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PREVALENCE AND MOLECULAR GENETICS OF METHICILLIN-RESISTANT *Staphylococcus aureus* FROM CLINICAL ISOLATES

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ABSTRACT

Background: The present study emphasized that prevalence and molecular genetics of methicillin- resistant *Staphylococcus aureus* from clinical isolates associate with hospital associated disease. **Materials and Methods:** *Staphylococcus aureus* was isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and cerebrospinal fluid (CSF). The clinical samples were collected from different pathology laboratories of Nagpur City. These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics and confirmed by 16s rRNA. **Results:** During this study, 1465 different clinical samples were tested in which 1255 (85.66%) samples showed growth of bacteria. Out of 1255 clinical samples, 1157 (92.19%) different strains of bacteria were isolated. Among the isolated organisms *Staphylococcus aureus* was found to be 201 (17.37%). For testing with getotyping of isolates by multiplex PCR detaction using *mec*A, *van*A, OXA gene. Among the 201 isolates, 195 strains of *S. aureus* harbouring single *mec*A gene and the percentage was found to be 97.01%. 102 strains of *S. aureus* showed resistance to two genes - *mec*A + OXA gene was found to be 50.75% whereas 74 (36.82%) strains of *S. aureus* showed resistance to three genes - *mec*A + *van*A + OXA gene. **Conclusion:** Identification of the genes is necessary for the surveillance of their transmission in hospitals.

Keywords: Molecular genetics, MRSA, Multiplex PCR, *mec*A gene

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* strains emerged soon after the introduction of methicillin into clinical practice but were generally rare until the 1980s. In the late 1970s, however, MRSA emerged as a major pathogen of hospital infection worldwide and in addition it had also become a community pathogen **[1]**, first detected in Europe in the 1960s shortly after the introduction of methicillin. Clinical infections are most common in patients in hospital intensive care units, nursing homes, and other chronic care facilities **[2]**. The increased frequency of community-associated MRSA infection has been associated with reports of increased morbidity and mortality—specifically, a longer duration of fever, prolonged hospitalization, a higher incidence of pulmonary complications along with bone and joint infections, and the re-emergence of a severe staphylococcal sepsis syndrome **[3]**. The key determinant of methicillin resistance is the *mecA* gene, which encodes in a novel penicillin-binding protein (PBP2/PBP2a) which reduces the binding of all β-lactam drugs to the cell wall and therefore, mediates cross-resistance to all these compounds **[4]**. The *mecA* gene is carried on a mobile genetic element designated the "staphylococcal cassette chromosome *mec"* (SCC*mec*) **[5]**, which is inserted into the chromosome at a unique site (*attBscc*) located near the *S. aureus* origin of replication **[6]**. SCC*mec* is composed of the *mec* gene complex, which confers resistance to methicillin, and the *ccr* gene complex, which encodes recombinases responsible for its mobility. There are two distinct types of MRSA. Each has a slightly different genetic makeup. To categorize the type of the bacteria and how the condition is spread, MRSA infections are classified as either hospital-acquired or community-acquired MRSA infections (HA-MRSA or CA-MRSA).

Molecular diagnosis plays an increasing role in fast detection of microbial pathogens and identification of drug-resistance determinants. Results can be obtained within a few hours **[7]**. Over the past several years, a variety of DNA-based tests have been developed to detect MRSA carriage more quickly **[8]**. Most of these assays are based on the detection of an *S. aureus* specific gene and the *mecA* gene, which encodes methicillin (oxacillin) resistance. Identification techniques based on molecular probing were recently established. One of these techniques relies on fluorescent detection of 16S rDNA with a peptide nucleic acid probe (peptide nucleic acid fluorescence *in situ* hybridization [PNA-FISH]). It identified *S.*

aureus in positive blood cultures in less than 3 hrs with a more than 95% sensitivity and specificity **[9]**. The method allowed rapid discrimination between *S. aureus* and potential contaminant CoNS **[10]**. In addition to PNA-FISH, multiplex real-time PCR is being developed to quantify organisms directly in clinical samples. Genes representative of both species and resistance mechanisms are amplified simultaneously. For MRSA, the resistance gene sought is *mecA* gene, which encodes low-affinity PBP2a. However, *mecA* is also present in methicillin-resistant CNS and thus detects simultaneously both MRSA and methicillin-resistant CNS **[11]**. Molecular epidemiology data and the observation that MRSA spread has been successfully managed by the use of rigorous infection-control practices clearly demonstrate that transmission is the main factor contributing to the increasing prevalence of MRSA infection or colonization in patients **[12]**.

mec DNA:

mec DNA is a large (approximately 30–50 kb) DNA fragment that does not occur in MSSA, and is always situated at a fixed site in the *S. aureus* chromosome, specifically near the *pur-nov-his* gene cluster. *mec* DNA contains *mecA*, the structural gene for PBP2a; transcription; and 20–45 kb of *mec*-associated DNA. The *mec*-associated DNA has been found to contain transposons and insertion elements providing a mechanism for the considerable variability found within the region. IS431 is a common insertion sequence in the staphylococcal chromosome plasmids and is present within the resistance determinants with similar IS elements, accounting for the multiple drug resistance phenotype common in MRSA the gene encoding for inducible erythromycin resistance, is located upstream from *mecA* in over 90% of MRSA **[13]**.

***mecA* gene:**

mecA, a structural gene located on the chromosome of *Staphylococcus aureus*, charactierises methicillin-resistant *S. aureus*. The mecA gene can be located in one of seven major types of SCC elements that can be transferred horizontally between *Staphylococcal* species (Deurenberg and Stobberingh, 2008). Most MRSA (>90%) harbor *mecA,* the gene encoding the PBP, PBP2a. *mecA* is inducible and encodes the 76-kD PBP2a polypeptide. The *mecA* gene occurs inboth MRSA and methicillin resistant CNS and is highly conserved **[13]**.

MATERIALS AND METHODS

Bacterial isolates

In this study, bacteria were isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and

cerebrospinal fluid (CSF). The clinical samples were collected
from different pathology laboratories of Nagpur City. During this study, 1465 different clinical samples were collected for isolation of *S. aureus.* These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics.

Antibiotic Sensitivity test:-

Kirby – Bauer disc method:

The *in vitro* antibiotic sensitivity test was performed using Kirby-Bauer disc diffusion method. Six to eight hours cultures of the microbes in peptone water that had achieved the 0.5 McFarland standard turbidity were flooded over Muller-Hinton agar and antibiotic disc were carefully placed on the surface of the agar, allowed to dry, then placed in the incubator at 37 ºC for 16-18 hours. The zones of inhibition of the various antibiotics were measured with a meter rule by taking the diameter of the zones. They were compared with the standard antimicrobial sensitivity chart and recorded as sensitive or resistant to the antibiotics. The antibiotics that were tested included; Amikacin (30mcg), Amoxicillin (30mcg), Ampicillin (10mcg), Cefaclor (30mcg), Ciprofloxacin (5mcg), Clindamycin (2mcg), Erythromycin (15mcg), Gatifloxacin (5mcg), Gentamycin (10mcg), Linezolid (30mcg), Methicillin (5mcg), Ofloxacin (5mcg), Oxacillin (1mcg), Piperacillin (100mcg), Streptomycin (10mcg), Teicoplanin (30mcg), Tetracylin (30mcg), Tobramycin (10mcg) and Vancomycin (30mcg). The antibiotic susceptibility tests on the isolates were done according to the guideline set by the Clinical and Laboratory Standards Institute **[14]**.

E- strip method:

Tested colonies from overnight culture were suspended with 0.85% of normal saline (NaCl) to a turbidity of 0.5 McFarland's. A sterile cotton swab was used to produce a uniform layer on a Mueller-Hinton agar plate and the excess moisture was allowed to be absorbed for about 15 min before the E-test strip was applied. The plate was incubated for 16 to 18 h at 37º C and the MIC end points were read where the inhibition ellipses intersected the strip **[15]**.

Multiplex PCR for detecting *mec*A, *van*A and OXA genes DNA extraction from colony was done by alkaline lysis method

A single colony of each organism was inoculated from MacConkey agar into 5ml of Luria-Bertanii broth (LB) and incubated for 20 h at 37˚C. Cells from 1.5ml of the overnight
culture was harvested by centrifugation at 12,000 rpm for 5 min. 1.5 ml from LB

media containing cells was taken eppendorf tube, than 100 µl TNE buffer was mixed. The mixture was centrifuged for 1 min at 10000 rpm and supernatant was discarded. Again 100 µl NaOH (50 mM) was added to pellet. After heating at 40˚C in water bath for 1 min, 60 µl of IM Tris HCl (PH 6.7) was added. Vortex, centrifuge at 10000 rpm for1 min was done. Then supernatant was used as template (1µl) **[16]**.

Table below shows the list of primers used for detection and sequencing of resistant genes.

DNA amplification in thermal cycler [20]

PCR analysis for beta lactamase genes of the family *mec*A, *van*A and OXA genes were carried out.

- **Preparation of reaction mixture**

For PCR amplification, 1 µl of template DNA was added to 50 µl of master mixture containing 4 µl of dNTPs mixture (2.5mM of each), 10X PCR buffer 5 µl (Ex Taq),0.5 µl of *Taq* polymerase (250 U), 1 µl of each primer stock solution (50pmol/µl), and remaining 38.5 µl volume was fulfilled by neuclease free water.

- **Amplification**

The prepared PCR tubes with master mixture were placed in the eppendorf thermal cycler. Amplification was carried out according to the following thermal and cycling condition:

For *mecA, vanA* and OXA gene

Primers used for detection and sequencing of resistant genes.

Target	Primer name	Primer sequence (5`→3`)	Product size (bp)	References
mecA	*mecA*-F	ATG CGC TAT AGA TTG AAA GGA T	147	Zhang K et. al. (2005) [17]
	mecA-R	GTG AAG ATA TAC CAA GTG ATT		
*van*A	*van*A-F	GGGAAAACGACAATTGC	247	Dutka-Malen S et. al. (1995) [18]
	*van*A-R	GTACAATGCGGCCTTA		
BlaOXA	OXA F	ATTATCTACAGCAGCGCCAGTG	475	Junyoung Kim et. al. (2009) [19]

Initial denaturation at 94˚C for 3 minute

Denaturation at 94˚C for 30 sec
Annealing at 50˚C for 30sec } **35 cycles**
Extension at 72˚C for 2 min

Final extension at 72˚C for 10 minutes

The PCR products were analyzed after electrophoresis in 1.0% agarose gel to detect specific amplified product by comparing with standard molecular weight marker. One percent agarose gel was prepared by melting 2.0 gm agarose in 200 ml of diluted TBE Buffer using a microwave woven. The melted agarose was

allowed to cool to about 50ºC and 20 µl ethidium bromide was mixed and shacked and was poured into gel tray and combs were placed. After solidification of the gel, the comb was removed. During electrophoresis, the gel was placed in a Horizontal electrophoresis apparatus containing TBE buffer and ethidium bromide.

Loading and electrophoresis of the sample

Five µl of amplified PCR product was mixed with 2.0 µl of loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. Hundred bp molecular weight marker was loaded in one well to determine the size of the amplified PCR products. Electrophoresis was carried out at 100 volts for 35 minutes. The amplified products of the study samples were visualized by Gel-DOC system **[20]**.

RESULTS & DISCUSSION

In this study, bacteria were isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and cerebrospinal fluid (CSF). The clinical samples were collected from different pathology laboratories of Nagpur City. These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics. During this study, 1465 different clinical samples were tested in which 1255 (85.66%) samples showed growth of bacteria. Out of 1255 clinical samples, 1157 (92.19%) different strains of bacteria were isolated. Some clinical samples showed more than one bacterial colonies. Among the isolated organisms *Staphylococcus aureus* was found to be 201 (17.37%). *Staphylococcus aureus* identified on the basis of Morphological, biochemical & Cultural Characteristics and confirmed by 16s rRNA **(Table 1)**.

In the present study, out of 400 urine samples, 383 (82.36%) samples showed positive bacterial growth. Out of 383 samples, 379 (98.95%) different bacteria were isolated. In the urine samples, *S. aureus* found to be 18.79%. In addition, *Staphylococcus aureus* was the most common cause of UTI among Gram positive bacteria. In the present, the second most common bacteria found in urine sample was *Staphylococcus aureus* (18.79%). Recent studies have revealed the importance of coagulase negative *Staphylococcus* spp. in urinary tract infections **[21]**. In this study, out of 325 blood samples, 282 (86.76%) samples showed positive bacteria growth. Out of 282 samples, 257 (91.13%) different bacteria were isolated. In the blood samples, *S. aureus* was found to be 16.31%. The third predominant bacteria in this study was *S. aureus,* this result was

similar to other studies **[22, 23]**. In some study, it was showed that coagulase negative staphylococci (CONS) were the most commonly isolated bacteria and this has been also found in other studies **[22, 23, 24]**. Reports from Addis Ababa Ethiopia **[25]** and Zimbabwe **[26]** revealed that (43.3%) and (42.9%) CONS were isolated; respectively.

In many studies of wound and pus, *Staphylococcus aureus* was the most common pathogen. In the present study, 285 wound/swabs samples were screened for the isolation of bacteria, 250 (87.71%) samples showed positive bacterial growth. Out of 250 samples, 232 (92.80%) different bacteria were isolated. In the wound/swabs samples, *S. aureus* was found to 17.2%, Wound infections are one of the most common hospital acquired infections and are an important cause of morbidity and account for 70-80% mortality **[27, 28]**. In the present study, out of 225 sputum samples, 175 (77.77%) samples grew positive bacterial growth. Out of 175 samples, 140 (80%) different bacteria were isolated. In the sputum sample, the most predominant bacteria found to be *S. aureus* 10.28%. Respiratory tract infection (RTI) is considered as one of the major public health problems and a leading cause of morbidity and mortality in many developing countries **[29]**. It is a global problem accounting for over 50 million deaths of each year and occurs in both community and health care settings **[30]**. The most common bacteria implicated as causative agents of RTIs was included but not limited to *Pseudomonas* spp., *Streptococcus* spp., *Proteus* spp., *Klebsiella* spp., *Staphylococcus* spp., *Enterobacter* spp., *Acinetobacter* spp., and *Haemophilus influenza* **[31]**. Out of 165 samples, 140 (75.15%) different bacteria were isolated. In the CSF sample, the bacteria found in this study was *S. aureus* (13.33%), Meningitis is an inflammation of brain membranes and spinal cord. These membranes are collectively known as meninges and these membranes provide protection against external hazards and various microorganisms **[32]**. The common bacterial etiology reported for acute bacterial meningitis includes *Group B streptococcus, Escherichia coli, Klebsiella spp., Enterococcus spp., Listeria monocytogenes, Streptococcus pneumoniae;* in the age group of one to six months, *S. pneumoniae, Haemophilus influenzae, Neisseria meningitidis, E. coli, L. monocytogenes*; in age group above six months, the prevalent bacterial agents include *Neisseria meningitidis* and *S. pneumoniae* **[33]**.

Antibiotic susceptibility testing of clinically isolated bacteria:

The antibiotic susceptibility was tested by disc diffusion method according

to the Kirby - Bauer method by using Mueller-Hinton Agar [34]. The total 1157 bacteria were tested for their antibiotic susceptibility test. for Gram positive bacteria were Amikacin (30mcg), Amoxicillin (30mcg), Ampicillin (10mcg), Cefaclor (30mcg), Ciprofloxacin (5mcg), Clindamycin (2mcg), Erythromycin (15mcg), Gatifloxacin (5mcg), Gentamycin (10mcg), Linezolid (30mcg), Methicillin (5mcg), Ofloxacin (5mcg), Oxacillin (1mcg), Piperacillin (100mcg), Streptomycin (10mcg), Teicoplanin (30mcg), Tetracylin (30mcg), Tobramycin (10mcg) and Vancomycin (30mcg) The results were interpreted as per Clinical and Laboratory Standard Institute (CLSI) **[14]**.

Table 1: Identification of bacteria on the basis of Morphological, biochemical & Cultural Characteristics

Sr. No.	Test		Interpretation
1.	Morphological Characteristics		
		Gram Staining	Gram Positive
		Motility	Non-motile
2.	Sugar Fermenation		
		Glucose	A+G
		Lactose	A+G
		Maltose	A+G
		Mannitol	A+G
		Sucrose	A+G
3.	IMViC Test	Indole	-
		Methyl Red	-
		VP	+
		Citrate	+
4.	TSI	Acid	+
		H_2S	-
5.	Enzymatic Test	Urease	-
		Oxidase	-
		Catalase	+
6.	Cultural Characteristics		
		MSA	Yellow colonies
		MAC	Colorless colonies
		CLED	Golden Yellow colonies
		BPA	Black colonies

MSA – Mannitol Salt Agar; MAC – MacConkey Agar; CLED – C.L.E.D. Agar; BPA – Baired Parkar Agar

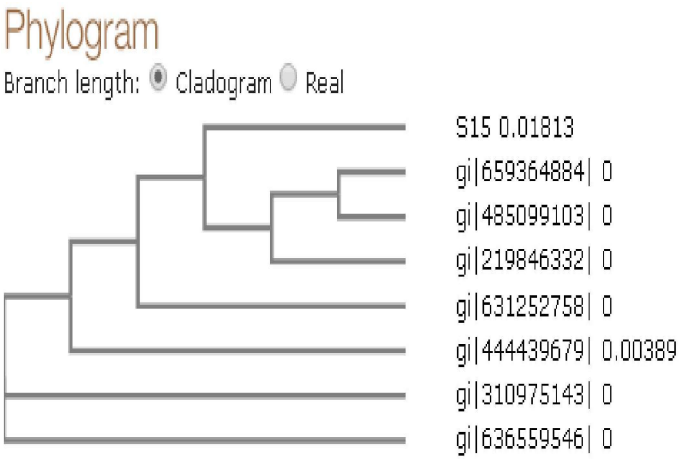
Confirmation of *Staphylococcus aureus* by 16s rRNA

Sr. No.	Sample ID	Identification (BLAST) Percentage	Similarity
1	S15*	*Staphylococcus aureus*	98

S15 (840 bp)

5'GTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC CGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCT GTTATTAGGGAAGAACATATGTGTAAGTAACAGAGCACATCTTGACGGTACCTAATCAGAAAGCCACG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAA AGCGCGCGTAGGCGGAATTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGTT TCTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATAT GGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTCGGAAAGCGTGGGG ATCAAACAGGATTAGATACCCTCCTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTC CGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACT CAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGAAAAATTCGAAGCAACGCGAAGAA CCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGAC AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC CTTAAGCTTAGTTGCCATCATTAAGTTG 3'

Gel Data:



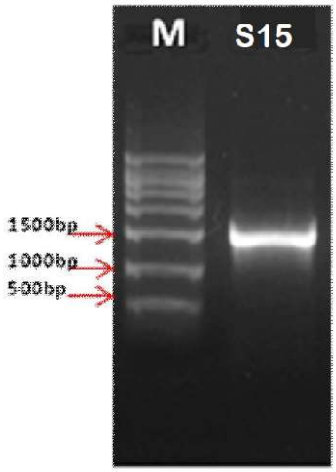


Table 2: Prevalence of *Staphylococcus aureus* in different clinical samples

Sr. No.	Sample	No. of Isolates	Percentage
1.	Urine (n=465)	72	18.79%
2.	Blood (n=325)	46	16.31%
3.	Wound/Pus (n=285)	43	17.2%
4.	Sputum (n=225)	18	10.28%
5.	Cerebrospinal fluid (n=165)	22	13.33%

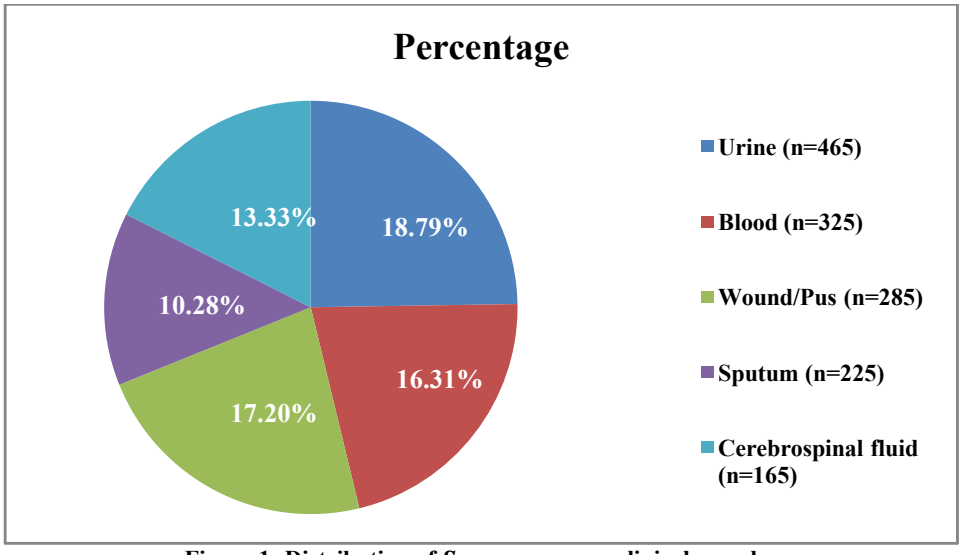


Figure 1: Distribution of *S. aureus* among clinical samples

Table 3: Antibiotic resistance of *Staphylococcus aureus* isolated from various clinical specimens

Sr. No.	Antibiotic Tested	*Staphylococcus aureus* (*n*=201)		
		S	R	%R
1	Amikacin	9	192	95.52
2	Amoxicillin	45	158	78.60
3	Ampicillin	36	165	82.08
4	Cefaclor	48	153	76.11
5	Ciprofloxacin	50	151	75.12
6	Clindamycin	59	142	70.64
7	Erythromycin	44	157	78.10
8	Gatifloxacin	77	124	61.69
9	Gentamicin	3	198	98.50
10	Linezolid	56	145	72.13
11	Methicillin	6	195	97.01
12	Ofloxacin	75	126	62.68
13	Oxacillin	13	188	93.53
14	Piperacillin	91	110	54.72
15	Streptomycin	45	156	77.61
16	Teicoplanin	87	114	56.71
17	Tetracylin	12	189	94.02
18	Tobramycin	27	174	86.56
19	Vancomycin	64	137	68.15

***n* - Number of Isolates; S – Sensitive; R – Resistant; %R – Percentage of Resistance**

On the basis of antibiotic susceptibility test, following multiple drug resistant bacteria were identified.

Table 4: Distribution of MDR pathogens in *Staphylococcus aureus* isolated from clinical samples

Sr. No.	Bacterial Isolates	Total Number	MDR Strains	MDR %
1.	*Staphylococcus aureus*	201	176	87.56%

Table 5: Pattern of multidrug resistance among *Staphylococcus aureus* isolated from clinical specimen

Sr. No.	Pattern of multidrug resistance	Organisms	Number of Strains
	Resistance to 17 antibiotics		
1.	M, VA, OX, LZ, CF, CD, E, TB, S, AK, AC, A, G, T, OF, CJ, TE	*Staphylococcus aureus*	17
	Resistance to 15 antibiotics		
2.	M, VA, OX, CF, E, TB, S, AK, AC, A, G, T, OF, CJ, TE	*Staphylococcus aureus*	23
	Resistance to 13 antibiotics		
3.	M, VA, OX, E, TB, S, AK, A, G, T, OF, CJ, TE	*Staphylococcus aureus*	34
	Resistance to 10 antibiotics		
4.	M, OX, E, TB, S, AK, A, G, T, OF	*Staphylococcus aureus*	37
	Resistance to 9 antibiotics		
5.	M, OX, E, TB, S, AK, A, G, T	*Staphylococcus aureus*	26
	Resistance to 8 antibiotics		
6.	M, OX, E, TB, S, AK, G, T	*Staphylococcus aureus*	39

In the current study, the next most common multiple drug resistant bacteria found to be *S. aureus*. *S. aureus* showed high resistance against Gentamicin (98.50%), Methicillin (97.01%), Amikacin (95.52%), Tetracylin (94.02%), Oxacillin (93.53%), Tobramycin (86.56%), Ampicillin (82.08%) and Vancomycin (68.15%) **(Table 3, Figure 2)**. The values of resistance to drugs such as gentamicin, tetracycline and oxacillin were similar to those described for hospitals in Brazil **[35]** and in Latin America **[36]**. Most of the strains of *S. aureus* were resistant to methicillin. The most common resistance mechanism of *S. aureus* to β-lactams is

penicillinase, which is encoded by the *bla* gene usually carried on a plasmid. The main mechanism of methicillin-resistance is not mediated by penicillinase but the newly acquired PBP2a, encoded by *mecA* gene **[37]**. The finding of the present study was methicillin (97.01%) and oxacillin (93.53%) was highly resistant to *S. aureus*. The prevalence of MRSA varies among different countries and different areas of a country. In a study performed by Alborzi *et al*. in Shiraz, Iran, 33% of all *S. aureus* isolates were reported as MRSA. It appears that MRSA has emerged as an important endemic pathogen in hospitals **[38]**. Fridkin *et al*. (2002) reported a median increase of 2.4% in the prevalence of oxacillin-resistant *S. aureus* in U.S. hospitals from 1996 to 1999 **[39]**. According to the reports, prevalence of MRSA is increasing in Europe. In Austria 21.6%, Belgium 25.1%, Spain 30.3%, and France 33.6% of isolated *S. aureus* strains are methicillin resistant **[40]**. In a survey performed in Pakistan, 61.29% of isolated *S. aureus* strains were resistant to oxacillin **[41]**. Methicillin resistant *Staphylococcus aureus* (MRSA) has emerged as a serious public health problem of global concern. Screening for methicillin resistant isolates in this study showed a prevalence rate of 97.10%. This was however higher than the studies conducted in other areas in Nigeria such as Ilorin **[42]** 34.7% and Jos **[43]** 43.0%. In contrast, the prevalence of MRSA was found to be low in France (6%), Ireland (5%) and United Kingdom (2%) **[44]**. However, a high prevalence of 83% MRSA was reported from Pakistan **[45]**. This confirms the high regional variations in the findings from different countries and cities. Amongst the MRSA in this study, more that 70% were resistant to erythromycin and ciprofloxacin, and more than 80% were resistant to tetracycline, and gentamicin. Regional differences were again common and this was due to the different MRSA clones circulating in Australia. In the 1980s and 1990s multi-resistant strains (later typed as ST239-III or Aus2/3 EMRSA) became epidemic in the eastern Australian states with some spread to hospitals in South Australia, the Northern Territory and Tasmania **[46]**. As a result, MRSA with tetracycline, and gentamicin resistance (characteristic of ST239-III) are rare in Western Australia—less than 3% in this survey. Erythromycin and ciprofloxacin resistance were more widespread in this survey with at least 30% of MRSA with this profile in any region. Erythromycin and ciprofloxacin resistance is common in ST239-III strains but is also characteristic of ST22-IV (EMRSA-15). ST22-IV is a common healthcare-associated MRSA (HA-MRSA) in Australia and is found in all regions **[47]**.

In this study, resistance was also detected for vancomycin, teicoplanin and linezolid. The predominant profile observed in this study just indicated sensibility to vancomycin and teicoplanin, corroborating the fact that these glycopeptides remain as the reference therapy for serious infections caused by multi-resistant Gram-positive strains **[35]**. Lineages with increased resistance to teicoplanin have been reported in Europe and United States **[48]**. Resistance to vancomycin is uncommon, but *S. aureus* with decreased susceptibility have been reported in the United States and Japan **[49]**. However, considering only the isolates of 2004, phenotype B proved to not be the predominant profile, signalling an important change in the resistance profile of the isolates. The resistance rate of MRSA isolates to vancomycin was found to be 68.15% **(see Table 3)**. This result was similar when compared to previous findings from different parts of the world, including Ethiopia. This variation may be due to timely emergence of resistance strains. However, the present study was higher in contrast to report from New York (10%). This finding may be explained by method variation, since assessment of the previous study was in a controlled clinical trial. This result was lower when compared to previous findings from different parts of the world, including Ethiopia. Some reports revealed that there is no MRSA isolate resistance to vancomycin **[50]**. This variation may be due to timely emergence of resistance strains.

The low percentage sensitivity of *S. aureus* observed in the present study against the following drugs; Cefaclor, Gatifloxacin, Ofloxacin and Piperacillin was in agreement with the reports published by some workers **[51]** from Nigeria and another researcher **[52]** in Eritrea. All MRSA isolates encountered in this study were highly resistant to antibiotics, such as Tobramycin and Tetracylin. A similar result was noted for erythromycin among MRSA strains from Trinidad and New York **[53]**.

In this study, high prevalence of multidrug resistant MRSA was observed. This may predispose patients to infection with intractable isolates and emphasizing the need for improved infection control practices and guidelines for use of antibiotics in this setting. Moreover, all MRSA strains isolated in the present investigation were resistant to more than 15 antibiotics tested shown in **Table 5**. In this study, out of 201 *S. aureus* strains, 176 (87.56%) strains were found to be MDR shown in **Table 4**. Arora *et al*. (2010) revealed that, 73% of MRSA strains were multidrug resistant **[54]**.

This indicated that resistant strains were emerged and the emergence of those resistant strains, especially for the most bactericidal anti-MRSA agents, may have further aggravated the emergence of multidrug resistant MRSA, and it may threaten the success of an MRSA control program. The burden of multidrug resistant MRSA is increasing over time **[55]**. The reports of recent studies are implicating the gut as an important reservoir of multidrug resistant *S. aureus* **[56]**. The increased prevalence of MDR-MRSA is due to a lack of sufficient knowledge on the danger of the wrong use of antibiotics, high proximity to a large number of unlicensed drug vendors, high poverty among the people which hinders them from completing the dosage regimen of the antibiotics, widespread and sometimes, the inappropriate use of broad spectrum antibiotics in the medical and the veterinary practice, antibiotic prophylaxis, high number of immune compromised patients, the increased use of invasive procedures and devices and inadequate infection control measures **[57]**.

Characterization of Multiple Drug Resistant (MDR) strains using molecular method: (Genotyping Resistance Testing)

Methicillin - resistant *Staphylococcus* strains possess the ability to grow in the presence of derivatives of β-lactams **[58]**. Methicillin-resistance is transferred to susceptible strains through horizontal transfer of *mecA* gene. The *mecA* gene encodes penicillin-binding protein 2a **[59]** and it is a useful molecular marker of methicillin-resistant strains. Isolates of *S. aureus* that carry *mecA* gene or that produce PBP2a should be reported as MRSA and isolates lacking *mecA* gene or do not produce PBP2a should be reported as MSSA **[60]**. In the presence study, *mecA*, *vanA* and OXA gene present in MRSA strains of *S. aureus*.

The bacteria resistant genes were identified by using primer. The results of primers amplified by PCR as shown in **Figure 3-5**.

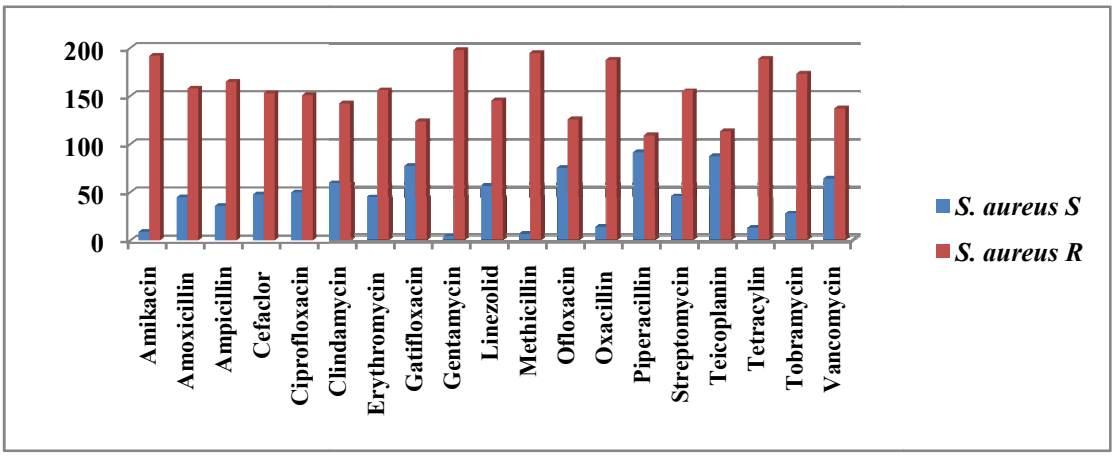


Figure 2: Antibiotic susceptibility testing of *S.arueus*

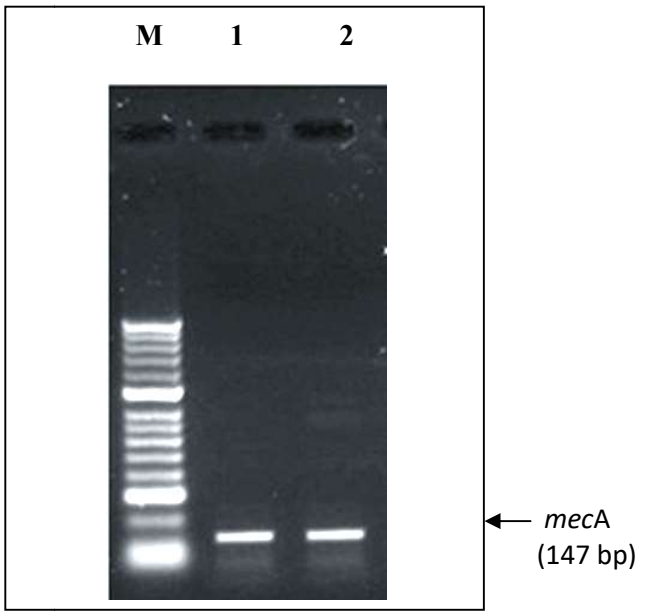


Figure 3: Multiplex PCR detection of *mec*A gene in *S. aureus*
Lane M – Marker; Lane 1 - *S.aureus* isolate; Lane 2 - Standard *mec*A gene

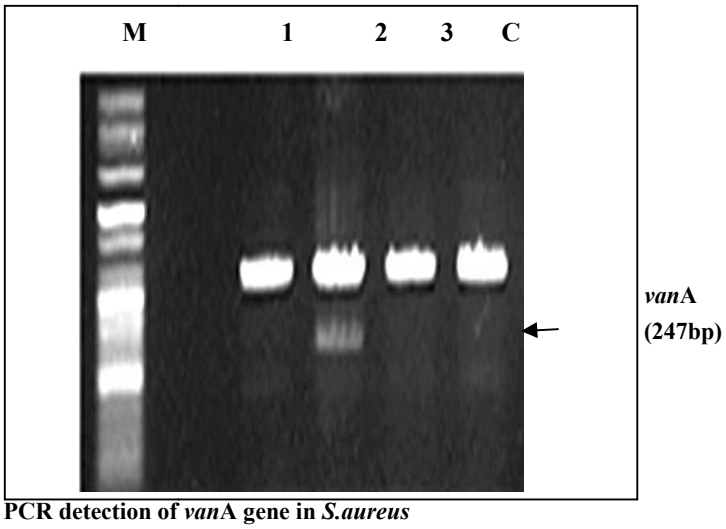


Figure 4: Multiplex PCR detection of *van*A gene in *S.aureus*
Lane M: Marker; Lane 1, 2, 3: *S. aureus* isolate, C – Control *van*A gene

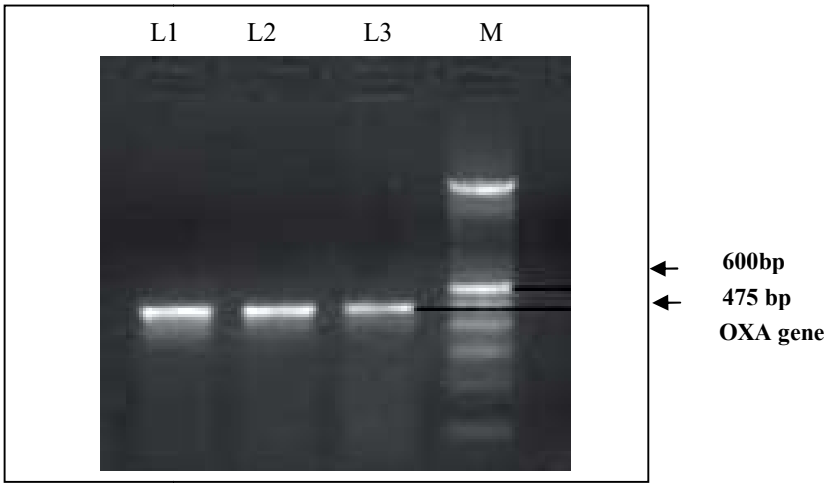


Figure 5: Multiplex PCR detection of OXA gene
Lane M: Marker; Lane 1,2,3 : *S. aureus;*

Table 6: Resistance genotypes in *Staphylococcus aureus* strains

Positive by PCR for ESBL genes	Number amplified
	***Staphylococcus aureus* (n=201)**
Three gene - *mec*A, *van*A, OXA gene	**74 (36.82%)**
Two genes - *mec*A, OXA gene	**102 (50.75%)**
One gene – *mec*A gene	**195 (100%)**

Genomic DNA isolated from 201 phenotypic confirmed MRSA producing organisms (*S. aureus*=201) were subjected to PCR using pairs of primers. All isolates was found to be MRSA positive and these isolates could be typed for one or more genes. Among the 201 isolates, 195 strains of *S. aureus* harbouring single *mec*A gene and the percentage was found to be 97.01%. 102 strains of *S. aureus* showed resistance to two genes *mec*A and OXA gene was found to be 50.75% whereas 74 (36.82%) strains of *S. aureus* showed resistance to three genes - *mec*A, *van*A, OXA gene. The molecular antibiotic resistance analysis of HA-MRSA isolates revealed that HA-MRSA isolates tested positive for the mec*A* gene, vanA and OXA gene. These findings support the report of previous studies in the country that MRSA isolates are much less resistant to non β-lactam antibiotics **[61]**. An increasing number of studies has shown that HA-MRSA isolates from certain areas have become resistant to multiple drugs, which have enabled them to invade hospitals and affect the distribution of MRSA clones **[62]**. A more widespread population study focusing on the collection and screening of HA-MRSA isolates for specific antibiotic resistance genes to detect more than one resistance gene in the country. The current study suggest that surveillance system of hospital acquired MRSA infections especially in healthcare centres in rural communities should be vigorous, timely, and efficient, so as to report their epidemiology in the country.

CONCLUSION

S. aureus was found in average 15.81% in tested urine, blood, pus/wound swan, stool and CSF samples. A diverse array of molecular types was isolated, and antibiotic resistance was common, including methicillin resistance and multi-drug resistant strains. The antibiotic resistance analysis of the isolates revealed that the *mec*A gene was detected in high frequency, whereas the *vanA* and OXA gene antibiotic resistance genes were detected in low frequency.

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There are no conflicts of interest.

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