infections was the leading cause of patient mortality (1).

Methicillin resistance is acquired by the bacterium due to *mecA* gene which codes for penicillin-binding protein (PBP2a) having low affinity for β-lactam antibiotics. *mecA* gene is located on a mobile genetic element called Staphylococcal cassette chromosome *mec* (SCC*mec*) (4). SCC*mec* genomic island comprises two site-specific recombinase genes namely *ccrA* and *ccrB* [cassette chromosome recombinase] accountable for mobility. Currently, SCC*mec* elements are classified into types I, II, III, IV and V based on the nature of the *mec* and *ccr* gene complexes and are further classified into subtypes according to variances in their J region DNA (5-7). SSC*mec* type IV has been found in community-acquired isolates with various genetic backgrounds.

Type I, II and III are large in size and are commonly found health care- associated MRSA (HA-MRSA) whereas Type IV and V are relatively smaller in size and are prevalent in community acquired MRSA (CA-MRSA) along with Panton Valentine Leukocidin (PVL) as a specific marker (3, 4, 10). Due to the smaller size, Type IV and V SSC*mec* variants can be easily transferred the methicillin-sensitive strains of

S. aureus [MSSA] (6).

The epidemiology and evolutionary relationships of MRSA can be better understood by molecular characterization of MRSA. Identification of bacterial genetic background is a crucial stage to decide control measures of MRSA infection. SCC*mec* typing technique is an effective guideline to detect and control the dissemination of MRSA (4, 6). Multiplex PCR, Multilocus Sequence typing, Antimicrobial susceptibility tests, DNA Microarrays are effectively utilized for the identification MRSA types and subtypes (4, 6, 7).There are very few reports available on characterization of the types of SCC*mec* types and subtypes I, II, III, IVa, b, c, d, and V and PVL genes among clinical MRSA isolates from India and Asian countries as well.

In the current study, we scrutinized MRSA, by Microbiological assays from COVID-19 confirmed patient samples. Those isolates that qualified as MRSA by microbial assays were further characterized by molecular techniques by amplifing *mecA* gene from genomic DNA. Bacterial isolates that showed presence of *mecA* gene in their genome were then subjected to typing and subtyping for SSC*mec* element using multiplex PCR technique. For the multiplex PCR assays 16 primer sets were used. The present study was undertaken to categorize the types of SCC*mec* types and subtypes I, II, III, IVa, b, c, d, and V and PVL genes among clinical MRSA isolates.

MATERIALS AND METHODS

Isolation and identification of MRSA were made by standard conventional and biochemical methods described previously (11-14). *S. aureus* ATCC 25923 strain was used as the quality control strain.

Medical records for the source patient were reviewed for demographic information, history of prior hospitalization, and presence of major comorbid conditions (e.g. Diabetes mellitus, renal dysfunction, post-surgical status, malignancy, solid organ or stem cell transplantation, neutropenia, trauma or burn injury) and antibiotic exposure within the preceding year.

MRSA isolates were designated as HA-MRSA if the source patient had any of the following risk factors: a history of hospitalization, residence in a long-term care facility (e.g. nursing home), dialysis, or surgery within one year to the date of specimen collection; growth of MRSA within 48 h or more after admission to a hospital, presence of permanent indwelling catheter or percutaneous device at the time of culture; or prior positive MRSA culture report. If none of the above risk factors were present, the isolates were considered CA-MRSA (12, 15).

Based on the Microbiological and Molecular (*mecA* gene PCR amplification) confirmation of MRSA isolated from 500 MRSA SCC*mec* clinical samples, 144 cultures were selected for multiplex analysis. The multiplex PCR method developed by Zhang et al. was adapted with some experimental alterations to determine the specific type of these isolates (8, 9).

DNA extraction. Genomic DNA of all 144 MRSA was extracted following standard extraction procedures with some modifications. Cells from overnight grown pure culture were harvested by centrifugation and were resuspended in TE buffer (pH 8). Cells were treated by lysozyme and were mechanically disrupted with glass bead lysis by vortexing. Cell lysate was centrifuged at and the supernatant was separated and further treated with 1% SDS and 100µg proteinase K (incubation at 50°C for 30 minutes). Post incubation, equal volume phenol: chloroform: isoamyl alcohol mix was added to remove the denatured protein. After centrifugation, the aqueous layer was collected and 50 µg of RNAse A was added and the sample was incubated at 37°C for 30min. Additional extraction step of Chloroform: Isoamyl alcohol was carried out and the aqueous layer was separated post centrifugation. Genomic DNA in aqueous layer was precipitated with 0.15M NaCl and 2 volume chilled absolute ethanol. Pellet obtained after centrifugation was subjected to 70% ethanol wash. Final pellet of genomic DNA post centrifugation was dissolved in sterile distilled water and stored at 4°C till further use. The quality of the DNA was checked on 0.8% Agarose gel.

Primer synthesis for multiplex PCR. Primer sequences (Zhang et al. 2005) (8) mentioned in the reference (Table 1) were commercially synthesized from Sigma and IDT.

Multiplex PCR. 25 µl reaction mixture for multiplex PCR had 1× PCR buffer, 0.02mM each deoxynucleoside triphosphate, different concentrations of primers as mentioned in the above Table, 1.25U Taq DNA polymerase and extracted genomic DNA as a template. A single reaction of negative control (with-

Table 1. Primer sequences as per standard reference Zhang et al. 2005

Sr. No.	Name of primer	Primer Sequences (5' – 3')	Concentration in PCR (µM)	Size of Amplicon (bp)	Specificity
1	PCD96 F	GCTTTAAAGAGTGTCGTTACAGG	1.02	613	Type I
2	PCD97 R	GTTCTCTCATAGTATGACGTCC			
3	PCD81 F	CGTTGAAGATGATGAAGCG	0.85	398	Type II
4	PCD82 R	CGAAATCAATGGTTAATGGACC			
5	PCD83 F	CCATATTGTGTACGATGCG	0.67	280	Type III
6	PCD84 R	CCTTAGTTGTCGTAACAGATCG			
7	PCD85 F	GCCTTATTCGAAGAAACCG	0.68	776	Type IVa
8	PCD86 R	CTACTCTTCTGAAAAGCGTCG			
9	PCD87 F	TCTGGAATTACTTCAGCTGC	0.66	493	Type IVb
10	PCD88 R	AAACAATATTGCTCTCCCTC			
11	PCD89 F	ACAATATTTGTATTATCGGAGAGC	0.74	200	Type IVc
12	PCD90 R	TTGGTATGAGGTATTGCTGG			
13	PCD92 F	CTCAAAATACGGACCCCAATACA	0.87	881	Type IVd
14	PCD93 R	TGCTCCAGTAATTGCTAAAG			
15	PCD94 F	GAACATTGTTACTTAAATGAGCG	0.83	325	Type V
16	PCD95 R	TGAAAGTTGTACCCTTGACACC			

out template) was also set up.

Multiplex PCR amplification program was set up in SureCycler 8800 (Agilent Technologies). The program was set as, initial denaturation at 94°C for 5 min followed by first 10 cycles of 94°C for 45 seconds, primer annealing at 65°C for 45 seconds and 72°C for 1.5 min and another 25 cycles of 94°C for 45 seconds, primer annealing at 55°C for 45 seconds and 72°C for 1.5 min, ending with a final extension step at 72°C for 10 min and followed by a hold at 4°C. Post completion of PCR, the amplicon was visualized on 1.5% agarose gel containing ethidium bromide.

Typing of MRSA with Multiplex PCR using primer sets described by Oliveira et al. Out of 144 selected MRSA cultures with *mecA* gene in their genome, 23 could be categorized to specific type and subtype using the primers described by Zhang et al. Hence typing of remaining 21 nontypeable MRSA was carried out using primers described by Oliveira et al by multiplex PCR.

Primers. Primer sequences (Oliveira et al. 2002) are described in Table 2 and were commercially synthesized from Sigma.

Multiplex PCR. The multiplex PCR reaction was set up with component concentrations as mentioned in the typing by Zhang et al. except the primer concentrations. All 8 pairs of primers were used in the reaction mixture with the concentration mentioned in the above Table 2.

Multiplex PCR amplification program was set up with some minor alterations in SureCycler 8800. The program was set as an initial denaturation step at 94°C for 5 min followed by 30 cycles of 94°C for 30 seconds, primer annealing at 53°C for 30 seconds and 72°C for 1 min, followed by a final extension step at 72°C for 5 min and hold at 4°C. Post completion of PCR, the amplicon was loaded on 2.5% agarose gel containing ethidium bromide.

RESULTS

A total of 500 MRSA strains were isolated from various clinical samples of COVID-19-positive patients. Distribution of MRSA was done with respect to clinical infections, age sex time of admission, and admission in the ICU. Among the 500 MRSA strains, 228 (46%) were isolated from sputum and tracheal aspiration; 158 (32%) were from pus samples. 86 (17%) strains were from blood samples and 28 (6%) were urine samples. Overall 361 (72%) patients were male, and 139 (28%) were female patients. Final outcome analysis revealed that 337 (67%) patients reported were dead of which 241 (72%) were identified as HA-MRSA while 96 (28%) were defined as CA-MR-

Table 2. Primer sequences as per references by (Oliveira et al. 2002)

Sr. No.	Name of primer	Primer Sequences (5' – 3')	Concentration in PCR (nM)	Size of Amplicon (bp)	Specificity
1	PCD98 F	TTCGAGTTGCTGATGAAGAAGG	400	495	I
2	PCD99 R	ATTTACCACAAGGACTACCAGC			
3	PCD100 F	AATCATCTGCCATTGGTGATGC	200	284	II
4	PCD101 R	CGAATGAAGTGAAAGAAAGTGG			
5	PCD102 F	ATCAAGACTTGCATTCAGGC	400	209	II, III
6	PCD103 R	GCGGTTTCAATTCACTTGTC			
7	PCD104 F	CATCCTATGATAGCTTGGTC	800	342	I, II, IV
8	PCD105 R	CTAAATCATAGCCATGACCG			
9	PCD106 F	GTGATTGTTCGAGATATGTGG	200	243	III
10	PCD107 R	CGCTTTATCTGTATCTATCGC			
11	PCD108 F	TTCTTAAGTACACGCTGAATCG	400	414	III
12	PCD109 R	GTCACAGTAATTCCATCAATGC			
13	PCD110 F	CAGGTCTCTTCAGATCTACG	400	381	IA
14	PCD111 R	GAGCCATAAACACCAATAGCC			
15	PCD112 F	CAGGTCTCTTCAGATCTACG	800 and 400	303	IIIA
16	PCD113 R	GAAGAATGGGGAAAGCTTCAC			

SA and 163 (32%) were discharged indicative of high mortality. MRSA is the major cause of nosocomial mortality and morbidity.

Identification and selection of unique and specific loci and primer designs for SCC*mecA* types and subtypes. For the typing and subtyping analysis of MRSA isolated from clinical samples, 144 genomic DNA (GDNA) were selected which had shown presence of *mecA* gene in their genome by PCR as per our earlier work (10). The mec element in these GDNA was determined by multiplex PCR using 8 primer sets described in the research work by Zhang et al. As per this article, MRSA could be classified into Type I to Type V (Table 1).

The multiplex reaction was carried out by grouping the primer sets in 3 groups based on the expected amplicon size for better resolution on agarose gel.

Group 1. had primer sets for the subtypes IVa (776 bp), II (398 bp) and IVc (200 bp),

Group 2. had the primers for the subtypes IVb (493 bp) and III (280 bp) whereas

Group 3. had the type/subtypes I (613 bp), IVd (881 bp) and V (325 bp) primers.

As it is evident from (Fig. 1) below when 44 GDNA were screened with multiplex PCR for the presence of type/subtypes using group1 set primers, 56 (56%) showed amplicons of size 776 bp which is the expected size for subtype IVa whereas 26 (18%) GDNA showed presence of type II which gives amplicon of size 398bp and none of them showed amplicon for presence of subtype IVc.

When the group 2 primer set was used for screening of type/subtype by multiplex PCR, none of the GDNA could give amplification indicating the absence of subtypes IVb and III. When GDNA were subjected for amplification using group 3 primers sets, only 11 (8%) samples gave the amplicon of size 325 bp indicating the presence of type V (Fig. 2). Whereas types I (613 bp) and IVd (881 bp) related amplicons were not seen even though the respective primer sets were present in the multiplex PCR reaction.

After screening all 144 GDNA of MRSA using multiplex PCR and 8 primer sets corresponding to 5 types as mentioned in Table 1, 97 GDNA got characterized into specific types and subtypes. 47 GDNA were non typeable using the primers suggested by Zhang et al. These 47 GDNA were hence screened by another 8 primer sets as suggested by Oliveira et al. A multiplex PCR was set up with all 8 primer sets in a single reaction and 47 GDNA were screened for typing analysis.

As depicted in (Fig. 3), when the multiplex PCR was carried out with 8 primer sets, 15 (34%) MRSA GDNA showed 2 amplicons. As per (Table 2) amplicon of 342 bp size is indicative of I, II and IV types but presence of 381 bp amplicon along with 342 bp classifies it as type IA (Oliveira et al.). Sample C7 showed amplicon of 243 bp size which could characterize it as type III.

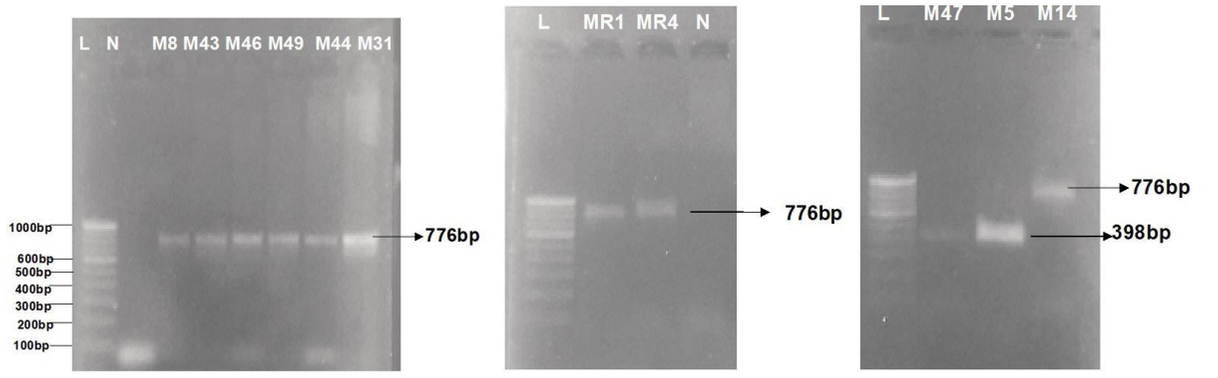

Fig. 1. Multiplex PCR using group1 primer sets to detect subtypes IVa (776 bp), II (398 bp) and IVc (200 bp). L; 100 bp ladder, N; Non template ontrol, M8-M31; MR1-MR4; M47-M14: Amplicons post multiplex PCR using MRSA genomic DNA isolated from pure colonies derived from clinical samples.

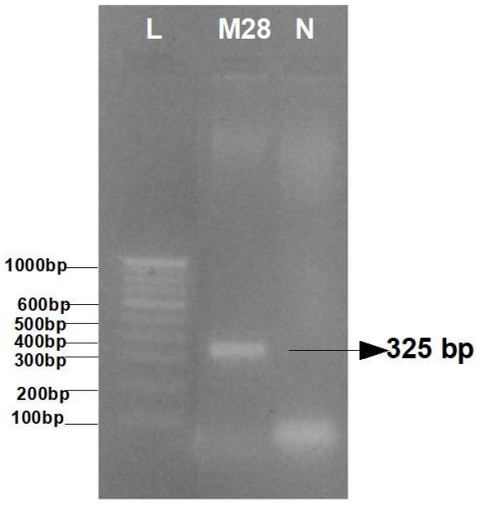

Fig. 2. Multiplex PCR using group 3 primer set to detect types I (613 bp), IVd (881 bp) and V (325 bp). L; 100 bp ladder, N; Non template control, M28: Amplicon from GDNA of MRSA.

15 (10%) MRSA also showed mixed types as evident form Fig. 4. MRSA isolate M33 showed multiple amplicons which could not be classified as a single type. Also MR6 and MR9 isolates showed amplicons of 2 sizes which is a mixed types indicative of IA and II. Whereas M6 gave amplicons of 2 sizes which fall into types I, II and IV. Type IA 25 (17%), Type III 11 (8%), were identified. There were no isolates that could not be typed. As mentioned in the Table 3, Type IVa , type II and type IA were the predominant subtype and type respectively for the selected MRSA.

Of the total 500 MRSA, 144 MRSA (60 were CA-MRSA and 84 were HA-MRSA) were selected for characterization of novel multiplex PCR assay for SSC*mec* Types I to V in MRSA. Molecular characterization of multiplex PCR analysis revealed results compare to the phenotypic results. Of the 60 CA-MRSA; in 56 MRSA strains type IVa was found and significantly defined as CA-MRSA while 4 strains showed mixed gens subtypes. Type II, III, IA, and

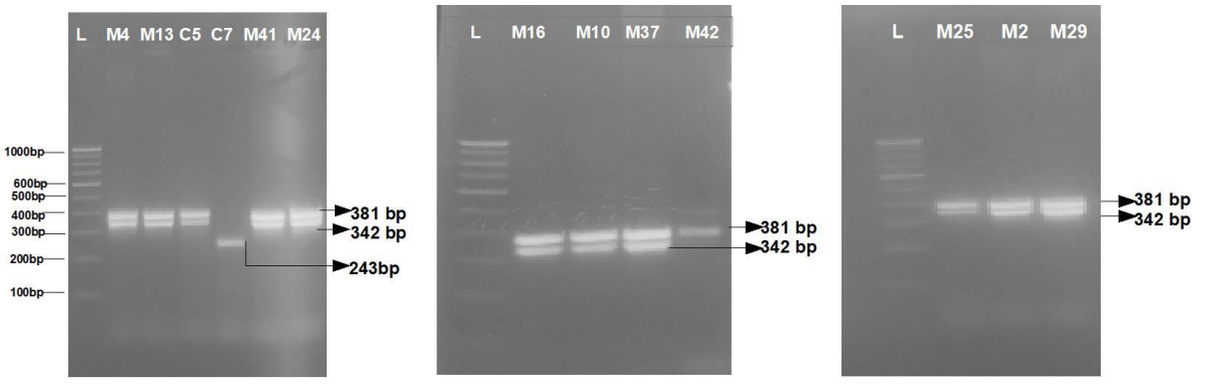

Fig. 3. Typing of MRSA with multiplex PCR using 8 primer sets. L: 100 bp ladder, M4-M24; M16-M42; M25-M29: Amplicon of respective GDNA of MRSA isolated from clinical samples

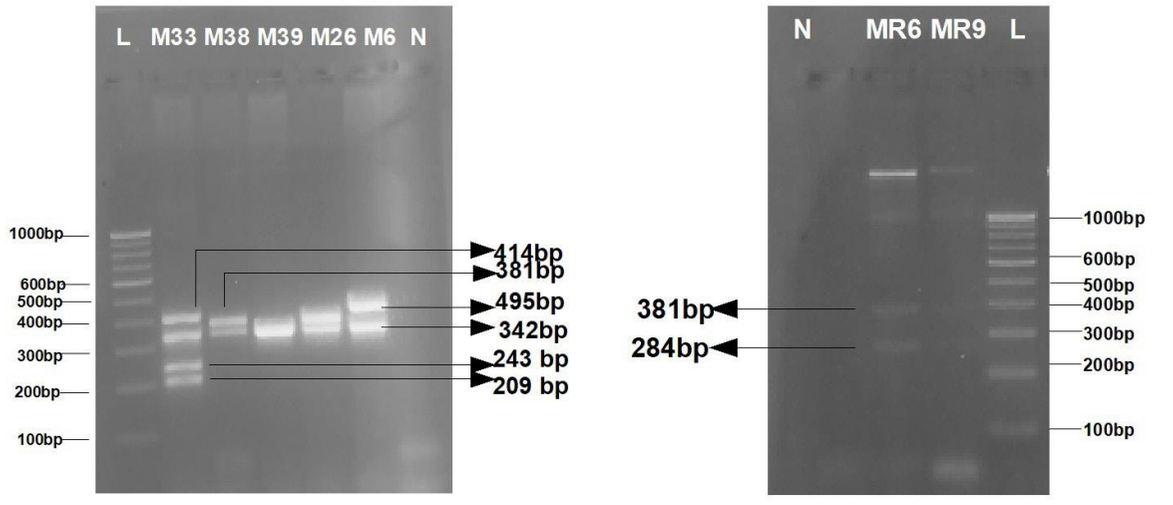

Fig. 4. Multiplex PCR for typing analysis using 8 primer sets. L: 100 bp ladder, M33-M6; MR6-MR9: Amplicon of respective GDNA of MRSA isolated from clinical samples

Table 3. Typing and subtyping analysis of 144 MRSA

Subtype	Positive isolates	Approximate %
IV a (Zhang et al.)	56	38.88
II (Zhang et al.)	26	18
V (Zhang et al.)	11	7.63
I A (Oliveira et al.)	25	17.36
III (Oliveira et al.)	11	7.63
Mixed (Oliveira et al.)	15	10.41
Total	144	

V were present in overall 84 HA-MRSA. Molecular subtyping was significantly correlated to define molecularly as CA-MRSA and HA-MRS; however, 15 (10%) strains showed mixed genes which indicates the alarming finding of changing epidemiology of CA-MRSA and HA-MRSA as well.

DISCUSSION

There are very few reports available on characterization of the types of SCC*mec* types and subtypes I, II, III, IVa, b, c, d, and V and PVL genes among clinical MRSA isolates from India and Asian countries as well.

During the hospital course of the hospitalized 500 COVID-19 patients co-infected with MRSA were foremost common complication was sepsis followed by pneumonia 203 (41%), systemic inflammatory response syndrome 172 (34%), acute respiratory distress syndrome 91 (18%), and multi-organ failure 34 (7%). Final outcome analysis revealed that 337 (67%) patients reported died, 163 (32%) were discharged indicative of high mortality. MRSA is the major cause of nosocomial mortality and morbidity.

In the past viral COVID-19 pandemic, MRSA was the leading cause of secondary bacterial pathogen that cause significant patient mortality rates. However, the impact of coinfection of MRSA with COVID-19 viral infection remains unclear. Adalbert JR et al. (2021) reported in a scoping review that of the 115 co-infected patients, there were a total of 71 deaths (61.7%) and 41 discharges (35.7%), with 62 patients (53.9%) requiring ICU admission. Patients were infected with methicillin-sensitive and methicillin-resistant strains of *S. aureus*, with the majority (76.5%) acquiring co-infection with *S. aureus* following hospital admission for COVID-19. Aside from antibiotics, the most commonly reported hospital interventions were intubation with mechanical ventilation (74.8%), central venous catheter (19.1%), and corticosteroids (13.0%) (1).

SCC*mec* types and subtypes I, II, III, IVa, b, c, d, and V and PVL genes among clinical MRSA typing is one of the most important molecular tools available for understanding the epidemiology and disseminated clonal strain relatedness of CA-MRSA and HA-MRSA, particularly with the emerging worldwide outbreaks. SCC*mec* element has a very complex and diverse structure and its detection (15-18).

After years of decreasing, the overall rate of MRSA cases stabilized in 2017 through 2020' the rate of hospital-onset cases increased 13% in 2020, while

the rate of community-onset MRSA cases decreased 5% compared with 2019. COVID-19: U.S. Impact on Antimicrobial Resistance, Special Report 2022, is a publication of the Antimicrobial Resistance Coordination and Strategy Unit within the Division of Healthcare Quality Promotion, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention. According to this report [www.cdc.gov/drugresistance/pdf/covid19-impact-report-508.pdf].

Hospital-onset MRSA bloodstream infections conceivably increasing and in the present study, we were found 86 (17%) cases were from blood stream infections and mortality was reported high. While this is a small segment of the overall MRSA cases, this increase, combined with the stabilization of overall MRSA cases, indicates that progress to prevent MRSA bloodstream infections in healthcare slowed likely due to challenges created by the pandemic. CDC partner studies identified interventions like MRSA decolonization to reduce the spread of pathogens in intensive care units and nursing homes, especially when combined with rigorous infection prevention and control.

John Chipolombwe et al. reported that A combination of three swabs from different body sites resulted in the highest detection rate for MRSA colonization (19). Present study indicates real burden of MRSA among health-care providers suggesting to prepare policy to design appropriate strategies for the control and prevention of MRSA at the study site and other similar settings. Kassu Desta et al. reported from Ethopia as health-care providers exhibit a higher burden of MRSA carriage than administrative staff. Xueying Yang et al. (2021) from China reported new spa type t19702 (20). We also revealed 3 types of SCC*mec*, namely, SCC*mec* type II, SCC*mec* type IVa, and SCC*mec* type V, with the most prevalent clonotypes being ST72 and ST59 (6, 21). Masafumi Seki et al. (2015) suggested active surveillance with PCR may be highly sensitive and useful for the early diagnosis of MRSA colonization to prevent nosocomial transmission from the emergency department to the regular inpatient wards of the hospital (22).

In the present study, all MRSA isolates were 100% susceptible to vancomycin and teicoplanin; however, MIC values plays a significant role in clinical outcome. Ching-Yen Tsai et al. (2018) from Taiwan reported the Impact of teicoplanin maintenance dose and MIC values on the clinical outcomes of patients treated for methicillin-resistant *S. aureus* bacteremia (23). Results of the present study and comparison through other reports of MRSA suggest that the differences between susceptibility and development of resistance pattern of MRSA isolates are influenced by the local environment, selective antibiotic pressure, acquisition and loss of plasmids carrying resistance genes and various mechanisms of antibiotic resistance. In the last decade, there are many reports about the emergence of the clonal complex of MRSA. Edet E Udo et al. (2020) from Kuwait reported the Emergence of MRSA Belonging to Clonal Complex 15 (CC15-MRSA) in Kuwait Hospitals (24).

MRSA infections are generally spread in the community by contact with infected people or through contact with a contaminated wound of MRSA or by sharing personal items, such as towels or razors, that touche infected skin. HA-MRSA infections usually are associated with invasive procedures or devices, such as surgeries, intravenous tubing or artificial joints; however, Azar Rahi et al. (2019) reported raw milk of dairy multidrug-resistant of multi-drug resistant MRSA which pose a hygienic threat concerning the consumption of raw milk in Iran (25).

Jeffrey C Peterson et al. (2019) reported clonal complexes CC5 and CC8 were the most frequent clones detected among the MSSA and the MRSA keratitis isolates. USA100 and USA300 clones were the dominant MRSA genotypes suggesting the changing epidemiology with the increasing prevalence of multidrug resistance among both MSSA and MRSA keratitis is a cause of concern (5, 26). In the present study, MRSA was found as an opportunistic pathogen caused by co-infections in combination with COVID-19 in hospitalized patients. The predominant underlying diseases were sepsis followed by pneumonia, systemic inflammatory response syndrome, acute respiratory distress syndrome, and multi-organ failure. However, even patients without underlying diseases and in all age groups may be at risk of co-infections as well. In the present study, the novel assay offers a rapid, simple.

In the present study, the recently described novel method of multiplex PCR offers a rapid, simple, and feasible method for SCC*mec* typing and subtyping of MRSA and may serve as a useful tool for clinical microbiologists, and epidemiologists in their infection control efforts to prevent hospital-acquired infections caused by these MRSA. Simultaneously it could be a very useful tool to do rapid molecular investigations

in pandemics like COVID-19 and control the further silent MDR pandemic.

In the present study, we acknowledge the potential limitations. It has been recently shown that some methicillin-susceptible *Staphylococcus aureus* [MSSA] and methicillin-susceptible coagulase-negative *Staphylococci* [CONS] could harbor SCC*mec* lacking *mecA* gene. Such SCC elements play a vehicle in the transfer of genes and/or genetic markers of antibiotic resistance and virulence factors making the strains more virulent and MDR. This genetic movement in Staphylococci is expecting further investigations. This multiplex PCR plays a crucial role in the same investigations. Secondly, we have investigated only MRSA as a co-infection in COVID-19 however multidrug resistant carbapenemase-producing *Klebsiella pneumoniae* and *Acinetobacter baumannii* were also the predominant secondary pathogens in COVID-19 patients. For the treatment of MDR there is need to develop more study for the efficacy of alternative medicines ((27, 28).

CONCLUSION

We have all witnessed of COVID -19 pandemic, and its mortality was mostly associated with co-morbid conditions and secondary infections of MDR pathogens. Rapid detections of causative agents of these superbugs with their changing epidemiology by investing in typing and subtyping clones are obligatory. We have described an assay designed for targeting SSC*mec* types and subtypes I, II, III, IVa,V according to the current updated SCC*mec* typing system. Changing patterns of molecular epidemiology has been observed by this newly described assay.

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