Title: SCC mec typing and detection of pvl gene in Methicillin resistant Staphylococcus aureus isolated from hospitalized patients in Guwahati city.

Abstract:

Background & objectives: Methicillin resistant staphylococcus aureus are known to cause hospital acquired as well as community acquired infections. They are one of the most common pathogens of nosocomial infections. In this study we tried to find out the presence of community acquired MRSA in hospital acquired infections. SCCmec typing PCR was done to differentiate MRSA to CA-MRSA and HA-MRSA. **Methods**: Total of 150 clinical strains of S.aureus isolated from various clinical samples of hospitalized patients were collected from A multiplex PCR was performed to carry out the SCCmec typing for the MRSA. Antibiotic resistance was done by Kirby Bauer method and the detection of LukS-PV and LukF-PV gene was done to see the ability of MRSA to produce panton valentine leukocidin. **Result:** We found that out of 53 MRSA 38(71.7%) were HA-MRSA and 13 (24.5%) were CA-MRSA. We also found the presence of pvl gene, it was more common among the CA-MRSA (69.2%) as compared to HA-MRSA (13.1%). We also found that the CA-MRSA showed more resistance as compared to HA-MRSA to antibiotics like, Ciprofloxacin(84.6%), Ampicillin(100%), Cefotaxime (61.53%), Erythromycin (53.8%). **Interpretation & conclusion:** In this study we found that there is a mixing between the MRSA associated with community and hospital infections. We also found that increased antibiotic resistance in CA-MRSA and presence of PVL gene in them. This could be an indication of exchange of virulence factor or antibiotic resistance among the two types of MRSA which can be a threat to the existing healthcare policy used for their treatment and control.

Keywords: Methicillin resistant staphylococcus aureus (MRSA), HA-MRSA, CA-MRSA, Panton valentine leukocidin, Antibiotics, Resistance.

1. Introduction:

Staphylococcus aureus is one of the most common pathogen that causes nosocomial infections in human being. It is also known for its ability to cause skin and soft tissue infections in community.[1] In the year 1959, Methicillin was introduced for the treatment of staphylococcal infection. In the year 1961, the first case of Methicillin resistant Staphylococcus aureus (MRSA) was reported. This case was reported from London.[2] The Methicillin resistance is acquired by the presence of the mec A gene that codes for an altered penicillin binding protein (PBP2a). The mec A gene along with its regulatory genes is carried by a mobile genetic element called Staphylococcus cassette chromosome mec (Sccmec).[3,4] Different types of Sccmec are reported, type I, II & III are considered to be hospital acquired MRSA (HA-MRSA) and type IV & V are mostly Community associated MRSA.[5] Increased rate of community acquired MRSA spreading is a serious problem for public healthcare system worldwide. As compared to the HA MRSA strains which are mostly responsible for hospital acquired infections, CA MRSA strains do not have a specific risk factor. They can cause infection in healthy individuals too. This shows that they are more virulent than the HA-MRSA.[6] Based on many studies that are conducted across the world, cases of CA MRSA strains are reported from patients who are hospitalised or do not have the risk factors associated with CA MRSA infection.[7] In India, overcrowding and poor hygiene has led to the spread of CA-MRSA causing bacteraemia that mostly affects neonates, people from lower economic area and breast abscess in lactating mothers.[8] A toxin named Panton valentine leukocidin (PVL) is produced by many CA-MRSA strains, this is an important virulence factor that can give infection in young children and immunocompetent people. PVL gene has two components lukS-PV and lukF-PV; it acts by lysing the polymorphonuclear cells thus affecting body's defence mechanism. [9] This toxin is frequently detected in S. aureus isolated from samples of skin and soft tissue infections. Strains of S. aureus that are PVL positive can cause infections severe than necrotizing pneumonia.[10]

Based on the literatures reviewed, it has been found that the CA- MRSA is isolated from a high percentage of isolates that are grown from patients who are hospitalised or have no risk factor of community infections. S. aureus species have also shown the ability of biofilm production in

many studies. This also is a challenge for the existing policies for the treatment of Staphylococcus aureus treatment. In this study, we have tried to find a correlation between the presence of PVL gene in CA-MRSA and HA-MRSA.

2. Materials and methods

Sample collection: A total of 150 isolates of Staphylococcus aureus isolated from various clinical samples were included in this study.

Identification of *S. aureus*:

Identification of the *Staphylococcus aureus* was done by microscopic examination of smears stained by Gram's stain. All the strains showing gram positive cocci were included for further examination. Catalase test was performed, only catalase positive organisms were included in this study. Further, tube and slide coagulase was performed and strains showing positive test were included and identifies as Staphylococcus aureus. All the tests done for identification were done along with control strains.

Antibiotic sensitivity testing:

After the identification of *Staphylococcus aureus*, antibiotic sensitivity pattern is determined by Kirby bauer disc diffusion method. Muller hinton agar is used to perform the test. After incubation at 37°celcius for 18-24 hours the zone of inhibitions are measured and interpreted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines.[11] Antibiotic discs from Himedia, India were used. The antibiotics include Gentamicin, Amikacin, Chloramphenicol, Ampicillin, Ciprofloxacin, Erythromycin, Cefotaxime, Trimethoprim-sulphamethoxazole, Levofloxacin, Linezolid, Penicillin, Rifampicin, Tetracycline, Cephalexin.

Identification of MRSA

Cefoxitin disc (30µg) was used to identify Methicillin resistant *Staphylococcus aureus* by Kirby bauer disc diffusion method. Muller Hinton agar (Himedia India) was used. After the

incubation at 37°Celcius for 18-24 hours, a zone size of ≤21mm was considered resistant (MRSA) and a zone size ≥22 was considered as sensitive.[12] All of the following tests were done using controls, where S. aureus ATCC 29213 was used as Methicillin sensitive control and ATCC 43300 as Methicillin resistant reference strain.[13]

Molecular identification of MRSA was done by Polymerase chain reaction by the detection of MecA gene that code for altered penicillin binding protein.

DNA isolation:

Bacterial DNA isolation was performed by using the Bacterial genomic DNA isolation kit (Himedia India). Fresh 24 hours old pure cultures of all the strains of MRSA were used to isolate the DNA. The presence of extracted DNA was checked by running the final product into agarose gel with ethidium bromide (ETBr). Visible band of DNA were seen using the Gel documentation system (Bio-Rad, USA).

Identification of HA-MRSA and CA-MRSA:

In order to classify HA-MRSA and CA-MRSA based on Sccmec type, a multiplex PCR was performed. Staphylococcus Cassette Chromosome (SCC) mec typing MRSA Detection Kit (Multiplex) by Himedia India, is a qualitative conventional PCR kit which focuses on simultaneous amplification of 7 targets specific for types I to VI and mecA gene. All the amplified targets are confirmed by using the agarose gel electrophoresis method.

Detection of *PVL* gene in MRSA:

Detection of Panton valentine leukocidin is done by polymerase chain reaction. Primers were designed from sequence used in a different study for the detection of LukS-PV and LukF-PV components of the gene. [14] The specificity of the primer was determined using similarity search in National centre for Biotechnology Information (NCBI) databases. The PCR product was then analysed by running on agarose gel along with by gel documentation.

3. Results

Result of Identification of S. Aureus

Total of 150 staphylococcus aureus strains grown from various clinical samples were collected from hospitals. The organisms were identified by Grams staining as gram positive cocci arranged in grape like clusters. Further the organisms were tested for the presence of the enzyme catalase and coagulase. Coagulase positive organisms are included in this study. The distribution of staphylococcus aureus as per the type of sample is given in Table I.

Table I: S. aureus isolated from different samples.

Sample type							
Pus	**Blood**	**Broncheoalveolar Lavage (BAL)**	**Tracheal aspirate**	**Discharge fluid**	**Sputum**	**Urine**	**Catheter tip**
75	23	17	16	7	5	5	2

Identification of Methicillin resistant staphylococcus aureus:

Out of the 150 staphylococcus aureus, we got 53 MRSA and 97 MSSA by Cefoxitin (30mcg, Himedia India) disc diffusion method. All the 53 MRSA were found to carry MecA gene by PCR. The entire test was performed using positive and negative controls. The results obtained by the controls were satisfactory.

The distribution of MRSA and MSSA in total 150 samples is shown in Table II.

Table II: MRSA & MSSA distribution in various samples

Sample	**MSSA**	**MRSA**	**HA-**	**CA-**	**Chi-**	**P value**

type		(Mec A gene)	MRSA	MRSA	square value	
Pus	46	29	19	9	2.985	0.88625
Blood	17	6	5	1		
BAL	10	7	5	2		
Tracheal aspirate	12	4	3	1		
Discharge fluid	5	2	1	0		
Sputum	3	2	2	0		
Urine	4	1	1	0		
Catheter tip	0	2	2	0		

Result of multiplex PCR for Scc Mec typing:

After the detection of all the Methicillin resistant staphylococcus the bacterial DNA of the organisms (MRSA) are isolated and subjected to multiplex PCR to detect the SCC mec type (figure 1) . Based on the findings of PCR the data is given in Table II & III. Out of the total 53 MRSA strains, 38, (type I-13, type II-7, type III-18) were hospital acquired strains and 13 (type IV-5, type IVa-5, type V-3) were community acquired. This shows out of total 53 MRSA that are obtained from hospitalised patients with no risk of getting community infection 13 (24.5%) are showing the genotypic traits of CA-MRSA.

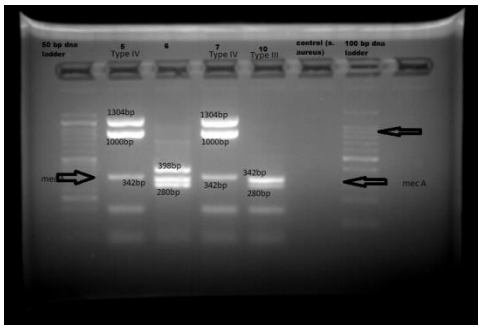

Figure 1: Sccmec typing PCR result showing amplification of various targets.

Table III: Results of SCCmec typing

Total isolates (150)	**SccMec type I**	**SccMec type II**	**SccMec type III**	**SccMec type IV**	**SccMec type Iva**	**SccMec type V**	**Unable to type**
MRSA (n=53)	13	7	18	5	5	3	2
Types of MRSA	HA- MRSA (total=38)			CA-MRSA (total=13)			

Table IV: Results of antibiotic resistance Pattern:

Antibiotics	MRSA(n=53)*			
	HA-MRSA (n=38) (Resistant %)	**CA-MRSA (n=13) (Resistant %)**	**Chi-Square**	**P- value**
Gentamicin	**13 (34)**	**4 (30.7)**	0.0516	0.82
Ampicillin	**38 (100)**	**13 (100)**	NA	NA
Ciprofloxacin	**30 (79)**	**11 (84.6)**	0.1974	0.6568
Erythromycin	**16 (42)**	**7 (53.8)**	0.5393	0.4627

Levofloxacin	**17 (44.7)**	**2 (15.3)**	3.7801	0.0488**
Cefotaxime	**21 (55.2)**	**8 (61.53)**	0.1555	0.6933
Trimethoprim-sulphamethoxazole	**14 (36.8)**	**4 (30.7)**	0.1564	0.692472
Linezolid	**1 (2.4)**	**0 (0)**	15.4942	0.000083**
Chloramphenicol	**2 (5.2)**	**0 (0)**	4.2801	0.038562**
Rifampicin	**5 (13.1)**	**1 (7.6)**	0.2787	0.597525
Teicoplanin	**1 (2.4)**	**0 (0)**	15.4942	0.000083**
Tetracycline	**7 (18.4)**	**2 (15.3)**	0.0615	0.804216
Cephalexin	**20 (52.6)**	**3 (23)**	3.4171	0.064523
Cefoxitin	**38(100)**	**13(100)**	NA	NA
*2 strains that we are unable to type are not included here **Significant NA not applicable				

Result of PVL gene detection

Polymerase chain reaction for the detection of *pvl* gene was performed for the 13 strains of CA-MRSA. Out of the total MRSA strains 14 MRSA showed the presence of the gene. The details are given in TableV.

Table V: PVL gene detection

Total MRSA (53)*	**PVL gene detected**	**PVL gene not detected**	**Chi-Square**	**P- value**
CA-MRSA (13)	9 (69.2%)	4	15.2923	0.000092**
HA-MRSA (38)	5 (13.1%)	33		
*2 strains that we are unable to type are not included here				

**Significant

4. Discussion:

Staphylococcus aureus is an important human pathogen. It is capable of causing both the hospital as well as community acquired infections. The nature of illness may vary from a simple infection to a fatal life threatening condition. It can spread from human to human and cause a wide spectrum of diseases.[15] Around the world Methicillin resistant staphylococcus aureus is known to cause skin and soft tissue infections however it is also known to cause many other fatal illnesses.[16] Although MRSA infections were initially identified as hospital acquired (HA-MRSA) only and community associated infection of methicillin resistant staphylococcus aureus were confined to community.[17] Many reports of CA-MRSA intruding the hospital settings and causing Hospital acquired infections has been reported worldwide.[18] In addition to that it is also found that CA-MRSA posses a toxin called Panton-valentine leukocidin that responsible for the lysis of leukocytes and necrosis of tissues.[19] In the present study we mainly focused on identifying the mixing of CA-MRSA in hospitals leading to their isolation from patients who are hospitalized and do not have any risk factors of community infections. We tried to detect the antibiotic resistance pattern of the CA-MRSA and compared it with the HA-MRSA isolated from hospitals. Out of the total 150 Staphylococcus aureus we got 53 (35%) MRSA and 94 (65%) MSSA. The highest number of MRSA was isolated from pus sample (n=29). Out of the 53 MRSA, 38 (71.6%) were HA-MRSA, 13 (24.5%) were CA-MRSA and 2 (3.7) were untypable strains as per the report of SCCmec multiplex PCR result. All the samples were collected from hospitalized patients without any risk of community infection; however we isolated 13 strains of CA-MRSA. The presence of PVL gene was predominantly seen in CA-MRSA 9(69.2%) as compared to 5(13.1%) in HA-MRSA, which was found to be statistically significant (p-value=0.000092). If we look at the antibiotic resistance pattern of the CA-MRSA, we can see the findings of our studies that are discussed above indicates the presence of CA-MRSA in healthcare setup which was similar to the findings of a study conducted in Mangalore, Amritsar, where CA- MRSA were isolated from hospitalized patients. [19,20] The CA-MRSA were predominantly resistant to Ampicillin (100%), Ciprofloxacin (84.6%), Erythromycin (53.8%), Cefotaxime (61.53%) as compared to HA-MRSA strains. However we found Linezolid,

Teicoplanin and Chloramphenicol to be sensitive to all the strains of CA-MRSA included in this study. The CA-MRSA also showed similar or low percentage of resistance as compared to HA-MRSA for antibiotics like Trimethoprim- sulphamethoxazole, Tetracycline, Cephalexin, Gentamycin, Levofloxacin etc. Drug resistance is not very common in CA-MRSA but most of the strains showed resistance to antibiotics which is common in an organism mostly associated with hospitals. In the current study, the presence of PVL gene in the MRSA strains were identified, a total of 9 out of 13 CA-MRSA (69.1%) were carrying the pvl gene which was much higher as compared to 5 out of 38 HA-MRSA carrying PVL gene (13.1%). This shows that PVL gene is more common among the CA-MRSA but also posses by the HA-MRSA. In a study conducted in west Bengal we found similar findings where the CA-MRSA were showing resistance to multiple antibiotics and the presence of PVL gene.[21]

Based on the studies conducted across the world we have seen an increasing trend of CA-MRSA in hospital settings, in addition to that CA-MRSA has also shown increasing resistance to antibiotics in many studies. In our study we found the presence of CA- MRSA in hospitalized patients, the high drug resistance pattern could be due to its adaption in the hospital environment for a long time, which could also be the reason for the presence of pvl gene among the HA-MRSA strains. These findings, as concluded in many studies, may differ based on various factors like geographical area, public health system and awareness, environmental conditions, healthcare planning etc. [21, 22] In our study, we found a significant number of isolates of CA-MRSA in the samples of hospitalized patients with no risk of community infection. This finding shows the mixing of community strains in hospitalized patients. We also observed multiple drug resistance in CA- MRSA, which is not very common in them but seen in HA-MRSA. This could be due to the mutual transfer of character among the two types as they encounter each other in hospital environment. We also have found strains that are HA-MRSA carrying PVL gene and multidrug resistant, these organisms are having multiple virulence factors like pvl gene producing the Panton valentine leukocidin that destroys the phagocytic activity of the immune system and resistance to multiple antibiotics. This makes the treatment of the organism difficult for the clinicians. We found that antibiotics like Levofloxacin, Linezolid, Chloramphenicol and Teicoplanin can still be an effective treatment options provided there are strict rules to stop over the counter selling of antibiotics.

5. Conclusion:

Staphylococcus aureus can be a threat to the healthcare system due to its developing resistance to antibiotics and other virulence factors. In our study we found the mixing up of community type MRSA into hospital setup. The presence of PVL gene makes it more virulent as they can lead to necrosis by destroying the leukocytes. More studies should be conducted and routine diagnosis pattern needs to be updated so that genotypic picture of the organism is revealed. The health care systems must run a surveillance program specifically for staphylococcus aureus outbreaks or screening of carriers among hospital personnel. This will help in formulating advanced ways of dealing with the pathogen.

Conflicts of Interest:

The authors declare no conflict of interest.

Reference:

1. Elston JW, Barlow GD. Community-associated MRSA in the United Kingdom. *J Infect*. 2009;59(3):149-155. doi:10.1016/j.jinf.2009.07.001
2. Buzaid N, Elzouki AN, Taher I, Ghenghesh KS. Methicillin-resistant Staphylococcus aureus (MRSA) in a tertiary surgical and trauma hospital in Benghazi, Libya. *J Infect Dev Ctries*. 2011;5(10):723-726. Published 2011 Oct 13. doi:10.3855/jidc.1701

3. Moosavian M, Shahin M, Navidifar T, Torabipour M. Typing of staphylococcal cassette chromosome *mec* encoding methicillin resistance in *Staphylococcus aureus* isolates in Ahvaz, Iran. *New Microbes New Infect*. 2017;21:90-94. Published 2017 Nov 28. doi:10.1016/j.nmni.2017.11.006
4. Berger-Bächi B, Strässle A, Gustafson JE, Kayser FH. Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in Staphylococcus aureus. *Antimicrob Agents Chemother*. 1992;36(7):1367-1373. doi:10.1128/AAC.36.7.1367
5. Matouskova I, Janout V. Current knowledge of methicillin-resistant Staphylococcus aureus and community-associated methicillin-resistant Staphylococcus aureus. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2008;152(2):191-202. doi:10.5507/bp.2008.030
6. Funaki T, Yasuhara T, Kugawa S, et al. SCC*mec* typing of PVL-positive community-acquired *Staphylococcus aureus* (CA-MRSA) at a Japanese hospital. *Heliyon*. 2019;5(3):e01415. Published 2019 Mar 28. doi:10.1016/j.heliyon.2019.e01415
7. Buck JM, Como-Sabetti K, Harriman KH, et al. Community-associated methicillin-resistant Staphylococcus aureus, Minnesota, 2000-2003. *Emerg Infect Dis*. 2005;11(10):1532-1538. doi:10.3201/eid1110.050141
8. Francis JS, Doherty MC, Lopatin U, et al. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant Staphylococcus aureus carrying the Panton-Valentine leukocidin genes. *Clin Infect Dis*. 2005;40(1):100-107. doi:10.1086/427148
9. Niveditha Sundar Poojary, et al, Application of monoclonal antibodies generated against Panton-Valentine Leukocidin (PVL-S) toxin for specific identification of community acquired methicillin resistance Staphylococcus aureus,Microbiological Research, Volume 169, Issue 12, 2014.
10. Monecke S, Müller E, Buechler J, et al. Rapid detection of Panton-Valentine leukocidin in Staphylococcus aureus cultures by use of a lateral flow assay based on monoclonal antibodies. *J Clin Microbiol*. 2013;51(2):487-495. doi:10.1128/JCM.02285-12.
11. CLSI . *Performance Standards for Antimicrobial Susceptibility Testing*. 30th ed. Clinical and Laboratory Standards Institute; Wayne, PA, USA: 2020. CLSI supplement M100.
12. Broekema NM, Van TT, Monson TA, Marshall SA, Warshauer DM. Comparison of cefoxitin and oxacillin disk diffusion methods for detection of mecA-mediated resistance in

Staphylococcus aureus in a large-scale study. *J Clin Microbiol*. 2009;47(1):217-219. doi:10.1128/JCM.01506-08

13. Khan A, Wilson B, Gould IM. Current and future treatment options for community-associated MRSA infection. *Expert Opin Pharmacother*. 2018;19(5):457-470. doi:10.1080/14656566.2018.1442826
14. Gerard Lina, Yves Piémont, Florence Godail-Gamot, Michèle Bes, Marie-Odile Peter, Valérie Gauduchon, François Vandenesch, Jerome Etienne, Involvement of Panton-Valentine Leukocidin—Producing *Staphylococcus aureus* in Primary Skin Infections and Pneumonia, *Clinical Infectious Diseases*, Volume 29, Issue 5, November 1999, Pages 1128–1132
15. Stefani S, Goglio A. Methicillin-resistant Staphylococcus aureus: related infections and antibiotic resistance. Int J Infect Dis. 2010; 14:S19-S22.
16. Afsharian M, Hemmati M, Mansouri F, Azizi M, Zamanian MH, Mohseni Afshar Z, et al. Frequency of class I and II integrons in methicillin-resistant and methicillin-sensitive Staphylococcus aureus isolates in the City of Kermanshah. Arch Clin Infect Dis. 2019; 14(4).
17. Naimi TS, LeDell KH, Como-Sabetti K, et al. Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. *JAMA*. 2003;290(22):2976-2984. doi:10.1001/jama.290.22.2976
18. David, M.Z.; Cadilla, A.; Boyle-Vavra, S.; Daum, R.S. Replacement of HA-MRSA by CA-MRSA infections at an academic medical center in the midwestern United States, 2004-5 to 2008. *PLoS ONE* **2014**, *9*, e92760.
19. Preeja PP, Kumar SH, Shetty V. Prevalence and Characterization of Methicillin-Resistant *Staphylococcus aureus* from Community- and Hospital-Associated Infections: A Tertiary Care Center Study. *Antibiotics (Basel)*. 2021;10(2):197. Published 2021 Feb 18. doi:10.3390/antibiotics10020197.
20. Type IV, S. (2019). American Journal of Clinical Microbiology and Antimicrobials. *Am J Clin Microbiol Antimicrob*, *2*(1), 1032.
21. Karmakar, A., Jana, D., Dutta, K., Dua, P., & Ghosh, C. (2018). Prevalence of Panton-Valentine leukocidin gene among community acquired Staphylococcus aureus: a real-time PCR study. *Journal of pathogens*, *2018*.

22. Bhowmik, D., Chetri, S., Das, B. J., Dhar Chanda, D., & Bhattacharjee, A. (2021). Distribution of virulence genes and SCCmec types among methicillin-resistant Staphylococcus aureus of clinical and environmental origin: a study from community of Assam, India. *BMC research notes, 14*(1), 1-7.