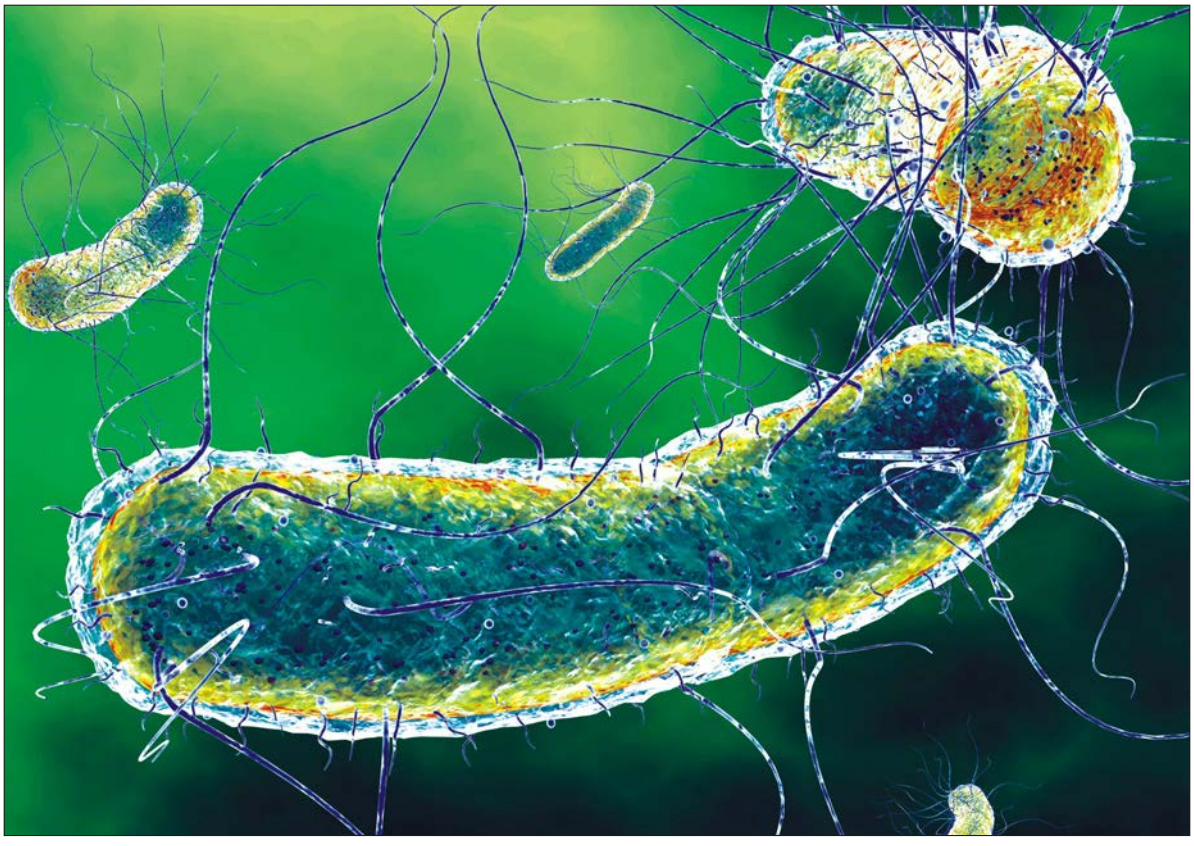

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Molecular Characterization of Methicillin Resistant *Staphylococcus aureus* Strains Isolated in Kerala, South India

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Abstract: The aim of the present study is to report the prevalence, antimicrobial susceptibility pattern molecular characteristics of methicillin resistant *Staphylococcus aureus* strains and the emergence of vancomycin intermediate *Staphylococcus aureus* (VISA) strains in Kerala, India. The study was conducted during January 2006 to December 2007 on 70 strains obtained from pus cultures of patients from various hospitals in Kerala, India. Organisms were isolated, cultured and identified as per standard routine procedures. Susceptibilities to thirteen commonly used antibiotics were tested by agar diffusion method as recommended by CLSI. Minimum inhibitory concentrations of oxacillin, ciprofloxacin and vancomycin were determined using standard protocol. Plasmid profile analysis of the strains carried out and the central resistance determinant *mec*A and internal control gene *fem*A were isolated and sequenced. Cassette chromosome typing carried out as per standard procedures. Among the 70 strains isolated 13 of them showed reduced susceptibility to vancomycin and two isolates were resistant. All the strains were resistant to oxacillin and ampicillin and uniformly sensitive to gentamycin. *mec*A gene was isolated from 88% strains and sequence analyzed. The strains were found to be Hospital Associated-MRSA (HA-MRSA) with type III cassette chromosome. This study reveals the high prevalence of MRSA and a gradual emergence of VISA strains in Kerala. This is greatly due to the irrational and overuse of antibiotics like vancomycin and partly due to negligence on the part of health care workers in acknowledging the prevalence of MRSA and VISA strains and initiating appropriate strategies to control their spread. Careful use of existing antibiotics and regular monitoring of strains circulating in a particular hospital at regular intervals is necessary to control the spread of multidrug resistant strains and to prevent the emergence of even more serious strains.

Key words: HA-MRSA, VISA, staphylococcal cassette chromosome, *mec*A, vancomycin

INTRODUCTION

Staphylococcus aureus is a pathogen of major concern because of its ability to cause a diverse array of diseases ranging from minor infections to life threatening septicemia and its ability to adapt to adverse environmental conditions (Franklin Lowy, 2003). Within two years of introduction of methicillin into therapy, *S. aureus* strains resistant to methicillin were detected. About 70% of *S. aureus* isolates are found to be methicillin resistant (Arakere *et al*., 2005). In India the prevalence and spread of MRSA has been recognized late which led to its emergence as a real threat to community and hospital settings. MRSA is associated with higher mortality than MSSA strains. The resistance pattern of MRSA strains varies from region to region depending on the genetic background and

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vancomycin was the only antibiotic found to give uniform sensitivity so far. So, when antibiotics such as vancomycin are used for treatment, every strain of MRSA must be tested in the laboratory for their *in vitro* susceptibility patterns (Rajaduraipandi *et al*., 2006).

Methicillin resistant *Staphylococcus aureus* has an additional genetic material known as the *mec*DNA, not found in methicillin sensitive strains, which encodes penicillin binding protein PBP2a having reduced affinity for beta lactam antibiotics. The *mec*A is found as a part of a mobile genetic element found in all MRSA strains designated as SCC*mec* (Staphylococcal cassette chromosome mec). SCCs have been found to show great geographical variation which makes cassette chromosome typing essential for complete characterization of MRSA. Majority of the *Staphylococcus aureus* strains isolated in Kerala are multidrug resistant but have not yet been characterized. This study was undertaken to analyze the molecular characteristics of these strains for the first time.

MATERIALS AND METHODS

Bacterial Isolates

A collection of 70 methicillin resistant *Staphylococcus aureus* isolates obtained from pus cultures of patients from various hospitals in Kerala during the period 2006-2007 were identified using standard microbiological methods (Health Protection Agency, 2007) such as Gram staining, catalase test, slide coagulase and mannitol fermentation. Methicillin resistance was confirmed by disk diffusion with oxacillin disks in Mueller Hinton agar (Himedia laboratories, Mumbai, India) according to CLSI (National Committee for Clinical Laboratory Standards, 2000) recommendation (National Committee for Clinical Laboratory Standards, 2006).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed according to CLSI standards using commercially available disks (HiMedia Laboratories, India). The antibiotics used were Ampicillin (25 μg), ciprofloxacin (10 μg), cotrimoxazole (25 μg), ceftriaxone (30 μg) chloramphenicol (30 μg), ofloxacin (2 μg), tetracycline (10 μg), gentamycin (50 μg), oxacillin (1 μg), erythromycin (15 μg), vancomycin (30 μg), fusidic acid (10 μg) and mupirocin (5 μg). *Staphylococcus aureus* strain ATCC 25923 was used as the control strain. The strains were classified into a different antibiotype if at least one difference was observed. Homogeneous and heterogeneous resistance to methicillin was differentiated by incubation of plates with a 5 μg oxacillin disk at 30 and 37°C as described earlier (Lemaitre *et al*., 1998).

MIC Determination

Minimum inhibitory concentrations of oxacillin, ciprofloxacin gentamycin and vancomycin (ranging for each antibiotic from 0-512 μg mL^{-1}, by serial two-fold dilution) were determined for each strain by broth macrodilution methodology as recommended by CLSI. The tubes were incubated at 37°C and read for turbidity after 24 h.

Plasmid Profile Analysis

Plasmids were isolated by alkaline lysis method by Birnboim as described earlier (Sambrook *et al*., 1989) and separated by agarose gel electrophoresis.

Transformation Studies

Chromosomal DNA isolated from the MRSA strains using standard procedure by adding lysozyme to lyse the cell wall and digestion with EcoR1 restriction endonuclease carried out.

Table 1: Primers used in present study

No.	Gene		Primer sequence	Amplicons (bp)	References
1	*mec*A	(f)	ACTGCTATCCACCCTCAAA	160	Arakere *et al.* (2005)
		(r)	CTGGTGAAGTTGTAATCTGG		
2	*fem*A	(f)	AAAAAAGCACATAACAAGCG	133	Arakere *et al.* (2005)
		(r)	GATAAAGAAGAAACCAGCAG		
3	*bldZ*	(f)	CCTGCTGCTTTCGGTAAGAC	226	Present study
		(r)	GTTCAGATTGGCCCTTAGGA		
4	*cif*2	(f)	TTCGAGTTGCTGATGAAGAAGG	495	Oliveira *et al.* (2002)
		(r)	ATTTACCACAAGGACTACCAGC		
5	*kdp*	(f)	AATCATCTGCCATTGGTGATGC	284	Oliveira *et al.* (2002)
		(r)	CGAATGAAGTGAAAGAAAGTGG		
6	*dcs*	(f)	CATCCTATGATAGCTTGGTC	342	Oliveira *et al.* (2002)
		(r)	CTAAATCATAGCCATGACCG		
7	*rif*4	(f)	GTGATTGTTCGAGATATGTGG	243	Oliveira *et al.* (2002)
		(r)	CGCTTTATCTGTATCTATATCGC		
8	*rif*5	(f)	TTCTTAAGTACACGCTGAATCG	414	Oliveira *et al.* (2002)
		(r)	GTCACAGTAATTCCATCAATGC		

Transformation of MSSA strain with total chromosomal DNA from the MRSA strain was performed using Calcium chloride method as described earlier (Sambrook *et al*., 1989). Transformants were selected on plates containing 30 mg L^{-1} of ampicillin.

Amplification Studies

Template DNA was prepared using a procedure described earlier (Hanssen *et al*., 2004). The central determinant responsible for methicillin resistance *mec*A and the internal control gene *fem*A were isolated by PCR amplification using published primers (Primers and References in Table 1). The reaction mixture consisted of 200 µM dNTPs, 10X reaction buffer, 500 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase and 20 pmol of each primer (Mehrotra *et al*., 2000). The amplification conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1.5 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 3 min which is a slight modification from previously published work (Mehrotra *et al*., 2000). Primers for *fem*B and *bla*Z genes were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3-www.cgi) and synthesized at the oligo synthesis department, Bangalore Genei, India. Cassette chromosome typing was carried out using multiplex PCR according to the procedure of Oliveira and DeLencastre (2002).

Sequencing and Comparison

The PCR products for *mec*A and *fem*A were purified using standard product purification procedures (Sambrook *et al*., 1989) and the purified samples were sequenced in both directions using automated ABI 3100 Genetic Analyser. The nucleotide sequences obtained were analysed using Chromas Software and compared to the sequences in GenBank, EMBL and DDBJ databases using BLASTn.

RESULTS

All the strains were heterogeneously resistant to methicillin and uniformly sensitive to gentamycin. Strains showed 100% resistance to ampicillin and oxacillin (Table 2). The strains were classified in 21 antibiotypes using a panel of 12 antibiotics (Table 3).

Oxacillin MICs ranged from 32-256 µg mL^{-1}. Most of the strains showed high level resistance to ciprofloxacin with MICs ranging from 64-128 µg mL^{-1}. MIC of vancomycin for VISA strains ranged from 8-16 µg mL^{-1}. The two strains resistant to vancomycin by disk diffusion showed MICs of 16 µg mL^{-1}. Sensitivity of the strains to gentamycin was confirmed by their MICs which ranged from

Table 2: Antimicrobial resistance pattern of the strains as determined by disk diffusion

Antibiotics	Resistant	Intermediate	Sensitive
	(%)		
Ampicillin	70 (100.0)	-	-
Oxacillin	70 (100.0)	-	-
Ceftriaxone	30 (42.8)	40 (57.1)	-
Ciprofloxacin	57 (81.4)	6 (8.5)	7 (1.0)
Ofloxacin	36 (51.4)	17 (24.2)	17 (24.2)
Chloramphenicol	17 (24.2)	1 (1.4)	52 (74.2)
Tetracycline	31 (44.2)	17 (24.2)	22 (31.4)
Gentamycin	-	-	70 (100.0)
Erythromycin	32 (45.7)	3 (4.2)	35 (50.0)
Vancomycin	2 (2.8)	13 (18.5)	55 (78.5)
Fusidic acid	41 (58.5)	22 (31.4)	7 (1.0)
Mupirocin	48 (68.5)	-	22 (31.4)
Cotrimoxazole	50 (71.4)	-	20 (28.5)

Table 3: Classified strains in 21 antibiotypes

No.	Antibiotype	Resistance pattern	Strains
1	BT1	Amp, oxa, cip, co, chl, ofl,ery, tet, fu, mu, van	6
2	BT2	Amp, oxa, cip, co, chl, ofl, tet, fu, mu	3
3	BT3	Amp, oxa, cip, co, ofl, ery, tet, fu, mu, van	5
4	BT4	Amp, oxa, cip, co, ofl, ery, tet,fu	3
5	BT5	Amp, oxa, cip, co, ery, tet, fu, mu	4
6	BT6	Amp, oxa, cip, co, chl, ofl, fu, mu	3
7	BT7	Amp, oxa, cip, co, chl, ofl, ery, mu	3
8	BT8	Amp, oxa, cip, co, ofl, tet, fu, mu, van	6
9	BT9	Amp, oxa, cip, co, ofl, tet, fu, mu	4
10	BT10	Amp, oxa, cip, co, ofl, tet, fu	2
11	BT11	Amp, oxa, cip, tet, fu, mu, van	3
12	BT12	Amp, oxa, cip, co, ofl, ery, fu	4
13	BT13	Amp, oxa, cip, ofl, ery, fu	5
14	BT14	Amp, oxa, cip, ofl, tet, fu	3
15	BT15	Amp, oxa, cip, fu, mu, van	2
16	BT16	Amp, oxa, chl, ofl, tet, fu	2
17	BT17	Amp, oxa, tet, fu, mu	1
18	BT18	Amp, oxa, co, mu	2
19	BT19	Amp, oxa, co, tet, fu, mu	2
20	BT20	Amp, oxa, ery, fu, mu, van	3
21	BT21	Amp, oxa, cip, ofl, ery, tet	2

BT: Biotype, Amp: Ampicillin, Oxa: Oxacillin, Cip: Ciprofloxacin, Co: Cotrimoxazole, Ofl: Ofloxacin, Chl: Chloramphenicol, Tet: Tetracycline, Ery: Erythromycin, Fu: Fusidic acid, Mu: Mupirocin, Van: Vancomycin

Table 4: Minimum inhibitory concentrations of the isolates

	Minimum inhibitory concentrations (μg mL^{-1})										
AB	512	256	128	64	32	16	8	4	2	1	<1
CIP	0	3	22	26	7	5	0	0	6	1	0
OXA	0	2	3	7	28	29	1	0	0	0	0
GEN	0	0	0	0	0	0	1	10	15	18	26
VAN	0	0	0	0	0	8	5	24	18	9	6

AB: Antibiotics, CIP: Ciprofloxacin, OXA: Oxacillin, GEN: Gentamycin, VAN: Vancomycin

<1-4 μg mL^{-1} (Table 4). High molecular weight plasmid corresponding to cadmium acetate and mercuric chloride resistance was isolated only from the VISA strains. Transformation studies revealed that the mec DNA was not transformed from MRSA strains to MSSA strains. 160 bp *mec*A gene was isolated from 88% strains. The phenotypically methicillin resistant *mec*A negative strains were positive for *blaZ* PCR confirming that they are beta- lactamase hyper producers. All the strains were HA-MRSA (hospital associated MRSA) and carried type III cassette chromosome. The sequencing and comparison of *mec*A gene revealed that the sequences showed 98% sequence homology to *mec*A gene of reference strain MRSA252 from Genbank database. The sequence was submitted to Genbank (Acc. No. EU552505).

DISCUSSION

Antibiogram analysis has been shown to be a good epidemiological marker for MRSA phenotyping. The results of this study demonstrated that majority of the *S. aureus* strains isolated from Kerala were multidrug resistant. All the strains were resistant to ampicillin and oxacillin. The high level resistance to ampicillin, ciprofloxacin, ofloxacin and cotrimoxazole can be attributed to the fact that these broad spectrum antibiotics are frequently used in treatment of common infections. The uniform sensitivity of the strains to gentamycin was rather unexpected. Earlier MRSA strains were known to be resistant to gentamycin. So, the use of this antibiotic gradually decreased until it was no longer used for therapy, while the use of fluoroquinolones increased which explains the high level ciprofloxacin resistance and gentamycin sensitive phenotype. The spread of GS-MRSA has been reported by Lemaitre *et al*. (1998). On the contrary, resistance to gentamycin has been reported as being increased since 1996 (Price *et al*., 1998). Studies by Rajaduraipandi *et al*. (2006) also reported 63.6% resistance to gentamycin. These studies have also reported lower resistance rates to ciprofloxacin and ofloxacin compared to the present study. Heterogeneous resistance is a common trait of MRSA with the level of resistance being strain specific and varying according to their genetic background. Contrary to the earlier studies that reported the presence of high molecular weight plasmids encoding mupirocin and chloramphenicol resistance (Udo *et al*., 2006), multidrug resistance in the tested strains were solely due to chromosomal genes.

In the present study 88% of MRSA isolates were found to encode and express the *mec*A gene. This kind of discrepancy in the correlation between the presence of *mec*A gene and oxacillin resistance has been reported earlier (Martineau *et al*., 2000). The sequence of *mec*A gene was found to be well conserved with only 1 to 2 bp differences as reported earlier (Hanssen *et al*., 2004). Another unexpected and rather alarming result is a small percentage of strains that showed intermediate resistance to vancomycin. The emergence of VRSA and VISA strains has been reported earlier in northern India (Assadullah *et al*., 2003; Tiwari and Sen, 2006). This is the first time VISA strains are being reported from Kerala.

The high prevalence of MRSA and VISA strains can be due to factors such as lack of awareness among health care workers and inappropriate use of antibiotics. Grouping of isolates with a common genetic background will help in understanding the spread of multidrug resistant strains of MRSA and at the same time predict the emergence of even more serious strains such as VRSA (Aviles *et al*., 2006). If preventive measures can be taken at the initial stages spread of VRSA can be controlled. But reluctance of health care workers in accepting the prevalence of MRSA in some hospitals is an important factor that obstructs the prompt institution of preventive measures. Vancomycin being the drug of choice for treatment of MRSA, the emergence of strains resistant to vancomycin is an alarming situation.

CONCLUSION

The study reports an increased prevalence of MRSA and the emergence of VISA strains in Kerala. Careful use of existing antibiotics and regular monitoring of strains circulating in a particular hospital at regular time intervals is essential to tackle the spread of highly resistant strains of *Staphylococcus aureus* and also to predict and prevent the emergence of even more resistant strains.

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