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Epidemiology and Molecular Variations in Methicillin Resistant *Staphylococcus aureus* Isolated from Different Clinical Samples of Private Hospitals of Salem District, India

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Abstract: Methicillin resistant *Staphylococcus aureus* is one of the major bacterial disease in humans and animals worldwide. It causes opportunistic infections acquired from different sources like patients and hospital staff mainly through their hands besides from their normal flora. The present investigation deals with the prevalence and molecular aspects of 50 *Staphylococcus aureus* isolates collected from various private hospitals of Salem, Tamil Nadu. The methicillin resistance screening by MIC method showed that 90% (40 strains) of the isolates were resistant to methicillin. Among the 40 strains 6 highly resistant ones were chosen and subjected to DNA sequencing so as to compare the sequence variation within the MRSA strains. Variation occurred in the base pairs among the six strains sequenced indicating that real mutation had occurred with the replacement of base pairs C by T in 1371thbp of S2 strain and A by G in 1376th bp of S5strain, a point mutation with the replacement of T by A in 8thbp of S6 and in 178thbp & 1333rd bp of S2strain and addition of a nucleotide A in 1397th bp of S3 strain and these changes might be responsible for their methicillin resistance.

Key words: *Staphylococcus aureus*

INTRODUCTION

Currently the frequently reported "Super Bug" or Methicillin-resistant bacterium, *Staphylococcus aureus* is a particular strain of *Staphylococcus aureus* that is resistant to many antibiotics. It is found on the skin of the body and in the nasal pathways but does not cause any problem to the body [1]. This bacterium becomes a threat once it enters the bloodstream, most often through a cut or abrasion. If these cuts or abrasions are unrecognized, or improperly cared for from lack of good hygiene or continual wound openings, the staphylococcal bacteria enter and can cause a MRSA infection in the body. The effective antibiotics available to treat MRSA infections are the glycolipid antibiotics like vancomycin and teichoplanin. Unfortunately from 1997 onwards, Vancomycin Intermediate Resistant *Staphylococcus aureus* (VISA) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) have started appearing in the hospitals as well as in the community at large. This type of emergence of multiple drug resistance is a challenging task to the clinicians and surgeons who handle serious patients in the ICUs [2]. The mortality rate due to MRSA bacteremia is two times more than that caused by MSSA. Recently some Pharmaceutical manufacturers are trying to produce many new antibiotics to meet the challenging problems created by MRSA infections [3]. Epidemiologic typing such as bacteriophage typing, antibiogram, [4, 5], Random Amplification of Polymorphic DNA (RAPD) [6, 7] and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) have been performed to identify the source of nosocomial infection by *S. aureus* and the epidemiology of outbreaks. The above techniques are also useful in tracking inter hospital spread of MRSA [8, 9]. Although many works on MRSA have been carried out in India, information is scarce on the molecular biology of MRSA. The present paper deals with the epidemiological and molecular aspects of 50 different *S. aureus* samples collected from various private hospitals of Salem district of Tamil Nadu.

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Table 1: Number of isolated MRSA from different clinical samples.

S.No	Clinical samples	No. of *S.aureus*	No. of MRSA
1	Pus	24	23
2	Sputum	10	8
3	Wound swab	7	5
4	Blood	5	2
5	Urine	4	1

Table 2: Number of isolated MRSA based on different categories.

S. No.		No. of MRSA	Percentage
	Age		
1.	<1Yr	2	3.4
2.	1-15yrs	4	6.9
3.	16-30yrs	9	15.5
4.	31-60yrs	16	27.6
	SEX		
1.	Male	43	74.1
2.	Female	12	25.9
	Hospital Stay		
1.	<10days	11	19
2.	11-20 days	10	17.2
3.	21-30 days	11	19
	Presence in ICU		
1.	Yes	27	46.6
2.	No	21	43.4
	Category of Infection		
1.	Respiratory	20	33
2.	Surgical site infection	8	11.75
3.	Skin,Subcutaneous	8	10.85
4.	Bacteremia	3	4.4
5.	Catheter related	1	1.5
6.	Urine	2	3.0

MATERIALS AND METHODS

Sample Collection: 50 different *S. aureus* samples were collected from different private hospitals located in Salem-city. The samples included were based on various categories like sex (male & female), clinical samples like pus, stool, urine, blood and sputum, based on category and risk factor of infection, based on different age groups, based on duration of hospital stay (Table 1 & 2). They were then carried safely in ice box to Laboratory and refrigerated.

Identification: The strains were inoculated on Brain Heart Infusion (BHI) Agar & Trypticase Soy Broth (TSB) (Himedia Laboratories, Mumbai, India) and grown for 24h at 37°C. The staphylococcal colonies were identified by examination under microscope by Gram staining. Catalase test was performed for characterization of the strains by established procedures for *S. aureus* [4].

Antimicrobial Susceptibility Testing

Disc Diffusion Method: Antibiotic susceptibility test was performed by Kirby-Bauer disk diffusion according to the guidelines recommended by NCCLS [10, 11].

Minimal Inhibitory Concentration Method: Minimum inhibitory concentration was determined for all the 50 *S .aureus* isolates according to the method of Sahm & Washington [12]. Muller Hinton broth (5ml) was inoculated with *S. aureus*. 0.001ml of cultures were then used for the inoculation of plates containing different concentrations of methicillin (0.5µg,2^1,2^2,2^3,2^4,2^5,2^6,2^7 and 2^8 µg). The plates were incubated at 35°C for 24h. The MIC was read as the lowest concentration of an antibiotic at which complete inhibition of growth occurred on the plate.

DNA Extraction from Bacterial Cells: The Genomic DNA was isolated using sonication method [13] and the isolated DNA was confirmed by Agarose gel electrophoresis method.

Polymerase Chain Reaction (PCR): The PCR was performed according to the procedures of [14]. Two primers were used for the detection of mecA gene.

mecA Forward 1: 1GACGATTATTCATCTATATCGTAT mecA Reverse 2: 1501 GAGATAGGCATCGTTCCAAAG: The PCR reaction mixture was prepared as follows:

2.5µl 10x buffer, 0.2Mm dNTPs,4 U Taq DNA polymerase, 2.5Mm $MgCl_2$, 50ng DNA template, 1.5µl of mecF1 primer and 1.5µl of mec R2 primer. The volume was made upto 25µl with 2.5µl of nuclease free water. DNA amplification was carried out to following cycling conditions: initial denaturation at 94°c for 4min followed by 35 cycles of amplification (denaturation at 94°C for 45s, annealing at 58°C for 45s and extension at 72°C for 60s), ending with a final extension at 72°C for 2 min. The presence of mecA gene was detected in 3% agarose gel electrophoresis in the presence of DNA marker.

DNA Sequencing: DNA sequencing for the mecA gene was performed using Applied Biosystems-3730 DNA Analyzer. The software used was Sequence analysis 5.2 version. Sequencing was carried out for only 7 methicillin resistant *S. aureus* isolated (including reference strain).

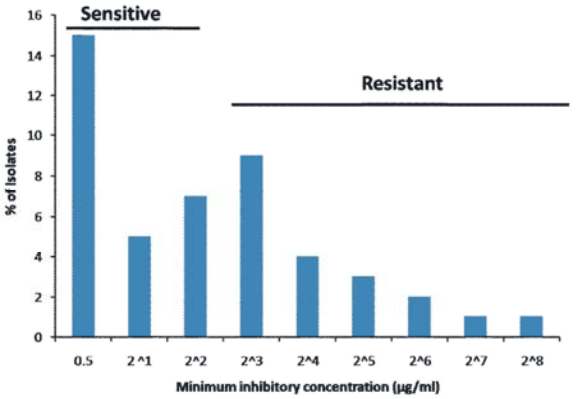
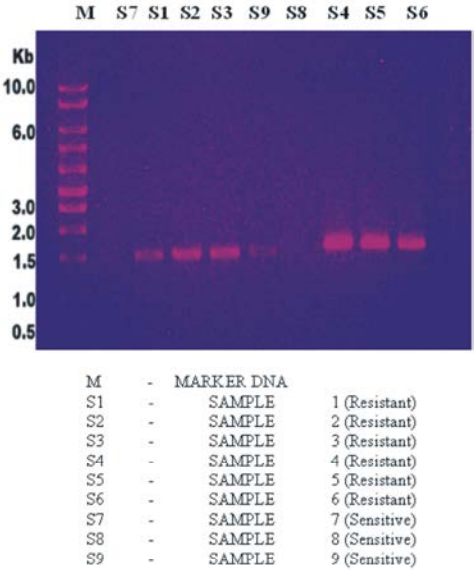


Fig. 1: Susceptibility of 50 nasal isolates of S. aureus to Methivillin by MIC



M	-	MARKER DNA	
S1	-	SAMPLE	1 (Resistant)
S2	-	SAMPLE	2 (Resistant)
S3	-	SAMPLE	3 (Resistant)
S4	-	SAMPLE	4 (Resistant)
S5	-	SAMPLE	5 (Resistant)
S6	-	SAMPLE	6 (Resistant)
S7	-	SAMPLE	7 (Sensitive)
S8	-	SAMPLE	8 (Sensitive)
S9	-	SAMPLE	9 (Sensitive)

Fig. 2: PCR Amplified product of MecA gene.

RESULTS

Susceptibility to Methicillin (MICs): The MICs of clinical *S. aureus* ranged between <0.5 and >256µg/ml. A total of 25% of isolates were found to be resistant to Methicillin (Fig. 1). About 7% were inhibited by 4µg/ml, 8% by 9µg/ml, 4% by 16µg/ml, 3% by 32µg/ml & 2% by 64µg/ml, 1%by 128µg/ml and 256µg/ml. In addition a total of 15% isolates were sensitive to methicillin (Fig. 1).

PCR Detection of mecA Gene: The isolated DNA was amplified using polymerase chain reaction for the molecular characterization of the isolated Bacillus species. Since, differentiation is difficult based on morphological and biochemical characteristics, genotypic characterization is essential to identify their variations.

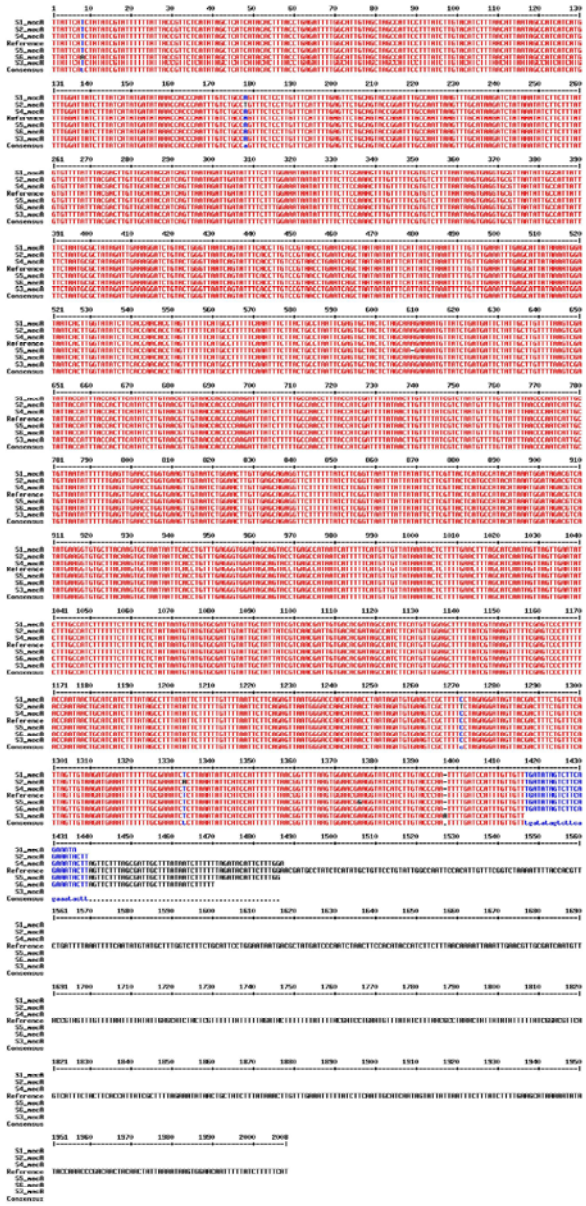


Fig. 3: MecA gene sequence analysis.

The isolated DNA along with random primers was used for the amplification purpose. The mecA-specific PCR product was detected in all MRSA clinical isolates. The products were run in 3% Agarose gel to see all different profiles and to group the isolates yielding similar amplified profiles. The image of gel was further analyzed by using non-linear dynamics software programme (Fig. 2).

MecA Sequence Analysis: The analysis of the mecA sequence showed that real mutation had occurred with the replacement of basepairs C by T in 1371thbp of S2

Table 3: List of mutated strains.

Strain No.	Base pair	Mutation type	Mutated nucleotide
S2	1371	Substitution	c by t
S5	1376	Substitution	a by g
S6	8	Point(transversion)	t by a
S2	178 & 1333	Point(transversion)	t by a
S3	1397	Addition	a

strain and A by G in 1376th bp of S5 strain, a point mutation with the replacement of T by A in 8thbp of S6 and in 178thbp & 1333rd bp of S2 strain and addition of a nucleotide A in 1397th bp in S3 strain (Table 3 & Fig. 3).

DISCUSSION

MRSA is a notorious bacterium which causes infections mainly in Health Care Institutions. Outbreaks of such infection in hospitals are also accelerated with marked increase in morbidity and mortality especially in ICU setting. This increase is accompanied with a high economic cost. Efforts have been made to control these outbreaks. Many institutions have reported an increase in the incidence of MRSA in recent years; meanwhile other studies reported a variation in the incidence. Zaman and Dibb [15] reported during a 3-year period study the prevalence of MRSA showed only minor variation between 6.5and 8.9% at King Khalid National Guard Hospital in Jeddah.

Staphylococcus aureus is an important pathogen both in the community and in hospital settings. The spectrum of diseases caused by this organism is extremely wide, ranging from superficial infections to deep seated and systemic infections such as pneumonia, endocarditis, osteomyelitis and sepsis [4, 16].

During the pre-antibiotic era, many staphylocoocal invasive infections resulted in the death of the patients from the systemic effects of toxin or septicemias. This devastating effect was relieved, to a great extent, by the introduction of penicillin in the 1940s. However, by the end of the 1950s; approximately 50% of hospital isolates of this bacterium were penicillin resistant. Subsequently, semisynthetic derivatives of penicillin (methicillin & others) were used to treat *S. aureus* infections.

Once again, within few years, methicillin resistant *S. aureus* was found in the clinical setting. Today, approximately 60% clinical isolates are MRSA. Recent research has revealed that colonization of MRSA pose a substantial threat for the hospital environment, resulting in nosocomial infections. The word colonization here does not mean the normal sites of colonization (nostrils and in the skin of the axilla/groin.), but rather unhygienic surfaces, such as the hands of the hospital personnel. According to NNIS data for the year 2004, 59.5% of *S. aureus* strains causing infections in hospitals were MRSA [17]. This is not only true for Europe and United States but for India as well. Initially, occasional reports on MRSA were available, but now it has become one of the established nosocomial pathogens. Although no surveillance system exists, the figures obtained from some large medical care facilities including tertiary care hospitals is alarming, with percentages as high as 51.6 to 54.8%. This is much higher than the range of 20 to 32.8% shown by earlier reports. Since our present sample size is very limited, we cannot represent widely however, at least in the premise of the hospital, our data has significance and it can be said that MRSA are emerging in the hospital environment.

Information is scarce on the molecular aspects of MRSA from Salem region. The prevalence rate of MRSA infection in our study was found to be 40.14%, which is in accordance with the reports of Udaya *et al*., (20%) and Mehta *et al*., (32.8%) [18, 19] from India. On the contrary, some of the reports show an alarmingly high incidence of MRSA infection. An important finding of the present study showed that the MRSA cases from ICU (Intensive Care Unit) accounted for 46.6% which is in agreement with the earlier report of Dominique *et al*. [20] from Switzerland.

Also a similar prevalence of MRSA in clinical isolates were reported in Jordan (57%) [21], 61% in Taiwan [22] and 65% in Kuwait [23]. Roghmann *et al*., [24] reported that the rate of subsequent MRSA infection after identification of MRSA colonization was 30%. This increased risk of infection with MRSA has led some to recommend screening all patients or only those who were at highest risk for colonization at admission to the hospital. Hence the organism has been considered as a major pathogen within hospital and in the community.

Treatment for *S. aureus* before 1950 involved the administration of Penicillin G were increasingly causing serious concern. Resistant strains typically produce an enzyme, called β-lactamase, which inactivates the β-lactam antibiotics. Efforts were made to synthesize penicillin derivatives that were resistant to β-lactamase hydrolysis. Crossley *et al.,* [25] have found that the resistance was not due to β-lactamase production but due to expression of additional penicillin binding protein (PBP2a), acquired from another species.

The present study also confirmed that *S. aureus* when subjected to methicillin antibiotic by means of disc diffusion method, 40 strains out of 50 were confirmed to be resistant to methicillin antibiotic, as there was no zone formation around the disc. This resistant pattern could be due to presence of the mecA gene in MRSA strains. These findings are in agreement with the observations of Song *et al*., [26] who have shown that the structural gene of PBP (mecA) was present only in resistant strains but not in the susceptible ones.

Using the presence of *mecA* as a defining standard, our results suggest that an increased sensitivity in the detection of methicillin resistance can be achieved by extending the incubation from 24 to 48 h with both agar MICs and disc diffusion tests. Addition of 5% salt to MHA was not beneficial in the disc diffusion method if the plates were read after 48 h incubation at 30°C.

The DNA sequencing analysis of MRSA revealed 4 base pair changes like C replaced by T in 1271th bp, T replaced by A in 1333rd bp,A by G in 1376th bp and addition of a nucleotide A in 1397th bp. This resulted in substitution and tranversion mutation which might have led to the change in amino acid sequence. These are the factors which could be responsible for methicillin resistance. In addition to good infection control practices, prudent use of antimicrobial agents is one of the major steps for reducing the growing problem of antibiotic resistance. The relationship between antimicrobial use and MRSA are complex and further studies addressing these issues are needed.

REFERENCES

1. Graham, P., S. Lin and E. Larson, 2006. A US population-based survey of staphylococcus aureus colonization. Annals of Internal Medicine, 144: 318-325.
2. Moellering Jr., RC., 2008. Current treatment options for community-acquired methicillin-resistant staphylococcus aureus infection. Clinical Infectious Diseases., 46(1): 032-1,037.
3. Howe, W.B., K.G. Harmon and A. Rubin, 2003. Preventing infectious disease in sports. Physician & Sportsmedicine, 31: 23-33.
4. Baird, D., 1996. Staphylococcus: cluster forming gram positive cocci, in J.G. Colke, A.G. Fraser, B.P. Marmion and A. Simmons (Eds) Practical Medical Microbiology, Newyork, NY: Churchill Livingstone, pp: 245-261.
5. Chambers, H.F. and M. Sachdeva, 1990. Binding of f-lactam antibiotics to penicillin binding proteins in MRSA,J.Infect.Dis., 161: 1170-1176.
6. Bertrand, X., M. Thouverez and D. Talon, 2000. Antibiotic Susceptibility and genotypic characterization of MRSA strains in Eastern France, J. Hosp. Infect, 46: 280-287.
7. Fueyo, J.M., M.C. Martin, M.A. Gonzalez Hevia and M.C. Mendoza, 2001. Enterotoxin production and DNA Finger printing in S.aureus isolated from human and food samples.Relations between genetic types & enterotoxins, Int. J. Food Microbiol., 67: 139-145.
8. Cloney, L., C. Marlowe, A. Wong, R. Chow and R. Bryan, 1999. Rapid detection of mecA in Methicillin resistant S.aureus among hospitals. Enver. Infec. Microbiol. Clin, 17: 498-505.
9. Gayathri, A., N. Savitha, S. Goete, M. Ragini, K.A. Satish, and R. Dasarathy, 2005. Genotyping of methicillin resistant staphylococcus aureus strains from two hospitals in banglore, south India, J. Clin. Microbiol., 43: 3198-3202.
10. National Committee for Clinical Laboratory Standards 1990a. Approved Standard M2-A4.Antimicrobial Disk Susceptibility Tests, 4th ed., Villanova, PA: National Committee for Clinical Laboratory Standards.
11. National Committee for Clinical Laboratory Standards 1990b. Approved Standard M7-A2Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, 2nd ed., Villanova, PA: National Committee for Clinical Laboratory Standards.
12. Sahm, D.F. and J.A. Washington, 1991. Antibacterial susceptibility tests: dilution methods. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors; Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of clinical microbiology. 5th ed. Washington, D.C.: American Society for Microbiology, pp: 1105-1116.
13. Lixin Zhang, U., Srinivasan, C.F. Marrs, D. Ghosh, J.R. Gilsdorf and B. Foxman, 2004. Library on a slide for bacterial comparative genomics. BMC Microbiol., 4: 12.
14. Geha, J., R. Uhl, A. Gustaferro and H. Pering, 1994. Multiplex PCR for identification of MRSA in clinical laboratory. J. Clin. Microbiol., 32: 1768-1772.
15. Zaman, R. and WL. Dibb, 1994. Methicillin resistant Staphylococ-cus aureus (MRSA) isolated in Saudi Arabia: Epidemiology and antimicrobial resistance patterns. J. Hosp. Infect., 26: 297-300.

16. Gang, K., C. Sanyal, I. Bang, E. Makaddas and R. Lari, 2000. Staphylococcal Septicemia in burns, Burns, 26: 359-366.
17. National Nosocomial Infections Surveillance (NNIS) System Report, 2004. data summary from January 1992 through June 2003, issued August 2004. Am. J. Infect Control., 31: 481-98.
18. Mehta, A.P., C. Rodrigue and K. Seth, 1998. Control of methicillin resistant Staphylococcus aureus in a tertiary care center: A fiveyear study. Indian J. Med. Microbiol., 16: 31-4.
19. Udaya, S.C., B.N. Harish and P.M. Umesh Kumar, 1997. Prevalence ofmethicillin resistant Staphylococcus aureus in JIPMER hospital. Indian J. Med. Microbiol., 15: 137-8.
20. Dominique S. Blanc, 2002. Epidemiology of methicillin resistant Staphylococcus aureus: results of a nation- wide survey in Switzerland. Swiss Med. wkly, 132: 223-9.
21. Al-Zu'bi, E., S. Bdour and A.A. Shehabi, 2004. Antibiotic resistance patterns of mecA-positive Staphylococcus aureus isolates from clinical specimens and nasal carriage, Microb Drug Resist. Winter, 10(4): 321-4.
22. Huang, S.S. and R. Platt, 2000. Risk of methicillin-resistant Staphylococcus aureus infection after previous infection or colonization. Clin Infect Dis., 36: 281-5.
23. Geberding, J.L., C. Miick, H.H. Liu and H.F. Chambers, 1991. Comparison of conventional Susceptibility tests with direct detection of penicillin-binding protein 2a in borderline oxacillin resistant strains of Staphylococcus aureus, Antimicrob. Agents Chemother., 35: 2574-2579.
24. Roghmann, M.C., A. Siddiqui, K. Plaisance and H. Standiford, 2001. MRSA colonization and the risk of MRSA bacteraemia in hospitalized patients with chronic ulcers. J. Hosp. Infect., 47: 98-103.
25. Crossley, K.D., B. Loesch, K.D. Landesman M.C. Mead and R. Strate, 1979. An outbreak of infection caused by strains of Staphylococcus aureus resistant to methicillin and aminoglyeosides 1. Clinical studies. J. Infect. Dis., 139: 273-279.
26. Song, M.D., S. Maesaki, T. Wachi, M. Takahashi, F. Doi, Y. Ishino, K. Maeda, A.I. Okongo and N. Matsuchashi, 1988. Primary structure and origin of the gene encoding the β-Lactam inducible pencillin-binding protein, responsible for methicillin resistance in Staphylococcus aureus. In: Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function. (Actore, P., Daneo-Moore., L., Higgins, M.L., Salton, M.R.J. and Shockman G.D. eds.), American Society for Microbiology, Washington. D.C., pp: 352-359.