"PREVALENCE AND MOLECULAR IMPACTS OF MRSA ISOLATES WITH IDENTIFICATION OF MECA AND ERMA GENES IN NORTH INDIA"

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Abstract

Background: Methicillin-resistant *staphylococcus aureus* (MRSA) infections have created big threats globally in the hospitals and communities due to their resistance with most of conventional antibiotics. To minimize infections of MRSA, continuous monitoring, knowledge of its prevalence and maintenance of hygienic condition have been required.

Aim and objectives: To study the prevalence and molecular impacts of MRSA isolates with identification of MecA and ErmA genes in north Indian region.

Methods: This was a cross sectional study performed in the Department of Microbiology and Central Research Laboratory of RMCH &RC (India) for a period of 3 years (Feb 2018 to Feb 2021). All the MRSA were collected mainly from blood and pus in different locations. All the standard phenotypic and genotypic protocols (disc diffusion, PCR etc) were followed to perform this study.

Results: A total of 90 MRSA isolates were identified by CX, OX, and E-test. The prevalence of MRSA was found to be around 41% in our study. MRSA isolates were found more in pus samples (61.11%) with the maximum age of 41-50 years. All MRSA isolates were found sensitive to linezolid, Teicoplanin, vancomycin, and Gentamycin however all the isolates were recorded resistant with Cefoxitin and Oxacillin. The presence of MecA gene was recorded in all the 90 isolates of MRSA while the presence of ErmA gene was found in 5 isolates only. The presence of MecA and ErmA gene was confirmed by the PCR followed by sequencing.

Conclusions:

This study has exposed a clear cut message to the clinicians for the better prescriptions, measurements of MRSA infections in communities and hospitals. The data generated in this study would be useful for the identification of MRSA isolates for future studies as well.

Keywords: MRSA, Disc Diffusion, Antibiotic Sensitivity, MecA gene, ErmA gene, NCBI data base.

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INTRODUCTION

There are several known pathogen including the *Staphylococcus aureus* (*S. aureus*) which causes infection in the skin, soft tissues, deep seated tissues, pneumonia, and post operative areas of human being. *Staphylococcus aureus* is a Gram-positive cocci bacterium and has mainly two types of strains; methicillin-susceptible *Staphylococcus aureus* (MSSA) and Methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. One of the highly infective strains of *Staphylococcus aureus* is MRSA which has multidrug-resistant recorded first in 1960. Further, MRSA has been found responsible to caused hospital acquired (HA-MRSA) and community acquired (CA-MRSA) infections [2]. In last few decades MRSA has been found to cause many severe infections such as nosocomial, necrotizing fasciitis, potential fatal diseases, pneumonia, osteomyelitis, endocarditis, severe sepsis, and toxic shock syndrome etc [3]. Many self-regulating risk factors have been reported due to MRSA infection such as hemodialysis, immune-suppression, peripheral mal-perfusion, diabetes and ulcers etc [4]. Along with HA-MRSA and CA-MRSA one more genetic variant of MRSA has been known as livestock-acquired MRSA (LA-MRSA) [5].

All these genetic variants of MRSA have created big challenges about to cure an infected person due to resistance to multiple classes of antibiotics including penicillin, and methicillin. Furthermore, MRSA has been found co-resistance with vancomycin, linezolid (oxazolidinone), and tigecycline [6]. Only new classes of antibiotic such as Rifampicin etc have been used to cure MRSA and biofilm infections. However, vancomycin-resistant *S. aureus* and rifampicin-resistant *S. aureus* strains have been recorded in China [7] may be due to mutations in rpoB gene. This gene regulates β-subunit of RNA polymerase and their modifications in aminoglycosides have been concerned to rifampicin resistance [8]. In the literature many antibiotic resistance genes of MRSA have been recorded such as mecA , ermA, ermB, ermC, mupA, msrA, and msrB [9-11]. From the above mentioned facts, antibiotic susceptibility patterns are being change continuously [12]. So, it is important to monitor continuously the prevalence, virulence factors, and antibiotics resistance patterns of MRSA for the control of its infections and to reduce extra in-hospital costs [13].

S. aureus infection has been mainly found on the skin, pus and blood [14]. In the global prospective, according to European Antimicrobial Resistance Surveillance Network Report 2018, it has been reported that the prevalence of MRSA vary from 16% to 44% in European countries [15-16]. On contrary, the prevalence of MRSA has been found around 65% in 2006 in Jordan [17]. In the report conducted by National Nosocomial Infection Surveillance System (NNIS), it has been found that in USA 50% of hospital acquired infections in ICUs are because of MRSA [18]. In many studies it has found that the prevalence of CA-MRSA is around 13-16% across various regions of Korea [19-20].

In the Asia countries, the prevalence of MRSA in varies largely among countries ST239-MRSA-III has been found more prevalent in the north while ST59-MRSA region [21].

In India, incidence of MRSA has been reported by many studies across the country [22-23]. In a report conducted by Indian Network for Surveillance of Antimicrobial Resistance (INSAR), it has been mentioned that in India during 2008 to 2009 MRSA prevalence was found around 41% [24]. Similarly, it has also mentioned that the prevalence of MRSA in India is reported to be 27% [25]. In another study, the overall prevalence of MRSA has been recorded around 37% in India during 2015 to 2019 [26]. The region wise prevalence of MRSA has been recorded around 41%, 43%, 33%, 34%, 36% and 40% in north, east, west, south, central and north east states of India respectively [26]. In this report it has been mentioned that maximum prevalence of MRSA has been found in Jammu & Kashmir with 55% while its minimum prevalence was 21% recorded in Maharashtra.

In north India,it has been recorded the prevalence of MRSA isolates around 33.7% during 2017 to 2019 and also dictated that most MRSA isolates were found from pus samples [27] . Similarly, in another the study it has been showed that the prevalence of MRSA was found to be 24.03% in north India. In this study, the distribution of MRSA among various clinical samples have been found to be 29.9% in pus samples, 12% blood culture samples, and 26.4% in respiratory samples respectively [28] [29].

The present study was undertaken to determine the prevalence, clinical characteristics and risk factors associated with MRSA infections in the north India region. This study also aims to determine the outcomes of patients infected with MRSA of this

region and the molecular profiling of MecA and ErmA genes.

Materials and Methods:

The Present study was a Cross sectional study performed in the Department of Microbiology and Central Research Laboratory of Rama Medical College Hospital & Research Centre (RMCH & RC), Mandhana, Kanpur (India), for a period of 3 years *i.e.*, February 2018 to February 2021. A total of 3950 clinical samples were collected. The samples were processed immediately to the laboratory and tested for their biochemical test for the identification according to the CLSI guidelines 2017 [30]. In case of delay the samples were kept at 4^0C.

Blood and pus samples were collected from the patients in the different wards of hospitals. Prior written consents were obtained from all the participants involved in this study. Ethical Clearance was taken from the ethical committee of RMCH & RC before the start of this study.

Screening of the MRSA:

Phenotypic screening:

On the basis of colony morphology, mannitol fermentation, Gram staining, catalase test, coagulase test and DNase activity MRSA isolates were identified. Blood and pus culture sensitivity test of all the isolates was also obtained by the use of BacT-Aelrt 3D automated microbial detection system (bio-Merieux, France) [29]. The phenotypic MRSA was performed using the cefoxitin, oxacillin disk diffusion test, E-test and MIC as per protocol of the Clinical and Laboratory Standard Institute guidelines (CLSI) [30]. History of family and surgery, hospitalization, dialysis etc was also considered of all the patients. Antibiotic susceptibility test was performed according to Kirby-Bauer disk diffusion (DD) method [30] [31].

All the isolates were tested by DD test by using 30 µg disc following making a lawn culture of 0.5 Mc Farland suspensions of isolates on Muller Hinton Agar (MHA) plate. Plates were analysed after incubation at 37°C for 18 h. The zone diameter of ≤19 mm was considered as antibiotic-resistant for MRSA [32] as per the CLSI guidelines [30].

Genotypic screening:

The molecular study was performed to detect MecA and ErmA genes in the isolates. These genes were identified as gold standard test for the identification of MRSA by using polymerase chain reaction (PCR) [33]. Cefoxitin was considered as an inducer of MecA and ErmA genes expression and the results were compared with Oxacillin as a standard [34] [30].

For the molecular work genomic DNA was isolated using Qiagen DNA extraction kit (Germany) with following standard protocol [35]. The primers (Table-1) for MecA and ErmA genes were got synthesized by Chromous Biotech. Pvt. Ltd. (Bangaluru).

The obtained DNA were amplified in PCR (BIO-RAD T100 Thermal Cycler, Singapore) (volume 20 µl) by mixing 10µl master mix (Takara), 5µl nuclease free water, 1 µl forward and reveres primer each and 3µl DNA as a template for PCR conditions with initial denaturation at 94ºC for 5 min, then for 34 cycle at 94ºC for 30 sec for cycle denaturation, 50ºC for 45 sec for annealing for *MecA* gene and 51ºC for 1 min for annealing for Erm *A* gene then extension was performed at 72ºC for 1min followed by final extension at 72ºC for 7 min. Amplified DNA were resolved in 1% agarose gel electrophoresis containing with standard procedure.

Table No.1: List of primers used for the identification of MRSA

MecA gene	**Forward primer**	5'- GTTGTAGTTGTCGGGTTTGG-3'	Tm-53.0ºC	**References**
	Reverse primer	5'- CTTCCACATACCATCTTCTTTAAC -3'	Tm-52.0ºC	[36]
ErmA gene	**Forward primer**	5'-TCTAAAAAGCATGTAAAAGAA -3'	Tm-55.0ºC	[37]
	Reverse primer	5'- TGATTATAATTATTTGATAGCTTC -3'	Tm-54.0ºC	

Statistical analyses: A suitable statistical analysis was conducted with SPSS 20 software (SPSS, Inc., Chicago, USA). Statistical variables were obtained using frequencies and compared by using the chi-square ($\chi 2$) test. P< 0.05 was considered statistically significant. Other statistical data such as mean, median and standard deviation were calculated whenever needed. OR value with 95% CI was obtained which indicated the strength of association between data [38].

Sample size calculation: Sample size was calculated by using following formula [39];

$$n \geq \frac{Z^2_{1-\alpha/2} \text{ X } p(1-p)}{d^2}$$

Alpha (α) = 0.05, Estimated proportion (p) = 0.174 Estimation error (d) = 0.051, Sample size was calculated = 220

Where n= required sample size, p= prevalence of study (50%), Z= 2.58 at 95% CI

RESULT:

A total of 3950 clinical samples were collected out of which 220 isolates were found to be *S.aureus* . The irrelevant samples were excluded by following above mentioned methodology. In the selected isolates there were 140 males and 80 females. There were 90 isolates observed to be MRSA after the phenotypic detection method and remaining 130 isolates were found in the category of MSSA. In this study, the prevalence of MRSA was found to be 41%.

Table No.2: Phenotypic Identification of *S.aureus* with the use of different test

Microscopic observation	Gram's test	Catalase test	Coagulase test	Urease test		Cefoxitin(cx) And Oxacillin(ox)	DNAase Test
Cocci form (For all 220 cases)	+	+	Slide +	Tube +	+	+	+

Table No. 3: Identification of staphylococcal strains with the use of different microbiological tests

Organism	Disc diffusion test	E-test	MIC test
MSSA	130 (59.09%)	-	-
MRSA	90 (CX, OX) (41%)	90	-
Total	220		

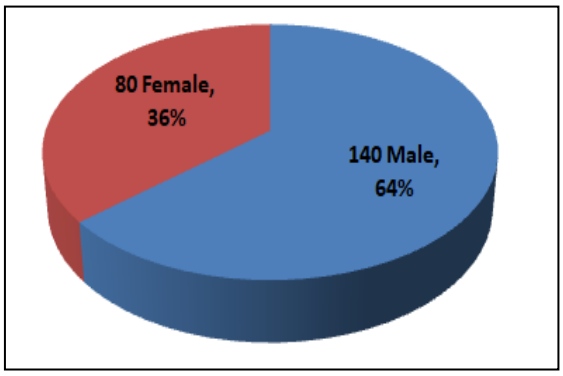

Fig.-1: Distribution of MSSA and MRSA

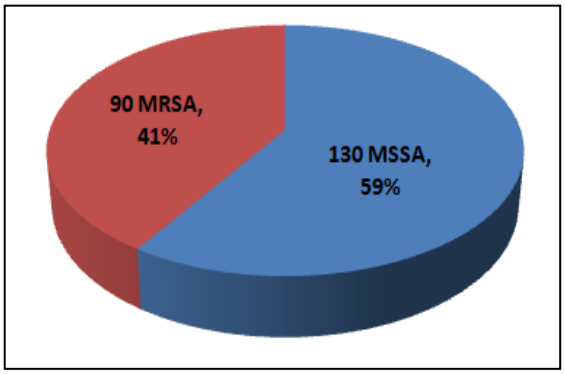

Fig.-2: Gender wise distribution of isolates

All the 90 MRSA isolates were screened by following test; 25 isolates were found D test positive (iMLSB), 20 isolates were confirmed by cMLSB, 25 isolates were decided by MS phenotype and 20 isolates were recorded phenotype sensitive.

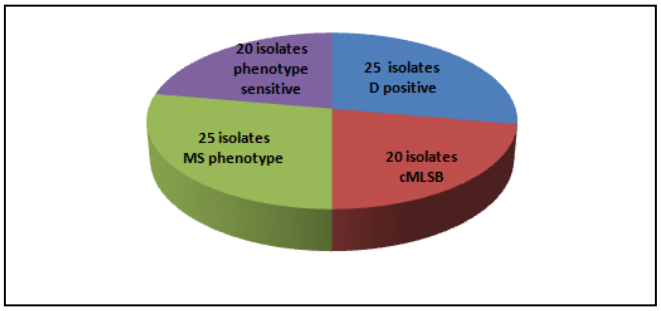

Fig.-3: Screening of MRSA (n=90) with phenotypic methods

During this study samples were collected from blood and pus and the distribution of isolates are mentioned in the following Table-4.

Table-4: Distribution of *S. aureus* from different tissues

S.N.	Sample tissue	*S. aureus* N=220	MRSA N=90	MSSA N=130	*p*-value
1.	Pus	105 (47.72%)	55 (61.11%)	45 (34.61%)	
2.	Blood	115 (52.28%)	35 (38.89%)	85(65.39%)	0.014

In this study the samples were collected from different department of hospitals and description of their tissues is mentioned in the following Table-5.

Table-5: Location wise distribution of *S. aureus*

S.N.	Location	Isolates N=220	Pus N=105	Blood N=115	OR (95%CI)
1.	Surgery ward	50 (22.7%)	25 (23.8%)	25 (21.7%)	0.78 (0.63-1.19)
2.	NICU	65 (29.5%)	20 (19.0%)	40 (34.8%)	0.82 (0.68-1.79)
3.	Medicine ward	40 (18.2%)	35(33.3%)	05 (4.3%)	0.91 (0.31-2.32)
4.	OPD	65 (29.5%)	25 (23.8%)	45 (39.1%)	0.602 (0.76-2.86)

The maximum number of isolates was obtained of the Blood from OPD of the hospitals.

For the study of age distribution of the patients, following Table was generated [Table 6].

Table-6: Age wise distribution of the *S. aureus* infected patients

S.N.	Age group (Years)	Male N=140	Female N=80	*p*-value
1.	0-10	15	08	0.056
2.	11-20	20	10	
3.	21-30	15	15	
4.	31-40	20	13	
5.	41-50	35	20	
6.	51-60	25	05	
7.	61-70	10	09	

It was observed that the maximum number of isolates was found in the age group of 41-50 years (males) and least in the age group above 61 years (females) .

Phenotypic Identification of MRSA

Antibiotic sensitivity pattern was obtained with disc diffusion test. The obtained resistance and sensitivity zone are recorded in the following Table-7. All methicillin-resistant *staphylococci* were analysed for their susceptibility against commonly used antibiotics. All MRSA isolates were found sensitive to linezolid, Teicoplanin, vancomycin, Gentamycin and were found resistance to Cefoxitin and Oxacillin.

Table-7: Antibiotic sensitivity pattern of *staphylococcus aureus* (N=220)

S.N.	Antibiotic	Disc potency	Resistance(mm)	Sensitive (mm)
1.	Deoxycycline (D)	30µg	40 (18.18%)	180 (81.82%)
2.	Erythromycin (ER)	15µg	85 (38.63%)	135 (61.36%)
3.	Gentamycin (GM)	10µg	30 (13.63%)	190 (86.36%)
4.	Linezolid	30µg	-	220 (100%)
5.	Oxacillin (OX)	1µg	90 (40.90%)	130 (59.09%)
6.	Penicillin (P)	10µg	190 (86.36%)	30 (13.63%)
7.	Teicoplanin (TEI)	30µg	-	220 (100%)
8.	Tetracyclin (TE)	30µg	40 (18.18%)	180 (81.82%)
9.	Vancomicin (VAN)	30µg	-	220 (100%)
10.	Ampicillin (AMP)	10µg	50 (22.72%)	170 (77.27%)
11.	Amoxicillin Clavunic acid (AMC)	20/10µg	35 (15.90%)	185 (84.09%)
12.	Cefoxitin (CX)	30µg	90 (40.90%)	130 (59.09%)
13.	Chloramphenicol (C)	30µg	50 (22.72%)	170 (77.27%)
14.	Ciprofloxacin (CIP)	5µg	35 (15.90%)	185 (84.09%)

15.	Clindamycin (CD)	2µg	95 (43.18%)	125 (56.81%)
16.	Co-Trimoxozole(COT)	25µg	45 (20.45%)	175 (79.54%)

Molecular analysis:

The authentic confirmation of MRSA was decided by the molecular analysis. The presence of MecA gene was detected in all the 90 isolates of MRSA while the presence of ErmA gene was found in only 5 isolates (3 were belonged to iMLSB and 2 were related to cMLSB) of MRSA. The gene sequences of MecA and ErmA gene was obtained and it was confirmed by homology of sequences.

Detection of Mec A and Erm A genes: In this study, 90 MRSA isolates were subjected for the molecular analysis. We obtained good quality of DNA of all the isolates. Gel photographs of the DNA samples are mentioned below observed by Gel documentation system.

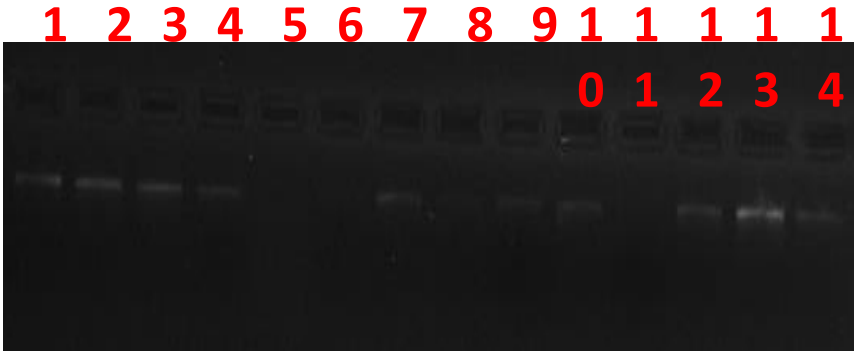

Fig. 4: Photograph of DNA isolated from *S. aureus* isolates

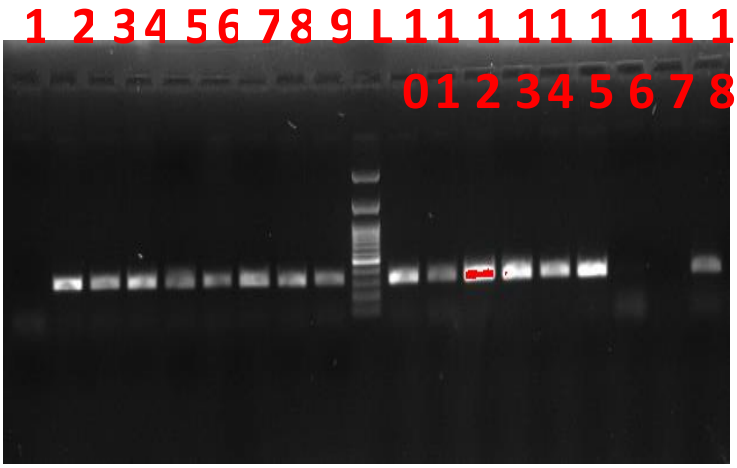

Fig. 5: Photograph of amplified Mec A gene in *S. aureus*, the amplified DNA band size was obtained 336 bp, L corresponding to 100bp ladder used, where Lane 15 is the positive control and Lane 17 a Negative control

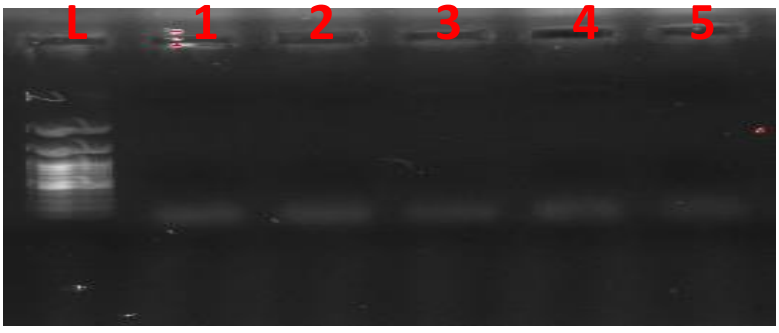

Fig. 6: Photograph of amplified Erm A gene in *S. aureus*, the amplified DNA band size was obtained 149bp, L corresponding to 100bp ladder

GTTGTAGTTGTCGGGTTTGGTATATATTTTTATGCTTCAAAAGATAAAGAAATTAATAATACTAT
TGATGCAATTGAAGATAAAAATTTCAAACAAGTTTATAAAGATAGCAGTTATATTTCTAAAAGC
GATAATGGTGAAGTAGAAATGACTGAACGTCCGATAAAAATATATAATAGTTTAGGCGTTAAA
GATATAAACATTCAGGATCGTAAAATAAAAAAAGTATCTAAAAATAAAAAACGAGTAGATGCT
CAATATAAAATTAAAACAAACTACGGTAACATTGATCGCAACGTTCAATTTAATTTTGTTAAAG
AAGATGGTATGTGGAAG

Fig.-7: Obtained gene sequences of MecA gene in *S. aureus*

TCTAAAAAGCATGTAAAAGAATTTGCGACCAGATTGCAAAATCTGCAACGAGCTTTGGGTTTAC
TCCCCCCGGTGGAGATGGATATAAAAATGCTCAAAAAAGTACCACCACTATATTTTCCTAAGAA
GCTATCAAATAATTATAATCA

Fig.-8: Gene sequences of ErmA gene in *S. aureus*

In the molecular study, we have obtained 100% prevalence of MecA gene while 5.55% prevalence of Erm A gene was recorded.

Discussion:

In the present study we have explored risk factors and outcome of MRSA infection in the north Indian population at a tertiary care centre. MRSA is a well known serious life threatening pathogen present in hospitals and populations. High prevalence of MRSA about 52% has been reported in other studies of India and in USA. However, the studies conducted in European countries have been reported less prevalence of MRSA which is 0.4% in Sweden and 48.4% in Belgium. In the present study we have found 41% prevalence of MRSA in north Indian population. Our finding is with accordance with others mentioned finding. There is big variations recorded in the prevalence of MRSA by different studies which may be due to different geographical area, different sample sizes, duration of study, and methods adapted etc.

In this study out of 90 MRSA isolates, 25 isolates were found as D test positive, 20 isolates were confirmed by cMLSB, next 25 isolates were identified by MS phenotype and remaining 20 isolates were belonged to sensitive phenotypes (Fig no. 3). Our finding strongly supported the study mentioned by Nezhad *et al*., 2017 [40] it which MRSA isolates were identified by similar methods and found identical ratio of isolates. In the comparison of gender wise distribution of MRSA in our study more prevalence was observed in males (64%) and in females it was found (36%) (Fig no. 2). Our study data shown similar trends with the data recorded by Rao *et al*., 2012 [41].

MRSA infection was found predominant in the patient's with age between the 41-50 years in our study (Table-4). Similar finding was recorded by Sharma *et al.,* 2011 [42]. MRSA isolates were found more in pus samples (61.11%) as compared to blood samples (38.89%) in this study (Table-2). The presence of more MRSA in pus may be due to direct exposure of wound with environmental microorganisms which makes the wound more prone for infection of *S. aureus*. Similar results were recorded by Mallick *et al.,* 2010 in Maharashtra (61.4%) [43] and Rao *et al*. 2012 in Andhra Pradesh (64%) [44].

In the literature, several studies have been exposed that disc diffusion testing method is superior to other phenotypic methods. In this study the sensitivity of antibiotics were screened (disc diffusion) and found that maximum sensitivity is Linezolid (100%), Teicoplanin (100%), Vancomicin (100%) and Gentamycin (86%) while least sensitivity antibiotics were Penicillin (13%) and Clindamycin (57%) (Table no.-5). Our finding is strongly supported by Lohan *et al*., 2021 [45], and many coworkers. Furthermore, the study conducted in Iran has been recorded similar resistance with gentamicin (60%), and kanamycin (57%), in the favour of our study.

Finally to the relevant confirmation of MRSA isolates obtained in this study was done with the use molecular techniques. Two genes namely MecA and ErmA were found in the MRSA isolates. For the further confirmation of MRSA, gene sequencing (Fig no. 7 and Fig no. 8) and with genes homology with the use NCBI data base (99% identical) were performed in this study. The presence of MecA gene (100%) (Fig no. 5) in all the 90 MRSA isolates and the presence of ErmA gene (5.6%) (Fig. 6) was found in 5 isolates among the screened MRSA. In the literature very few studies are available in the respect of MecA and ErmA genes identification for the confirmation of MRSA isolates. However, the prevalence of MecA and ErmA genes is strongly supported by the study Ramaite *et al*., 2021 [46].

Conclusion: Present study has focus on *S. aureus* especially MRSA based prevalence and associated risk factors to create awareness related to public. In this study, use of potent antibiotics to cure MRSA infection and proper monitoring of patients has exposed. This study is novel in the term of identification of MecA, ErmA genes with PCR and their sequencing in the north Indian region. Finally, this study would be helpful to control and cure of this dreaded organism in hospitals as well as in communities.

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Declarations:
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Conflicts of interest: There is no any conflict of interest associated with this study
Ethics approval: This study was approved by the Ethical committee of Rama University.
Consent to participate: We have consent to participate.
Consent for publication: We have consent for the publication of this paper.
Availability of data and material: The entire data and materials were self generated.
Authors' contributions: All the authors equally contributed the work at Rama University, Kanpur (India).

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