Surveillance of variant *mecA* gene and analysis of efflux pump in *mecA* negative MRSA infections

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Abstract: It has been known from the medical records of Kancheepuram district hospitals that there is a prevalence of Methicillin resistant *Staphylococcus aureus* strain. Now the recent epidemiology has shown the presence of Methicillin Resistant *Staphylococcus aureus* even in school going children community individuals. In view of the above situation, Methicillin resistant *Staphylococcus aureus* screening was undertaken in the above said region. Both samples from hospital sources and school going children were screened for Methicillin Resistant *Staphylococcus aureus*. The results have shown that the samples had multiantibiotic resistant property along with methicillin resistance. The genomic studies have revealed that there were of positive genomic sequence for *mec* A and 5 isolates for *mec*A negative gene sequence. This would assume that even negative *mec* A genome has the resistant expression. Out of the 5 isolates two isolates have shown the presence of both nor A and *qac* A/B genes. The above results are discussed with efflux of drugs and future proposal on proteomics.

Keywords: MRSA, MSSA, *mec*A, Efflux gene, *nor*A and *qac* A/B.

INTRODUCTION

The infection of Methicillin resistant *Staphylococcus aureus* (Herein referred as MRSA) is one of the priority disease of present time. It develops simple infective manifestation and may lead to life threatening diseases. Now it has a health problem at global level. Earlier it was thought that MRSA strains were identified from samples of nosocomial origin (Chambers, 2001; Salgado *et al., 2003).* Nevertheless since 1990 MRSA infections have also been proved for the presence even in patients do not have any previous hospital stay. Such isolates of MRSA are described as community MRSA. The most important aspect of MRSA is colonization in patients with diabetic mellitus; intravenous drug users; patients undergoing haemodialysis and AIDS. The previous studies that are carried out for the past 10 years have revealed that 47% of healthy school children with less than 10 years of age are carrier of *Staphylococcus aureus.* Among this infected strains 68% of colonies are penicillin resistant. However observations from Newzealand and Australia school of thought have disclosed that 6% to 16% of colonies are MRSA bacteremia (Khairulddin *et al.,* 2004). It has been well established by many microbiologists that beta lactum antibiotics are bind with penicillin binding proteins.

In MRSA the situation is different that beta lactum binds with PB2a protein which has low affinity with beta lactum antibiotic reactions. The reason is suggested that the MRSA has PB2a which is encoded by *mec*A gene. Because the PB2a catalyses all the reaction necessary for cell assembly for other pbs bound beta lactum antibiotics. Otherwise it is known as an analogue for the pencillin binding protein. Therefore the resistant characteristics are lies in S*taphylococcal* chromosomal (SCC) cassette which has the gene sequence of *mec* A (Wees, 2001).

The current studies by Udo *et al* (2006) have disclosed that isolated MRSA is highly resistant to ampicillin, amoxycillin, tetarcycline, erythromycin and gentamycin etc., due to its intrinsic resistance to all beta lactum antibiotics. The subject of MRSA and its eradication at clinical situation is interesting and gives scope for evolving new strategy to recover suscepbility for amenable to antibiotics. On the other hand the clinical practice with intensive fight against MRSA has resulted in development of several resistant mechanisms in MRSA. The various reasons suggested by research workers are enzymatic inactivation and depletion of intra cellular drug concentration by excess expression of efflux pump. Hence many more clear molecular epidemiological features of the antibiotic susceptible pattern of MRSA infection. Such study may provide to develop a potential efflux pump inhibitor and restore antibiotic sensitivity in MRSA strains.

MATERIALS AND METHODS

Clinical samples of nasal discharges, wound pus and skin tissues scraps were the materials for the present study. The samples were collected aseptically using cotton swabs and labelled for identification. They were obtained

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from school going children of 89 boys and 61 girls in the age group between 7 to 15 years. They were admitted in various hospitals located in the study area of Kanchipuram district, Tamilnadu. The collected samples were brought to the laboratory in sealed cartons and subject for the MRSA screening. This study was conducted with the approval of Institute Animal Ethical Committee of Karpaga Vinayaga Medical Sciences (KIMS).

Isolation and identification of MRSA strains

The clinical samples were processed for the isolation and identification of MRSA. The samples were inoculated in Mannitol salt Agar and incubated at 37°C for 18-48 hours and observed for growth. After the growth of *Staphylococci* identification was made on the basis of colonial characteristics. Yellow colonies on Mannitol salt agar, Gram staining and biochemical tests such as coagulase, catalase and beta hemolysis on Blood agar plate.

Antimicrobial susceptibility test

The conventional method of Kirby Bauer disc diffusion assay was followed. The screening was done on Muller Hinton Agar (Himedia) supplemented with the 4% NaCl at Methicillin (5µg) (Saravanan and Nanda, 2009) and other antibiotics disc used were Vancomycin (30µg) Ampicillin (10µg), Gentamycin (10µg), Cilndamycin (2µg), Oxacillin (1µg) and the results were interpreted with the standard instructions of clinical and laboratory standard institute (CLSI, 2009).

Isolation of genomic DNA

For the isolation of genomic DNA of *S.aureus* the technique suggested by Neumann *et al.,* (1992) was followed. The DNA was isolated by improved standard phenol/Chloroform. The extracted DNA analysed by electrophoresis. (75v, 4 Min) on 1.5% Agarose gel which had ethidium bromide. The purified DNA was stored at 20°C. Apart from above techniques the protocols adopted by Rallapalli *et al.* (2008) was also pursued.

Gene amplification

To amplify genes PCR was used. The primer for *mec*A, *nor* A and *qac*A/B was utilized to amplify the genes (table 1). The PCR parameters were included the initial denaturation at 95°C *for 5* minutes then followed by 5 cycles of denuration at 94°C for 1 min and continued with annealing at 40-60°C for 45 seconds and final elongation at 72°C for about 1 min after the end of the cycles followed by the final extension at 72°C for another 5 min and storage at 4°C for 1 min. The amplified genes were analysed by electrophoresis (75V, 40 Min) on a 1.5% agarose gel which have ethidium bromide and visualized in Gel documentation unit. The size of the PCR products was estimated with gene ruler of 1kb DNA ladder (Ferments, Canada).

STATISTICAL ANALYSIS

Statistical tools and Software (Chi square Analysis, SPSS 17.0 Ver.) were used to know the significance with parameters such as age (7-9, 1-12 and 13-15), gender (Male and female), sample type (Nasal, wound, Skin) and presence of respiratory infections.

RESULTS

The results on initial screening of MRSA in the present study location are furnished in tables 1and 2. The data for methicillin resistant strain is given in table 1 and the data exclusively for multi drug resistant is presented in table 2.

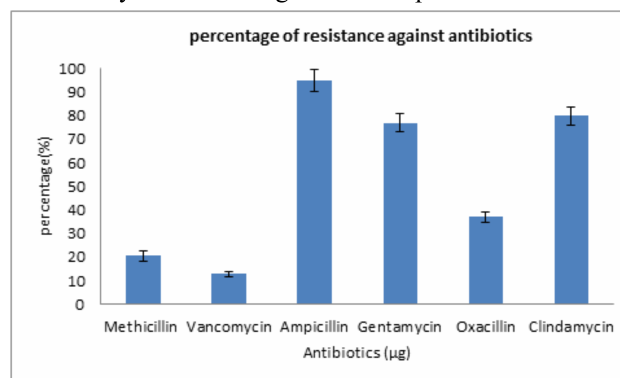

Fig. 1: Percentage of resistance of *Staphylococcus aureus* isolates towards tested antibiotics

Fig. 2: Yellow colored colonies of *S. aureus*

From the results recorded for methicillin resistance with regard to age, there were 2 positive MRSA cases under 7-9, 3 positive cases in 10-12 years age group and 4 infective children in 13-15 years batch. The rest of 141 individuals were noticed negative MRSA. When Gender was taken as criteria there was5 cases positive MRSA among Male children and 4 cases among female children. The distribution of Negative MRSA could be noticed as 80 and 61 respectively for male and female children. Similarly with relation to different schools the results have exhibit that there was 2, 5 and 2 positive MRSA and 30, 80 and 28 negative MRSA. Even in different nature of samples 2 positive MRSA in skin and 7 positive MRSA in Nasal. The interesting observation was in wound analysis there was an absence of MRSA bacteria (table 1).

Table 1: Incidences of methicillin resistance MRSA in Kanchipuram District during the study period.

Methicillin Resistance	Age			Gender		School			Sample Type			Respiratory Infection	
	7-9	10-12	13-15	Male	Female	1	2	3	Skin	Nasal	Wound	Yes	No
Positive	2	3	4	5	4	2	5	2	2	7	0	9	0
Negative	8	45	88	80	61	30	83	28	19	121	1	124	17
χ2	0.688			0.153		0.393			0.063			1.08	
P value	0.709^{NS}			0.696^{NS}		0.822^{NS}			0.969^{NS}			0.299^{NS}	

Table 2: Incidences of multi drug resistance MRSA in Kanchipuram District during the study period.

Multi Drug Resistance	Age			Gender		School			Sample Type			Respiratory Infection	
	7-9	10-12	13-15	Male	Female	1	2	3	Skin	Nasal	Wound	Yes	No
Positive	0	2	3	3	2	0	3	2	1	4	0	5	0
Negative	12	45	88	81	64	31	86	28	19	125	1	128	17
χ2	0.449			0.113		1.904			0.099			0.799	
P value	0.799^{NS}			0.737^{NS}		0.386^{NS}			0.952^{NS}			0.371^{NS}	

*-Significant Different at P<0.05 level (statistically significant) NS – Non significant different at P>0.05level * If P value was greater than 0.05 then it was considered to be non-significant.

Table 3: Sequences of oligonucleotide primers and their length

Oligo Name	Sequence 5′to 3′	Length
Sa *MecA*-FP	5′-ATCGATGGTAAAGGTTGGC-3′	19
Sa *MecA*-RP	5′-AGTTCTGCAGTACCGGATTTGC-3′	22
NorA-FP	5′-TTCACCAAGCCATCAAAAAG-3′	20
NorA-RP	5′-CTTGCCTTTCTCCAGCAATA-3′	20
QacA/B-FP	5′-CTATGGCAATAGGAGATATGGTGT-3′	24
QacA/B-RP	5′-CCACTACAGATTCTTCAGCTACATG-3′	25

In terms of percentage of MRSA occurrence knowing by microbiological method of mannitol salt agar has exhibited that there was 56.25% of MRSA and 43.75% negative MRSA yellow colonization of *S. aureus.* (Fig. 2 and 3). The same microbiological culture studies were also carried out for multi drug resistance character. The data is presented in table 2. From the table it is also evident that there is are at least 5 cases of positive multiantibiotic resistant MRSA *S. aureus* colonization in all the categories of analysis with the parameters of age, gender, school, sample type and respiratory infection, there was no any significant difference between the variable chosen. However there was variation in the percentage of resistance towards tested antibiotics.

The Results on the test for various antibiotic resistances have shown that there was 20% resistance upon methicillin treatment. On the other hand an extreme level of 100% resistance could be seen in experiments with ampicillin. There were presence of 80% resistance in the case of gentamycin, 40% resistance for oxacillin and 80%resistance for clindamycin (fig. 1). This would persumed that the antibiotic resistance by the MRSA strains is heterogeneous in nature.

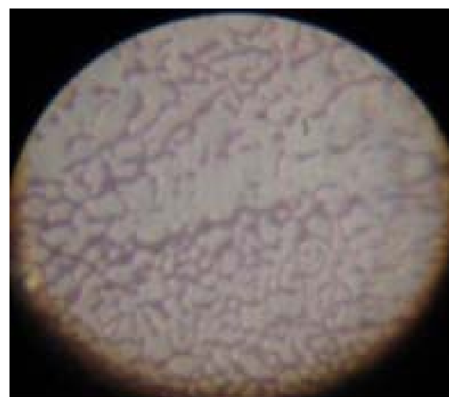

Fig. 3: Gram positive cocci in cluster *S. aureus.*

In this context one would expect to know the genetic variations on the resistant character. The same was carried out in this present work. The analysis of PCR with three different primers was used for the sequence amplification (table 3). Initially the genomic DNA from 14 MRSA isolates was done. The results are presented in figs. 4 and 5. Then the isolated genomic DNA of MRSA positive was subjected for the amplification, in order to ascertain whether the sequence had MecA positive genes. The above molecular experimental procedure had resulted that

there were 9 positive genomic sequences for *mec*A and the rest of 5 isolates containing *mec*A negative gene sequence (fig. 6).

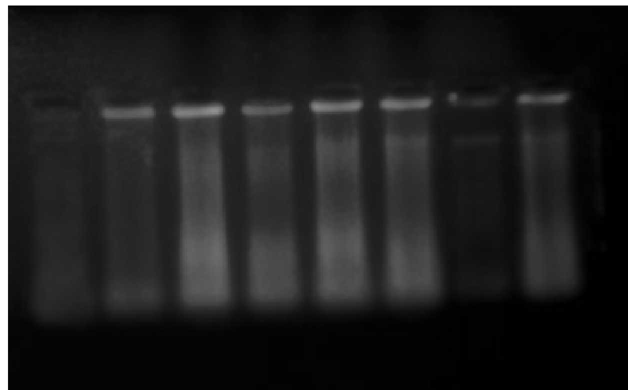

Fig. 4: Genomic isolation of DNA from MRSA (L2-SA1, L3-SA2, L4-SA3, L5-SA4, L6-SA5, L7-SA6 and L8-SA7).

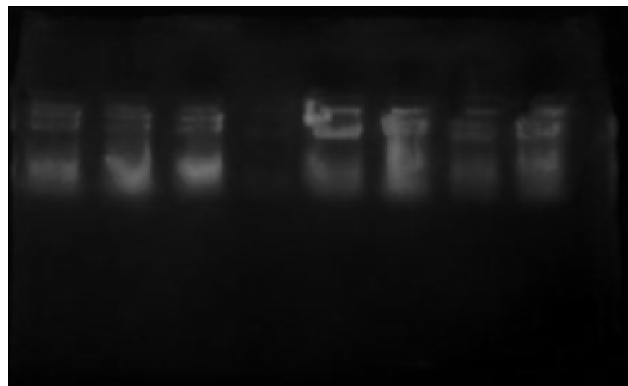

Fig. 5: Genomic isolation of DNA from MRSA (L1-(L1-1kb ladder, L2-SA8, L3-SA9, L4-SA10, L5-SAL6-SA11, L6-SA12, L7-SA13 and L8-SA14).

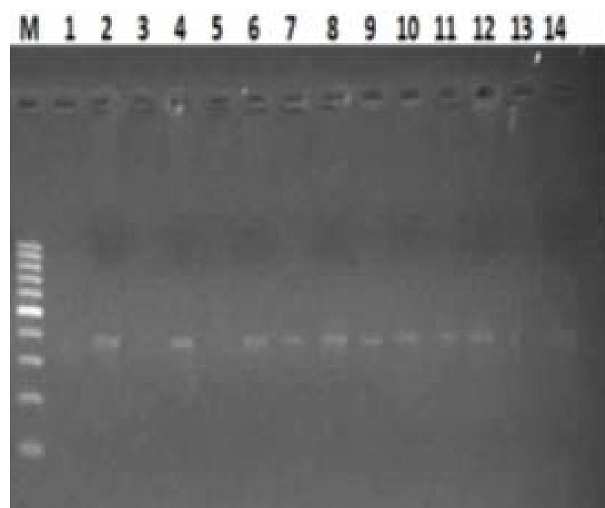

Fig. 6: 1 kb ladder, *mecA*; 52b bp pcr product size.

Fig. 7: 1 kb ladder, Nor A; 1 kb pcr product size.

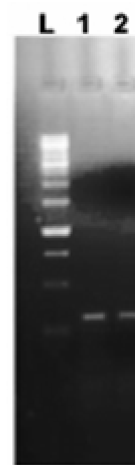

Fig. 8: 1 kb ladder, QacA; 1 kb pcr product size.

DISCUSSION

The results are closely agreeable with our earlier report of similar such observations (Karthikeyan *et al.,* 2006). A corresponding equal observation was also noticed by earlier works in this line (Ligozzi *et al*., 1991, Al-Zu'bi *et al*., 2004, Hafez et al., 2009). It is recalled here that there is comparable such work carried out by Sajithkahn *et al*., (2012). In their observations for 35 MRSA isolates they have noticed 33 strains of positive *mec*A genomes, In other words there was 94% of the positive *mec*A genes out of the total MRSA screening. Unlike to this in our present observations only 64% of MRSA isolates had *mec*A positive sequence. This would indicate that comparatively low incidence of *mec*A positive strains in Kanchipuram district. Subsequently equal attention for the negative isolates was also given. The five negative isolates known in the present study was looked for the presence of nor A and qac A genes. Out of 5 isolates two isolates have showed the presence of both nor A and *qac*A/B genes (fig. 7). It is inferred from the foregoing observation it could be observed that even in negative Mec A isolates have the resistance characteristics. It is

believed that the genes nor A and qac A/B genes are highly significant for the expression of multi drug resistance in *Staphylococcus aureus* (fig. 8). The above data was validated by statistical chi square analysis for the significance. Further the PCR method in this study may be one of the rapid techniques for the identification of resistance genes.

CONCLUSION

The present study on molecular epidemiological studies with screening of resistance gene sequence would allow thinking the role of proteins in rendering the resistant character or the efflux pump. Such information may require further full molecular analysis on proteomics. It is desirable to undertake proteomics studies on the protein molecules involved for efflux pump trait for multi drug resistance in MRSA bacterium in future.

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