

Comparative Analysis of *mecA* and *nuc* Gene Sequences of *Staphylococcus aureus* Strains Isolated from Human Urine Samples

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In the present study twenty five human urine samples of hospital patients were screened for *Staphylococcus aureus*. Twenty percent of the samples were positive for *Staphylococcus aureus* as yellow colonies were observed aerobically on mannitol salt agar, out of the five isolates two were catalase positive. On the basis of resistance against methicillin, out of the three catalase positive isolates, two were MSSA and one was MRSA. One MRSA and one MSSA strain was examined using PCR technique, for gene identification. The *mecA* and *nuc* gene were amplified using PCR technique. The presence of *mecA* gene and *nuc* gene in MRSA and MSSA respectively, confirmed the strains. Comparative gene analysis of *mecA* gene and *nuc* gene was performed using BLAST and CLUSTALW tools. Thereafter rooted phylogenetic tree was obtained for both *mecA* and *nuc* gene sequences explaining the genetic evolutionary lineage. Present study analyzed the resistance mechanism of MRSA and to identify the gene responsible in order to control the re-emerging methicillin resistant *Staphylococcus aureus* infection.

Keywords: MRSA, Antibacterial activity, *mecA* gene, *nuc* gene, PCR.

Staphylococcus aureus is one of the most important opportunistic pathogen among Staphylococci belonging to *Micrococaceae* family causing significant infections under appropriate conditions (Narmeen and Jubrael 2009). Methicillin-resistant *S. aureus*, abbreviated MRSA is one of a number of greatly feared strains of *S. aureus* which have become resistant to most β -lactam antibiotics. MRSA was first isolated in 1960 as a nosocomial

pathogen, now known as hospital-acquired MRSA (HA-MRSA). Another class of MRSA, designated community-acquired MRSA (CA-MRSA), emerged in the community from 1997 to 1999, posing a novel threat worldwide (Goldstein *et al.* 2012). Recently strains of multiple drug resistant *Staphylococcus aureus* have appeared and proven very difficult to treat. During the late 1950s and early 1960s, *Staphylococcus aureus* caused considerable morbidity and mortality as a nosocomial or hospital acquired pathogen and has become the head leading cause of nosocomial infection during the last two decades. Unfortunately, MRSA (methicillin resistant *Staphylococcus aureus*) strains isolated are resistant to multiple non- β -lactam containing antimicrobial drugs (Abraham *et al.* 2009).

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Resistance is conferred by the *mecA* gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that can function as transpeptidase (Lopez *et al.* 2012). In the absence of β -lactam antibiotics the *Staphylococci* utilize usual PBPs to synthesize the cell wall which is composed of peptidoglycan. In contrast, an additional low affinity penicillin binding protein PBP designated PBP 2a is encoded by a unique MRSA-PBP gene and is the main factor responsible for the expression of the β -lactam resistance of MRSA which is an intrinsic resistance of the cells to all β -lactam antibiotics is mediated by the methicillin resistance determinant (Haroun and Hameed 2011). Much of the research aimed at identifying novel therapeutic targets for the treatment of *Staphylococcus aureus* infections has been directed at identification of specific virulence factors and regulatory circuits that are relevant to the disease process. Some organisms rapidly acquire resistance e.g. coliforms and *Staphylococcus aureus*. The organisms exhibit remarkable versatility in their behaviour towards antibiotics, with some strains having overcome most commonly used drugs (Uwaezuoke and Aririatu 2004).

Comparative genomics is an attempt to take advantage of the information provided by the signatures of selection to understand the function and evolutionary processes that act on genomes (Chandrakanth *et al.* 2010). A typical alignment procedure involves the application of a program such as CLUSTAL W, followed by a tree building program. For further identification at genus level, bacterial isolates were identified through homology search with BLAST and FASTA using *mecA* and *nuc* gene sequences. Keeping in mind the increasing mortality rate due to MRSA infections in the last two decades, in the present study the sequence of the *mecA* gene which accounts for the resistance of *Staphylococcus aureus* towards β -lactam antibiotic methicillin and the sequence of *nuc* gene, which is found in all *Staphylococcus aureus* strains were subjected to phylogenetic analysis. This study suggests that further research must continue on analyzing the resistance mechanism of the genes responsible for imparting resistance in *Staphylococcus aureus* strains, in order to control the reemerging MRSA infections.

MATERIALS AND METHODS

Isolation and identification of *Staphylococcus aureus* Bacteria

Twenty five human mixed stream urine samples were collected from the pathology department, Jeevan Jyoti Hospital, Allahabad, INDIA. The urine samples were inoculated on mannitol salt agar (MSA) plates. The plates were incubated aerobically at 37°C for 24 h to obtain colonies (Abraham *et al.* 2009) that were identified on the basis of cultural, morphological and biochemical characteristics as described in the Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1984).

Antibiotic Susceptibility Pattern of *Staphylococcus aureus* against selected antibiotics

The test organism was tested for its sensitivity towards the given antibiotics using the Disk Diffusion Method (Bauer *et al.*, 1966). Overnight broth cultures were spread on the surface of Nutrient Agar Media plates with the help of sterile swabs. The antibiotic discs (Hi Media) were placed on the agar surface and plates were incubated aerobically at 37°C for 24 h. Zone of inhibition was measured in 'mm' and the result interpreted on the basis of CLSI standards (Wayne, 2003). Antibiotic discs of various concentrations i.e. Amoxicillin (AMX) 10 μ g, Ceftriaxon (CTX) 30 μ g, Cefuroxime (CFX) 30 μ g, Ciprofloxacin (CIP) 5 μ g, Gentamicin (GEN) 10 μ g, Methicillin (MET) 5 μ g, Norfloxacin (NOR) 10 μ g, Penicillin-G (P) 1 μ g, Tetracycline (TE) 30 μ g, Vancomycin (VA) 30 μ g were used in the present study.

Gene Amplification and Comparative Gene Analysis

DNA was isolated followed by PCR to amplify the gene sequences of *mecA* gene present in MRSA strain and *nuc* gene in MSSA strain and then gene sequencing was done. The gene sequences of *mecA* and *nuc* gene were analyzed using BLAST (basic local alignment search tool) to search the sequences available on the data base. Four sequences were selected having high similarity with *nuc* gene and six sequences were selected having high similarity with *mecA* gene. ClustalW tool was used for multiple sequence alignment of the *nuc* gene sequence along with the four related sequences. Then multiple

sequence alignment of the *mecA* gene sequence along with the six related sequences was performed. The data obtained as a result of multiple sequence alignment, was represented in the form of rooted UPGMA (unweighted pair group method of arithmetic mean) phylogenetic tree. The phylogenetic tree was interpreted in order to analyze the genetic evolutionary lineage.

RESULTS AND DISCUSSIONS

Culture Characterization

Out of twenty five samples, five samples (20%) were positive for *Staphylococcal* spp as yellow colonies were observed aerobically on mannitol salt agar, this yellow pigmentation was due to the mannitol fermentation by the organism. One colony was picked from each of the five plates

which were positive for *Staphylococcal* spp and noticed that these five isolates were gram-positive cocci, arranged in clusters, when screened for morphological characteristics.

On the basis of biochemical characterization the five isolates were found to be non-motile, also gave positive result for nitrate reduction test and methyl red test, while negative result was obtained for indole test. Tube Coagulase test was performed, which is the most commonly used method to identify *S. aureus* and the five isolates were coagulase positive. Another study showed comparable results in which thirty six out of hundred isolates (36%) were gram-positive cocci in cluster form, catalase positive and eighteen out of thirty six isolates fermented mannitol. Six out of eighteen isolates were tube coagulase positive, which was presumptive as MRSA (Amini *et al.* 2012). The carbohydrate fermentation test revealed that *Staphylococcus aureus* isolates demonstrated fermentation of glucose, galactose, sucrose, fructose, mannitol, whereas could not ferment raffinose (Table 1).

Antibiotic Susceptibility pattern of *Staphylococcus aureus*

On the basis of resistance against methicillin, out of the three catalase positive isolates, two were MSSA and one was MRSA. The MRSA strain was resistant towards the seven out of eleven antibiotics used, whereas MSSA was resistant towards three out of eleven antibiotics used. Among the eleven antibiotics, gentamicin possessed maximum inhibitory potential whereas penicillin showed least inhibitory effect against MRSA and MSSA strains. MRSA strain was multi-

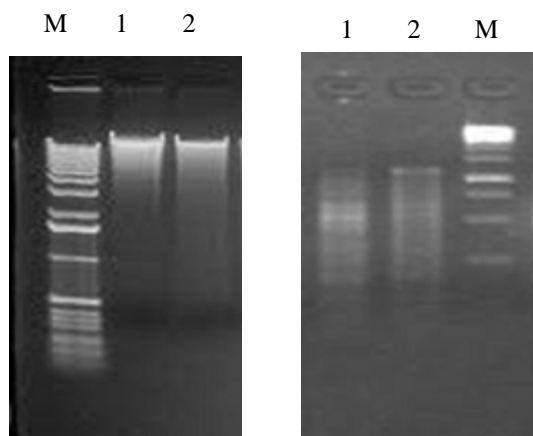


Fig. 1. Gel electrophoresis picture of (a) Genomic DNA isolation, (b) PCR amplification

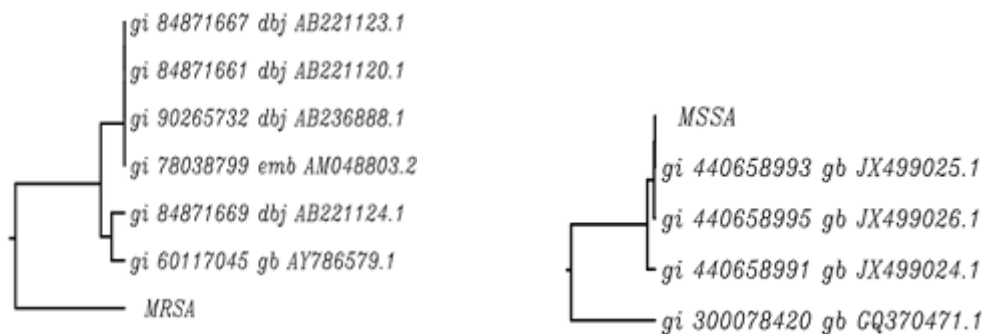


Fig. 2. Phylogenetic Tree for *mecA* gene sequence in (a) MRSA (b) MSSA

drug resistant as it was resistant to methicillin, penicillin, cefuroxime, ceftazidim, ceftriaxone, ciprofloxacin, tetracycline (Table 2).

In the present study the antibiotic susceptibility of the MSSA and MRSA isolates was tested. Results revealed that both MRSA and MSSA strains were sensitive to amoxicillin, gentamicin and this was comparable with the reports of other workers (Nwankwo *et al.* 2010; Khadri and Alzohairy 2010). In another study the isolates were most sensitive to gentamicin (91.7%), cloxacillin (85.4%) and most resistant to penicillin (95.8%) and ampicillin (89.6%). The capacity of *Staphylococcus aureus* to produce human diseases has not diminished with the introduction of antibiotics. The organisms exhibit remarkable versatility in their behaviour towards antibiotics, with some strains having overcome most commonly used drugs. In this study, a high sensitivity percentage to gentamicin (91.7%), cloxacillin (85.4%) was recorded. Also most of the strains of *Staphylococcus aureus* were sensitive to erythromycin (66.7%) and streptomycin (66.7%). This finding shows that staphylococcal infections could be treated with gentamicin, cloxacillin, erythromycin (Uwaezuoke and Aririatu 2004).

Amplification and comparative gene analysis of *mecA* gene and *nuc* gene sequences

Specific gene identification of MRSA and MSSA strains were performed. PCR was performed to amplify the *mecA* and *nuc* gene sequences. After gene amplification gel electrophoresis was performed for both MRSA and MSSA genome sequences for *mecA*, *nuc* gene sequences respectively (Fig. 1). *mecA* is the gene responsible for resistance towards methicillin while *nuc* gene is responsible in detecting the presence of *S. aureus*, thus this sample can be considered as MRSA. Those samples which lack *nuc* gene were not *S. aureus*. In the previous study five isolates of *Staphylococcus aureus* were positive for the presence of *mecA* gene and only one of the samples was positive to *nuc* gene (Amini *et al.* 2012).

Following sequences were obtained for *nuc* gene in methicillin susceptible and *mecA* gene sequence in methicillin resistant *Staphylococcus aureus* respectively.

nuc gene sequence in methicillin susceptible *Staphylococcus aureus*:

>giAAAGGTGAAGATAAAGTGCATGTTCAA

GAGTCGTCGATGGAGATACTTTCATAGCGAA
TCAAAACGGCAAGGAAATCAAGGTTAGA
CTCATTGGAGTGCATACGCCTGAAACA
G T A A A A C C G A A T A C G C C T G T A C
A G C C A T T T G G T A A G G A A G
C A T C A A A T T A T A G T A A G A A
G A C T T T A A C G A A T C A A G A C G T T T A T T T A
G A A T A C G A T A A A G A A A A G C A A G A C C G C T A T G
G T A G A A C A T T G G C G T A T G T A

mecA gene sequence in methicillin resistant *Staphylococcus aureus*:

>giATGAAAAAGATAAAAATTGTTCCACT
TATTTTAATAGTTGTAGTTGTCGGGTTT
GGTATATATTTTTATGCTTCAAAAAGATA
A A G A A A T T A A T A A T A C T A T
T G A T G C A A T T G A A G A T A A A A A T T T
A A A C A A G T T T A T A A A G A T A G C
A G T T A T A T T C T A A A A G C G A T A A
T G G T G A A G T A G A A A T G A C T G A
A C G T C C G A T A A A A A T A T T A A T A
G T T T A G G C G T T A A A G A T A T A A
A C A T T C A G G A T C G T A A A A T A A A
A A A A G T A T C T A A A A A T A A A A A A
C G A G T A G A T G C T C A A T A T A A A A T T A
A A A C A A A C T A C G G T A A C A T T G A
T C G C A A C G T T C A A T T T A A T T T T
G T T A A A G A A G A T G G T A T G T G G A A
G T T A G A T T G G G A T C A T A G C G
T C A T T A T T C C A G G A A T G C A G A A A
G A C C A A A G C A T A C A T A T T G A A
A A T T T T A A A A T C A G A A C G T G G T A A
A A T T T T A G A C C G A A A C A A T G T
G G A A T T G G C C A A T A C A G G A A C A
G C A T A T G A G A T A G G C A T C G T T
C C A A A G A A T G T A T C T A A A A A A G A T T A T A
A A G C A A T C G C T A A A G A A C T
A A G T A T T T C T G A A G A C T A T A T C A
A A C A A C A A A T G G A T C A A A A T
T G G G T A C A A G A T G A T A C C T T
C G T T C C A C T T A A A A C C G T T A A
A A A A A T G G A T G A A T A T T T A A
G T G A T T T C G C A A A A A A T T T C A T C T T A
C A A C T A A T G A A A C A G A A A G T C G T

On submitting the *mecA* and *nuc* gene sequences to nucleotide BLAST (basic local alignment search tool), information about the two test sequences were retrieved (Nuc gene sequence in MSSA: Query ID lcl|1011 Database Name: nr; Program: BLASTN 2.2.28; Query Length: 250) and (*mecA* gene sequence in MSSA: Query ID

lc|17517 Database Name: nr; Program: BLASTN 2.2.28; Query Length: 712). As a result of BLAST the sequences available on the database showing high sequence similarity with the *mecA* and *nuc* gene sequences respectively, were retrieved. Four sequences were selected having high similarity with *nuc* gene and six sequences were selected having high similarity with *mecA* gene. As a result of multiple sequence alignment using ClustalW tool, the *nuc* gene sequence along with the four related sequences and the *mecA* gene sequence along with the six related sequences were aligned

on the basis of sequence identity (Higgins *et al.* 1996) and then rooted UPGMA phylogenetic tree was obtained for both *mecA* and *nuc* gene sequences explaining the genetic evolutionary lineage (Fig. 2 a,b).

As a result of the phylogenetic tree retrieved for the *mecA* gene sequence in MRSA (Fig. 2a) it was concluded that the *mecA* gene sequence showed sequence similarity with two clusters of sequences *viz* gi 84b71667 dbj AB221123.1; gi 84b71661 dbj AB221120.1; gi 90265732 dbj AB236888.1; gi 78038799 dbj

Table 1. Cultural, morphological and biochemical identification of *Staphylococcus aureus* isolates

Characteristics		<i>Staphylococcus aureus</i>	
		MRSA	MSSA
Cultural characteristics	Colour of colony	Golden Yellow	Golden Yellow
	Shape of colony	Small, spherical	Small, spherical
	Elevation	Convex	Convex
	Margin	Regular	Regular
Morphological characteristics	Gram's reaction	Gram positive	Gram positive
	Arrangement of cells	Clusters	Clusters
	Spore formation	Non –sporing	Non –sporing
Biochemical characteristics	Indole test	Negative	Negative
	Methyl red test	Positive	Positive
	Catalase test	Positive	Positive
	Motility test	Negative	Negative
	Nitrate reduction test	Positive	Positive
	Coagulase test	Positive	Positive
Carbohydrate fermentation	D-Glucose	A ⁺ G ⁻	A ⁺ G ⁻
	D- Galactose	A ⁺ G ⁻	A ⁺ G ⁻
	Sucrose	A ⁺ G ⁻	A ⁺ G ⁻
	Maltose	A ⁺ G ⁻	A ⁺ G ⁻
	Lactose	A ⁺ G ⁻	A ⁺ G ⁻
	D-Mannitol	A ⁺ G ⁻	A ⁺ G ⁻
	Raffinose	A ⁺ G ⁻	A ⁺ G ⁻

A⁺ : Acid Positive ; A⁻ : Acid negative; G⁺: Gas positive; G⁻: gas negative; WA⁺ :Weakly Positive.

Table 2. Antibiotic susceptibility profile of *Staphylococcus aureus* strains (MRSA, MSA)

<i>S. aureus</i> strains	Antibiotics										
	MET (5mg)	P (10mg)	VA (30mg)	GEN (10mg)	NOR (10mg)	AMX (10mg)	CXM (30mg)	CAZ (30mg)	CTR (30mg)	CIP (5mg)	TE (30mg)
MRSA	-	-	++	+++	++	++	-	-	-	-	-
MSSA	++	-	+++	+++	++	++	++	++	++	-	-

(+++ Sensitive, (++) Intermediate, (-) Resistant, Methicillin (MET), Amoxicillin (AMX), Penicillin (P), Cefuroxime (CXM), Vancomycin (Va), Cefazidim (CEZ), Gentamicin (GEN), Ceftriaxone (CTR), Norfloxacin (NX), Ciprofloxacin (CIP), Tetracycline (TE)

AM048803.2 and gi 84871669 dbj AB221124.1; gi 60117045 dbj AY786579.1. The three sequences gi 84871667 dbj AB221123.1, gi 84871661 dbj AB221120.1 and gi 90265732 dbj AB236888.1 belong to one clade, thus these genes include the most recent common ancestor of all of its members and all of the descendants of that most recent common ancestor. The two sequences gi 84871669 dbj AB221124.1 and gi 60117045 dbj AY786579.1 are called a clade. Gene transfer is observed to occur from *mecA* gene sequence in MRSA to gi 84871669 dbj AB221124.1 and gi 60117045 dbj AY786579 gene sequences (Felsenstein 1985).

While the phylogenetic tree retrieved for the *nuc* gene sequence in MSSA (Fig. 2b) revealed that the *nuc* gene sequence showed sequence similarity with three clusters of sequences *viz* gi 440658993 dbj JX499025.1; gi 440658995 dbj JX499026.1 and gi 440658991 dbj JX499024.1 and gi 300078420 dbj GQ37047.1. The three sequences gi 440658993 dbj JX499025.1, gi 440658995 dbj JX499026.1 and gi 440658991 dbj JX499024.1 were one clade, these gene sequences include the most recent common ancestor of all of its members and all of the descendants of that most recent common ancestor. Gene transfer is observed to occur from the *nuc* gene sequence in MSSA to gi 440658993 dbj JX499025.1 (Saitou, 1996).

According to the MRSA genome information it was revealed that a circular chromosome of 2809422 bp length and pT811 plasmid of 4440 bp length was present.

CONCLUSION

Results from present study showed that among the eleven antibiotics used, gentamicin possessed maximum inhibitory potential whereas penicillin showed least inhibitory effect against MRSA and MSSA strains. MRSA strain was multi-drug resistant as it was resistant to methicillin, penicillin, cefuroxime, ceftazidim, ceftriaxone, ciprofloxacin, tetracycline. *mecA* and *nuc* gene sequences were retrieved using PCR from MRSA and MSSA strains respectively, followed by BLAST. Evolutionary lineage and genetic diversity of the bacterial strains isolated from different ecological niche, also giving information regarding the horizontal gene transfer was obtained from phylogenetic tree. This study suggests that

comparative genomic analysis can be used to identify the molecular genetic basis of 99.8% of the antimicrobial resistance phenotypes of the isolates, highlighting the potential of pathogen genome sequencing as a diagnostic tool and also screen the genetic changes associated with adaptation to the hospital environment and with increasing drug resistance over time, and how MRSA evolution likely has been influenced by country-specific drug use regimens.

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