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Prevalence of Methicillin Resistant *Staphylococcus aureus* in Community and Hospital Environments in Assam

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Abstract

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is an important public health concern globally. Co-resistance to multiple antibiotics in MRSA renders treatment options limited. Resistance to 23 antibiotics as per CLSI guidelines, 2011 was tested in one hundred and forty samples of *Staphylococcus aureus* isolates collected from both out and in patients in a tertiary care centre in Assam. Bacteria was isolated form clinical samples like urine, Pus, Blood, Throat swab and Sputum. Prevalence of MRSA was 39.68% in community and 60.32% in hospital acquired infections. High frequency of isolates was MDR. Overall, high resistance to commonly prescribed antibiotics were documented ranging from Oxacillin 117 (83.57%) followed by Cefpodoxime 104 (74.29%), Kanamycin 99 (70.71%), Cefoxitin 95 (67.86%), Ceftazidime 76 (54.29%), Ertapenem 74 (52.86%) and finally Methicillin 63 (45%). Isolates were most sensitive to Vancomycin 140 (100%) followed by Imipenum 134 (95.71%), Chloramphenicol 130 (92.86%), Meropenem 128 (91.43%), Gentamucin 119 (85%), and Pipericillin/ Tazobactam 114 (81.43%). PCR detection proved useful with 100% concordance with phenotypic detection methods of *S. aureus. mecA*gene was found to be prevalent in 37.86% of the total isolates. Our study advocates routine uses of molecular detection techniques as a complementary tool with the conventional tools of diagnosis.

Keywords
Staphylococcus aureus, MRSA, MDR, *mecA, femB.*

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INTRODUCTION

Staphylococcus aureus, a gram positive bacterium is known for its ability to cause wide range of infection ranging from simple skin infection to necrotizing pneumonia. The chronological evolution of resistance against methicillin is a major cause of worry form global health perspective. Its ability to perpetuate continuously among the healthy population has caused a global outbreak. Methicillin

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Resistant *Staphylococcus aureus* (MRSA) is at a rise in both community and hospital settings.

Prevalence pattern of MRSA amongst *S. aureus* varies from country to country. US has experienced a two-fold increase in MRSA detections between 2003 to 2008[1-2] where prevalence was documented to be maximum in bloodstream infections followed by pneumonia across the country. On the contrary, the European Union has witnessed a lesser infiltration of MRSA in some countries while some countries have a significant number of cases registered[3]. The developing countries of the African continent have showcased a higher risk of MRSA, with Ethiopia carrying for the highest percentage of prevalence and South Africa with the least. Studies report a steep rise in MRSA detection in the new millennium with an average cap of 50% in Africa[4-5]. A similar rise has also been seen in the Asian countries. Mainland China has witnessed an increase up to 51.7%[6-7]. Among all the Asian countries, India has been a subject of maximum MRSA prevalence ranging up to 74% in selected areas. The countries sharing border with Indian subcontinent has a witnessed lesser prevalence of MRSA in both communities associated isolates and healthcare associated isolates[8-11].

The diverse health care system across the country with a diverse socio-economic stature has played a significant role in boosting the MRSA prevalence in India. With a very less awareness about the super bug among the commons have also resulted in an increased fatality due to MRSA infections. The north-east Indian population has been least studied upon for the MRSA prevalence in both CA and HA infections. This study has been focused on the prevalence of MRSA in different clinical samples collected in the state of Assam from both community and healthcare setups. We are also reporting here the antibacterial sensitivity pattern of *S. aureus* isolates circulating in Assam and genotype of MRSA isolates.

MATERIALS AND METHODS

Bacterial isolates

Following ethical clearance (GU/ ACA/ Ethics/ 2012/ 3993 dated 10th January, 2012) a total of 140 consecutive isolates of *S.aureus* collected from clinical specimens in between March 2012 and November 2014 in the Bacteriology laboratory of Department of Microbiology, Gauhati Medical College, Guwahati, Assam were included in the study. The isolates were consecutive and non-repetitive. Out of 140 isolates, 11(7.86%) were from throat swab, 13 (9.29%) were from sputum, 15 (10.71%) from blood, 41 (29.29%) from pus and 60 (42.86%) were isolated from urine. One of each duplicate sample was inoculated directly onto Luria Bertani Broth, incubated for 24 hours at 37°c while the other sample was used to make a smear for Gram staining and other further biochemical tests.

The isolates were identified as *S. aureus* by standard methodology [12]. All the isolates were subjected to phenotypic including isolation and identification in MeReSA media and genotypic testing. Furthermore, *S. aureus* isolates were screened for methicillin resistance using 1µg/ml oxacillin disc in Mueller Hinton agar plate supplemented with 4% sodium chloride.

Antibiotic Susceptibility Test

For the detection of MRSA, Kirby-Bauer's disc diffusion method was used. 1 µg oxacillin disc on 5% Muller-Hinton agar was incubated overnight at 35°C according to the Clinical Laboratory Standards Institute (CLSI) guidelines, 2011[13].The Susceptibility towards the following antibiotics were tested as per CLSI guideline-Oxacillin (1µg), Methicillin (5µg), Pipericillin (100µg), Cefepime (30µg), Cefotaxime (30µg), Ciprofloxacin (5µg), Cefdinir (5µg), Cefpodoxime (10µg), Ertapenem (10µg), Imipenum (10µg), Cefoperazone (75µg), Ceftazidime (30µg), Tetracycline (30µg), Nitrofuranatoin (300µg), Gentamicin (10µg), Kanamycin (30µg), Vancomycin (MIC Interpretive standard), Quinupristin-dalfopristin (15µg), Clidamycin (2µg), Meropenem (10µg), Erythromycin (15µg), Cefoxitin (30µg), Chloramphenicol (30µg). All the antibiotics were procured from Hi-Media Laboratories, Mumbai, India.The plates were interpreted after incubating for 24 hours at 34°C. The diameter of the zones around each disc and was interpreted based on the same CLSI guidelines.

Detection of MRSA

For the identification of the MRSA among the isolates of *Staphylococcus aureus*, HiChromeMeReSa Agar Base (Himedia laboratories Mumbai, India) was used as per manufacturer's protocol. *Staphylococcus aureus* strains were streaked onto the Hi ChromeMeReSa agar and incubated at 33°C ±2 for 24 hours. Culture showing bright blue colour growth was taken as MRSA strains, while all others were recorded as MSSA strains. Based on the colour identification, 65 isolates were prone being positive. Further, MRSA was also tested by antibiotic susceptibility test as per CSLI guidelines[13]. *mecA* gene PCR was performed on all isolates identified as MRSA by phenotypic methods.

Genomic DNA Extraction

The isolation of genomic DNA from S. aureus strains was done using HiPurATM bacterial genomic DNA purification kit (HiMedia Lab, Mumbai). The isolated DNA was stored at -20^0C if a second PCR was necessary to resolve discordances.

Multiplex PCR for the detection of *femB*and *mecA*genes

Five microliters of the extracted DNA were transferred to 20 µl of PCR amplification mix consisting of 1X PCR buffer, 2.5 mM $MgCl_2$, 1.25U of Taqpolymerase, 0.25mM of dNTPs and 0.5 µl of each primer. The primers published elsewhere were used for the detection of *femB* (F- 5´ TTA CAG AGT TAA CTG TTA CC 3´ and R- 5´ ATA CAA ATC CAG CAC GCT CT 3´) and *mecA* (F- 5´ GTA GAA ATG ACT GAA CGT CCG ATA A 3´ and R - 5´ CCA ATT CCA CAT TGT TTC GGT CTAA 3´) yielding 700bp and 310 bp PCR products respectively[15].

Phylogenetic analysis

Unambiguous characterization was carried out by using Multi Locus Sequence Typing (MLST) of the seven housekeeping genes. Internal fragments of 450-500 bp of the genes were sequenced on both strands, which was then used to identify and establish distinct allelic profile.

The genes and the primers for the phylogenetic analysis were taken from the source: http://saureus.net/misc/info.asp. The PCR amplification was standardized as per the protocol mentioned in the link

Primer	Sequence
arcC F	5´-TTGATTCACCAGCGCGTATTGTC-3´
*arcC*R	5´ -AGGTATCTGCTTCAATCAGCG-3´
aro E F	5' ATC GGA AAT CCT ATT TCA CAT TC -3'
aroE R	5' GGT GTT GTA TTA ATA ACG ATA TC -3'
glp F	5' CTA GGA ACT GCA ATC TTA ATC C -3'
glpR	5' TGG TAA AAT CGC ATG TCC AAT TC -3'
gmkF	5' ATC GTT TTA TCG GGA CCA TC -3'
gmkR	5' TCA TTA ACT ACA ACG TAA TCG TA -3'
ptaF	5' GTT AAA ATC GTA TTA CCT GAA GG -3'
ptaR	5' GAC CCT TTT GTT GAA AAG CTT AA -3'
tpiF	5' TCG TTC ATT CTG AAC GTC GTG AA -3'
tpiR	5' TTT GCA CCT TCT AAC AAT TGT AC -3'
yqiF	5' CAG CAT ACA GGA CAC CTA TTG GC -3'
yqiR	5' CGT TGA GGA ATC GAT ACT GGA AC -3'

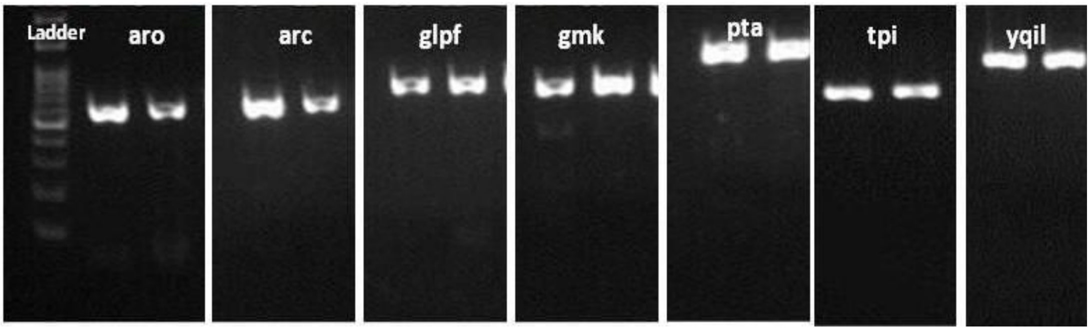

Figure 1: PCR Standardisation of MLST genes for phylogenetic analysis

RESULTS

Antibiotic susceptibility test

As per CLSI guidelines, antibiotic susceptibility of all the isolates against 23 antibiotics were carried out.[12] All the isolates were sensitive to Vancomycin 140 (100%) followed by Imipenum134 (95.71%), Chloramphenicol130 (92.86%), Meropenem128 (91.43%), Gentamucin119 (85%) and Pipericillin/ Tazobactam114 (81.43%). However, the highest resistance was observed for Oxacillin117 (83.57%) followed by Cefpodoxime104 (74.29%), Kanamycin99 (70.71%), Cefoxitin95 (67.86%), Ceftazidime76 (54.29%), Ertapenem74 (52.86%) and finally Methicillin63 (45%). Table 1 presents a comparative antibiotic resistance profile of MRSA and MSSA isolates.

Antibiotic		***S. aureus***				**Total**	
		MRSA	**N=63**	**MSSA**	**n=77**		
		No	**%**	**No.**	**%**	**No.**	**%**
Oxacillin	Susceptible	10	15.87	13	16.88	23	16.43
	Resistant	53	84.13	64	83.11	117	83.57
Pipericillin/ Tazobactam	Susceptible	46	73.01	68	88.31	114	81.43
	Resistant	17	26.98	9	11.68	26	18.57
Cefepime	Susceptible	9	14.28	66	85.71	75	53.57
	Resistant	54	85.71	11	14.28	65	46.43
Cefotaxime	Susceptible	20	31.74	71	92.20	91	65.00
	Resistant	43	68.25	6	7.79	49	35.00
Ceftazidime	Susceptible	4	6.34	60	77.92	64	45.71
	Resistant	59	93.65	17	22.07	76	54.29
Cefoperazone	Susceptible	33	52.38	68	88.31	101	72.14
	Resistant	30	47.61	9	11.68	39	27.86
Cefpodoxime	Susceptible	2	3.17	34	44.15	36	25.71
	Resistant	61	96.82	43	55.84	104	74.29
Cefdinir	Susceptible	9	14.28	67	87.01	76	54.29
	Resistant	54	85.71	10	12.98	64	45.71
Imipenem	Susceptible	58	92.06	76	98.70	134	95.71
	Resistant	5	7.93	1	1.29	6	04.29
Ertapenem	Susceptible	33	52.38	33	42.85	66	47.14
	Resistant	33	52.38	41	53.24	74	52.86
Meropenem	Susceptible	52	82.53	76	98.70	128	52.86
	Resistant	11	17.46	1	1.29	12	08.57
Gentamicin	Susceptible	50	79.36	69	89.61	119	85.00
	Resistant	13	20.63	8	10.38	21	15.00
Kanamycin	Susceptible	18	28.57	23	29.87	41	29.29
	Resistant	45	71.42	54	70.12	99	70.71
Vancomycin	Susceptible	63	100	77	100	140	01.00
	Resistant	-	-	-	-	-	-
Quinupristin-dalfopristin	Susceptible	19	30.15	63	81.81	82	58.57
	Resistant	44	69.84	14	18.18	58	41.43
Ciprofloxacin	Susceptible	34	53.96	69	89.61	103	73.57
	Resistant	29	46.03	8	10.38	37	26.43
Tetracycline	Susceptible	31	49.20	68	88.31	99	70.71
	Resistant	32	50.79	9	11.68	41	29.29
Nitrofurantoin	Susceptible	21	33.33	74	96.10	95	67.86
	Resistant	42	66.66	3	3.89	45	32.14
Chloramphenicol	Susceptible	54	85.71	76	98.70	130	92.86
	Resistant	09	14.28	1	1.29	10	07.14
Cefoxitin	Susceptible	05	7.93	40	63.49	45	32.14
	Resistant	58	92.06	37	48.05	95	67.86
Clidamycin	Susceptible	38	60.31	71	92.20	109	77.86
	Resistant	25	39.68	6	7.79	31	22.14
Erythromycin	Susceptible	23	36.50	66	85.71	89	63.57
	Resistant	40	63.49	11	14.28	51	36.43

Table1: Antibiotic susceptibility patterns of *S. aureus* isolates

Prevalence of methicillin-resistant *S. aureus*

Of these 140 isolates, 63(45%) were MRSA and 77(55%) were MSSA. The most common sample from which MRSA isolated was urine 29 (46.03%), followed by 12 (19.04%) in pus, 9 (14.28%) in blood samples, 7 (11.11%) in sputum and 6(9.52%) in throat swab. Out of these 63 methicillin resistant *Staphylococcus aureus* isolates 25 (39.68%) were from out-patients whilst 38 (60.32%) were isolated from in-patients. Table 2 summarizes the prevalence of MRSA in different clinical specimens. Both community and hospital isolates of MRSA were resistant to penicillin, cephems (Parental and Oral), streptogramins, nitrofurantoins and other classes of antibacterial drugs as shown in Table 3.

Isolates Source	Total infected	MRSA	MSSA
Urine	60	29	31
Pus	41	12	29
Blood	15	9	6
Throat swab	11	6	5
Sputum	13	7	6
Total	**140**	**63**	**77**

Table 2: **Prevalence of MRSA in different clinical specimens**

Test/ Report Group	Antimicrobial Agent	Minimum number of MRSA isolates Resistant to specific Test group (n=63)
Penicillin	Oxacillin; Methicillin	**53**
β lactam/ β- lactamase inhibitor combinations	Pipericillin-Tazobactam	**17**
Cephems (parental)	Cefoperazone; Cefotaxime; Ceftazidime; Cefepime; Cefoxitin	**30**
Cephems (oral)	Cefpodoxime; Cefdinir	**54**
Carbapenems	Imipenem; Ertapenem; Meropenem	**05**
Glycopeptides	Vancomycin	**00**
Aminoglycosides	Gentamicin; Kanamycin	**13**
Macrolides	Erythromycin	**40**
Fluoroquinolones	Ciprofloxacin	**29**
Nitrofurantoins	Nitrofurantoin	**42**
Lincosamides	Clindamycin	**25**
Phenicols	Chloramphenicol	**09**
Streptogramins	Quinupristin-dalfopristin	**44**
Tetracyclines	Tetracycline	**32**

Table 3: Multiple drug resistance pattern of MRSA isolates

Detection of *femB*and *mecA*

The *S. aureus* isolates were subjected to *femB*and *mecA*PCR. The presence of *femB* gene confirms the isolate to be *S. aureus* and presence of *mecA* gene indicates the particular strain to be methicillin resistant. PCR analysis through *femB* detection confirmed phenotypic data. Duplex PCR generated staphylococcus-specific amplification products in 140 clinical isolates.

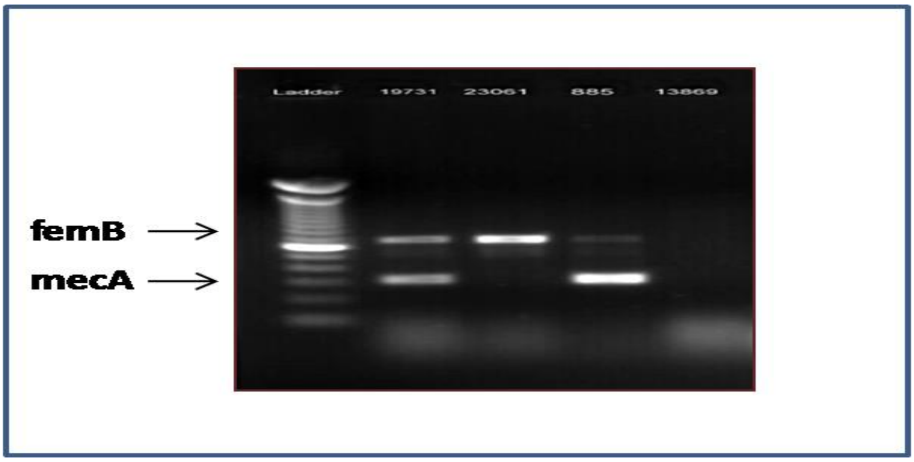

Figure 2: Agarose gel electrophoresis for *mecA* (310 bp) and *femB* (700 bp) genes. Lanes: 1 Molecular weight ladder; Lane1:100bp ladder molecular size marker, Lane 2: Positive Control, Lane 4: Negative Control, Lane 2-3: genes in *S. aureus* isolates

MLST:

MultiLocus Sequence Typing was carried out on four representative isolates with different pattern of susceptibility towards antibiotics. Similar primers were used for PCR amplification, followed by sequencing.

	Allele										
	arrc	aroe	Glpf	gmk-1	yqil	pta	tpi	ST	Antibiotics resistant	Genes present	Source
S.aureus (Isolate# 5991)	2	2	2	2	6	179	2	New	OX; MET; PIT; CPM;CTX;CAZ;CPD; CDR; ETP; GEN; K; RP; TE; NIT; CX; CD;E	MecA fem B	Pus
S.aureus (Isolate# 11377)	New	3	1	1	11	72	New	New	OX; MET; PIT; CPM; CTX;CAZ;CPZ; CPD; CDR; IPM; ETP;MRP; GEN; K; RP; CIP; TE; NIT; C; CX; CD;E	mec A, fem B e	Urine
S.aureus (Isolate# 25458)	1	1	1	1	12	4	3	683	OX; MET; CPM; CTX;CAZ;CPD; CDR; ETP; K; RP; CIP; TE; NIT; CX; CD;E	mec A, fem B	Pus
S.aureus (Isolate# 464)	1	1	1	1	22	1	1	772	OX; MET; CPM; CTX;CAZ;CPZ; CPD; CDR; GEN; K; CIP; NIT; CX	mec A, fem B	Blood

Table 4: Selection of MLST isolates

Pair wise detection matrices were calculated to score homologies or divergence value and PAM substitution model available in the software MEGA was used. Neighbour joining method by using Kimura-two-parameter of MEGA was used to create distance matrix dendogram. Statistical significance of Neighbour Joining Tree topology was tested using Bootstrapping (1000 pseudo replicas). Branches with bootstrap values greater than 50% were considered statistically significant.

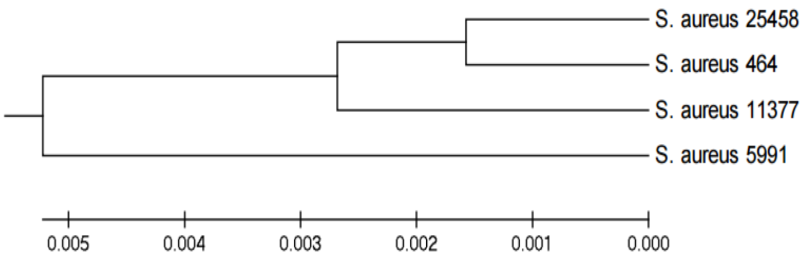

Figure 3: Phylogenetic divergence of clinical *S.aureus* isolates

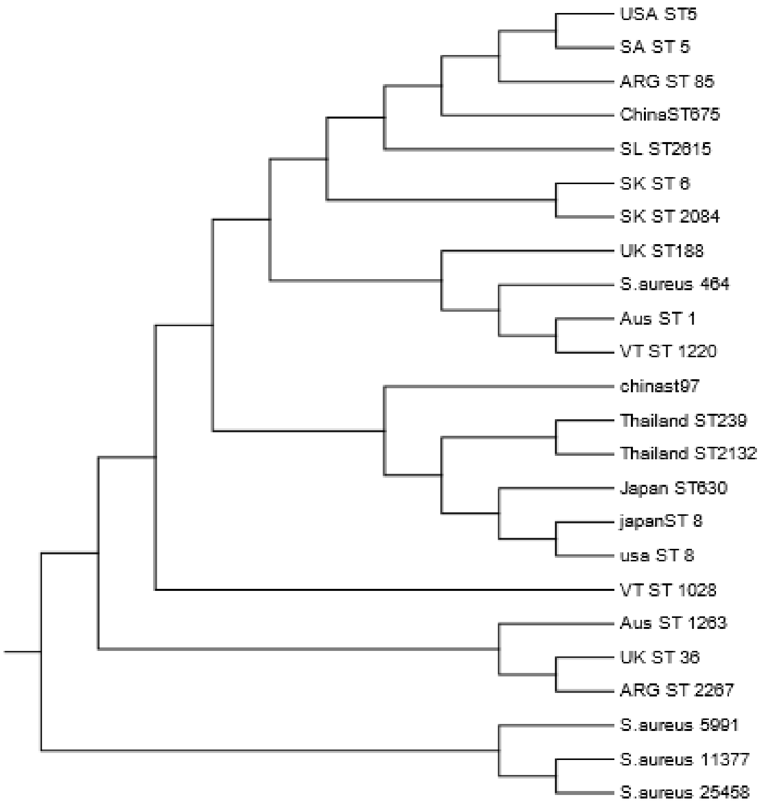

Figure 4: MLST comparison with isolates from other countries

DISCUSSION

45% higher prevalence of MRSA was found in this region indicating a rise in infections[16-17]. There should be strict drug policies so that the overuse of antibiotics can be limited leading to resistant isolates. Antibacterial surveillance studies are required to be done periodically to document the prevalent sensitivity pattern. Our results also warrant need of development of newer antibacterial agents.

Of the 140 isolates, 59 (42.14 %) were Community Acquired and 81 (57.86%) were Hospital Acquired. Gender wise, it was observed that 56% of the males and 44% of the females were infected of which 17% were children (2-13 yrs). Our study also documented considerable multi drug resistance in MRSA as compared to MSSA isolates. Higher proportion of isolates were found to be resistant to most of the commonly prescribed antibiotics as per CLSI guideline 2011 which poses a great challenge for clinical management of disease. Co-relation between phenotypic and PCR detection strategies indicates that molecular detection could be useful where culture isolation is difficult. In addition, higher sensitivity and specificity along with rapid detection time make PCR an attractive option for routine diagnostic application. The allelic profile and phylogenetic analysis suggests genetic diversity among the isolates found in different environments across Assam.

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