

RESEARCH ARTICLE

## Genetic Profiling of Methicillin-resistant *Staphylococcus aureus* in An African Hospital by Multiplex-PCR

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### ABSTRACT

**Objectives:** Methicillin resistant *Staphylococcus aureus* (MRSA) nasal carriage often precedes infection. This work aims to genetically profile MRSA carriage of resistant genes (*mecA*, *blaZ* and *tetM*) and certain virulence genes (*nuc*, *pvl*, *spa* and *scn*).

**Methods:** Phenotypically confirmed *Staphylococcus aureus* were Screened for oxacillin resistance and assessed for  $\beta$ -lactamase production. Further confirmation of identity was performed by multiplex PCR of the 16SrRNA, *mecA*, *tetM*, *pvl*, *nuc*, *spa* and *scn* genes. DNA gel fingerprint analysis was carried out and the bands were clustered in a dendrogram.

**Results:** The study analysed 81 *Staphylococcus aureus* isolates for Methicillin resistance out of which 38 (46.9%) were MRSA. The DNA extraction method was effective for all MRSA isolates evaluated. All phenotypic MRSA isolates (100%) were positive for the *S. aureus* specific 16SrRNA gene confirming that these isolates were *S. aureus* strains. The *nuc* gene was detected in 19/38 (50%) of the MRSA isolates, *blaZ* gene in 14/38 (36.8%) and *tetM* in 18/38 (47.4%).

**Conclusions:** The need for improved vigilance to recognize MRSA strains cannot be overemphasized. If MRSA control is not tracked, these strains will increase exponentially. *J Microbiol Infect Dis* 2020; 10(3):144-153.

**Keywords:** Methicillin resistant *Staphylococcus aureus*, PCR simulation, *mecA*, Electrophoretogram

### INTRODUCTION

The hallmark of Methicillin resistant *Staphylococcus aureus* (MRSA) is the proliferation of antimicrobial resistance and MRSA nasal carriage often precedes infection [1]. The feat of this bacterium as a pathogen is directly linked to its range of virulence and immune evasion factors that enable the bacterium to dodge host defenses [2].

MRSA, as we have come to accept, brought about a tumultuous antibiotic's resistance era in hospital and community sceneries worldwide, owing to its increased intransigency to several  $\beta$ -lactam antibiotics engendered by the acquisition *mecA* gene that codes for the production of modified penicillin binding protein 2a (PBP2a) [3].

Nasal secretions are mixtures of proteins, sugars and salts, containing e.g. lysozyme and immunoglobulins (IgA and IgG), as well as defensins and complement proteins [4]. *S. aureus* is resistant to lysozyme due to the cell wall modifying enzyme. Expression of several factors, including staphylococcal protein A (*spa*) and chemotaxis inhibitory protein of *S. aureus* (CHIPS) can limit opsonization by binding to the immunoglobulins, changing conformation bacterial cells to one not recognized by neutrophils. CHIPS and the staphylococcal complement inhibitor (SCIN/*scn*) are innate immune modulators, known to interfere with the complement system [5]. Other virulent factors possessed by this organism includes Hemolysin and Panton-Valentine leucocidin cause killing of white blood cells and red blood cells [5].

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The most common housekeeping genetic marker is the 16SrRNA gene because it is present in all bacteria [6]. PCR amplification from these genetically conserved parts of the bacterial genome is a sturdy procedure for the identification.

Nasal carriage is important for endogenous infections and for transmission to other individuals, as colonization of extra nasal sites often originates from the nasal reservoir. In this regard, special attention was paid to methicillin-resistant (MRSA) which has become a foremost issue during the past three decades [7].

Data on molecular characterization of methicillin resistant *Staphylococcus aureus* of nasal origin is scanty in this part of the country (North West), and there are only few documentations of the situation in Sokoto.

This work aims to genetically profile the MRSA carriage of resistant genes (*mecA*, *blaZ* and *tetM*) and some selected virulence genes (*nuc*, *pvl*, *spa* and *scn*).

## METHODS

### *Staphylococcus aureus* Isolates

This study was approved by Ethics Committee of Sokoto state Ministry of health (SMH/1580/V. IV). Eighty-one phenotypically confirmed *S. aureus* (Microgen Staph ID kit) isolates collected randomly from the anterior nares of Patients and Staff of State-owned hospitals in Sokoto state Nigeria were used in this study.

### Screening for Oxacillin resistance

A standardized suspension (0.5 McFarland) of *S. aureus* isolates were prepared and streaked onto Oxacillin resistance screening agar base (ORSAB) medium pre-supplemented with 6 µg/ml oxacillin and 4% sodium chloride and incubated at 35 °C for 24 hours. The emergent bluish colonies from the overnight cultures were considered methicillin resistant [8].

### Rapid chromogenic test for β-lactamase detection

The production of β-lactamase enzymes was verified by the chromogenic cephalosporin method using nitrocephin impregnated sticks (beta-lactamase stick) (BR0066A, Oxoid® UK) according to the manufacturer's instructions. A

typical pure colony of *S. aureus* was selected from the growth medium. The colonies of interest were touched with the impregnated end of the stick. The stick was then rotated to pick up a minute slice of pure colony and was observed for color changes within 10 minutes up to an hour. A color change from yellow to pink-red indicated positive β-lactamase test [9].

### Primer design

The primer design was based on partial coding sequences of genes of interest obtained from GenBank/NCBI. The open reading frames of these sequences were identified and noted. The regions of the template sequence to be excluded and included in the design were marked and the primers were calculated using Primer3Plus®. The forward and reverse primer sequences obtained were compared with other sequences in NCBI primer blast to rule out overlaps. Primers were designed to nest in the nonhomologous regions, to certify specificity and allow species-specific amplifications; to further verify this, the sequences were evaluated in a virtual PCR simulation before ordering for its production.

### PCR simulation

PCR simulation was executed using Snap Gene™ software (version 1.1.3) to determine the efficiency and the validity of the expected amplicon sizes of the proposed primers before ordering (Assumption-free). The target gene nucleotide sequences were retrieved from Gene bank and used as a matrix on which the chosen primer sequence was evaluated. Upon evaluation, the operational primers yielded oligonucleotides, PCR fragment length and a virtual agarose gel with observed band showing the expected amplicon size [10].

### Primer reconstitution

The received primers were centrifuged briefly (8000 rpm for 30 s) before reconstitution to get all the lyophilized DNA to the bottom of the tube. 100 micromolar of each stock primer was prepared as described by the manufacturer. Furthermore 10 micromolar of the working stock concentration of each primer were prepared by diluting 10 microliters of each stock primer with 90 microliters of nuclease-free water in 1.5ml

nuclease-free microtubes. The primers used in this study is shown in Table 1.

#### **DNA extraction (Lysis, precipitation and purification and elution)**

An overnight culture of each of the *S. aureus* isolates in brain-heart infusion broth was suspended in 1.5 µl Eppendorf tube, centrifuged at 12000 rpm for 5min. The supernatants were discarded into a disinfecting jar and the pellets were re-suspended in a 200 µl nuclease-free water (molecular grade). To the suspension, 20 µl of proteinase K and 200 µl Qiagen™ lysis buffer was added to each tube and vortexed for 15 s and incubated at 56 °C for 20 min. The tubes were then centrifuged and transferred to the Qiagen spin column in a 2 ml collection tube. The whole content of the Eppendorf tubes was transferred into QIAamp™ spin column inserted into 2 ml collection tube, centrifuged at 8000 rpm for 30 s. Five hundred microliter of buffer AW1 was added to the spin column and centrifuged again at 8000rpm for 1 min and the flow through was discarded. 200 µl of buffer AW2 was added and centrifuged at 14000 rpm for 3 minutes. The tubes were further centrifuged for 14000 rpm for 1min to avoid carryover of wash buffer. The spin column was transferred into a freshly labelled 1.5 µl nuclease-free microtubes. To unbind the attached DNA from silica membrane, 100 µl of Qiagen™ elution buffer was added to the silica membrane in the spin column, incubated at room temperature for 5min and centrifuged at 8000 rpm for 1 minute [11]. The flow through (extracted DNA) was capped and stored at -20 °C until required.

#### **Multiplex PCR for 16SrRNA, nuc, blaZ and tetM genes**

PCR was performed using the thermal cycling conditions as stated by Qiagen Research UK [11]. A master mix was prepared in an Eppendorf tube and the total volume was determined by the number of samples to be analyzed.

A PCR cocktail was prepared containing 12.5 µl of Qiagen master mix. In accordance with the manufacturer's instruction (Qiagen), Lyophilized primers were reconstituted using molecular grade water and 0.25 µM of each primer (0.5 µl of each primer pair, forward and reverse) was added to the cocktail. In addition, 3 µl nuclease-

free water and 2.5 µl of Q-reagent was added to the cocktail. Overall, 20 µl of the mixture was then introduced into nuclease-free microtubes (PCR tubes) containing 5 µl of DNA template. The tubes were then transferred into Applied Biosystems® 9700 thermocycler. The machine was programmed thus; an initial denaturation step at 94 °C for 15 minutes followed by 35 cycles of denaturation (at 94 °C for 30 s), annealing (57 °C for 1 minute and 30 s) and extension (72 °C for 2 minutes). The reaction was completed by final extension time of 10 minutes at a temperature of 72 °C.

#### **Multiplex PCR for mecA, pvl, spa and scn**

Multiplex PCR was performed for detection of *mecA*, *pvl*, *spa* and *scn* genes specific using primers shown in Table 3.2. The PCR was performed in a final reaction volume of 25 µL consisting of; 12.5 µl of Qiagen™ master mix, 5 µL of DNA template, 2.5 µL of Q-reagent, 0.5 µL of each primer pair (2 µL entirely) and 3 µl nuclease-free water. DNA was amplified on a thermocycler (Applied Biosystems® 9700), and PCR conditions were as follows: Initial denaturation for 15 min at 94 °C, 30 cycles of denaturation for 10 s at 94 °C, annealing for 30 s at 59 °C, extension for 1 min at 72 °C, and a final extension of 10 min at 72 °C.

#### **Gel Electrophoresis**

The PCR product (Amplicons) was electrophoresed on a 1.5% agarose gel pre-stained with ethidium bromide. A 100 bp plus ladder was loaded in the in the first well using 10x Qiagen™ loading dye [12]. The agarose gel was transferred into Bio-Rad gel documentation device and viewed using a UV trans-illuminator. Gel-lane and band analysis as well as point to point regression analysis of the electrophoretogram was carried out using Bio-Rad™ image lab software version 3.0.

#### **DNA Gel-fingerprint analysis**

Phylogenetic relatedness of MRSA strains was determined by analysis of DNA fingerprints via BioNumerics™ version 7.6 software (Applied Maths, Kortrijk, Belgium). Normalization steps were involved in the analysis to certify suitable gel-to-gel banding pattern contrast. A procedure of "band scoring" identified bands in each lane that combine to make the fingerprint based on

the threshold of stringency and optimization settings. The band positions in the electrophoretograms were standardized for variation from lane-to-lane and gel-to-gel. Subsequent processes were performed using the unweighted pair group method with arithmetic averages (UPGMA) and cluster analysis with dice coefficient (18).

### Statistical analysis

All statistical analysis was conducted with the SAS version 9.4 software package (SAS Institute Inc., Cary, NC, USA).

## RESULTS

We analysed 81 *Staphylococcus aureus* isolates for methicillin resistance out of which 38 (46.9%) were MRSA. The DNA extraction method was effective for all 38 MRSA isolates evaluated, Figure 1A shows electrophoretograms of multiplex PCR (M-PCR) assay for 16SrRNA (956 bp), *nuc* (421 bp), *blaZ* (216 bp), *tetM* (158 bp) genes of 38 MRSA strains. The assay showed (Table 2) that all 38 MRSA isolates (100%) were positive for the *S. aureus* specific 16SrRNA gene, further confirming that these isolates were *S. aureus* strains. The *nuc* gene was detected in 19/38 (50%) of the MRSA isolates, *blaZ* gene in 14/38 (36.8%) and *tetM* in 18/38 (47.4%).

DNA gel finger print analysis was carried out and the bands were clustered in a dendrogram using dice coefficient based on band pattern similarity and intensity with a minimum band tolerance of 3%. As evident in Figure 2A, five clusters with a similarity of 70% were identified. Four profile groups (A, B, C, and E) were the most predominant among the population when compared to group D. Profile A was the most common with 34.2% (n=13) of the isolates belonging to this group and this was followed by profiles A (9.9%, n=8), and B, C with 7.4% (n=6) on each group. Group D was the least populated group with only 5 isolates (6.2%). The profiles of the groups are as follows; A= 16SrRNA + *tetM* /16SrRNA + *nuc* + *tetM* (n=8), B= 16SrRNA+ *blaZ* +*tetM* /16SrRNA+*nuc* + *blaZ* +*tetM* (n=6), C= 16SrRNA + *nuc* /16SrRNA + *nuc* + *blaZ* (n=6), D=16SrRNA + *tetM* /16SrRNA+ *blaZ* + *tetM* (n=5) and E= 16SrRNA only/ 16SrRNA + *blaZ* (n=13).

Figure 1B shows the electrophoretogram of multiplex PCR amplicons *mecA* (533 bp), *pvl* (433 bp), *spa* (350 bp), *scn* (130 bp) for 38 MRSA. The *mecA* gene was detected in 16 isolates, *pvl* in 17 isolates, *spa* 18 isolates and *scn* in 17 isolates. Table 2 summarizes the distribution of amplified genes across the MRSA isolates. The dendrogram clustered five major clades at 70% similarity (Figure 2B). The five clades were designated by A–E. Clade E was the most populated with 26.3% of the isolates and this was followed by profiles A, B, D and C with 23.7%, 18.4%, 18.4% and 10.5% of the MRSA isolates respectively. The profiles of the groups are as follows; Group A = 9 isolates (were positive for *spa*, *spa*+ *scn* only, *pvl* + *scn*), Group B=7 isolates (*mecA*, *mecA*+ *pvl*, + *mecA* +*spa*), C=4 isolates (predominantly *mecA*-*scn* positive isolates), D=7 isolates (in addition to being *mecA* positive, were predominantly *pvl* positive isolates), E=10 isolates (predominantly *pvl* and *spa* positive isolates) and a singleton (*pvl*, *spa*, *scn*).

## DISCUSSION

All 38 MRSA isolates were positive for 16SrRNA genes and 42.1% (16/38) of the MRSA isolates carried the *mecA* gene, with 22 isolates not expressing the *mecA* gene. The 16SrRNA gene, the *nuc* gene and *mecA* gene PCR was used to identify and confirm isolates as MRSA. Elhassan et al [15] explained that the inability to detect *mecA* gene in some studies may not be unconnected with the fact that some *mecA*-containing isolates might have lost the gene on prolong storage and probably due to higher temperatures between the preliminary characterization to the time of final molecular characterization as a result of inconsistent power supply in the environment as supported by some studies.

$\beta$ -lactam resistance is a significant phenotype of MRSA and the amount of  $\beta$ -lactam resistance gene harbored and expressed differ among strains. The *blaZ* gene is a common  $\beta$ -lactam (penicillinase) resistant mechanism [16]. In this study 53.1% of the isolates were beta-lactamase positive, but not all the MRSA were positive for the beta-lactam resistance gene (36.8%). A higher rate of 94.2% has been reported by (20) in contrast with our documentation.

Table 1. Nucleotide sequences of Primer used in PCR.

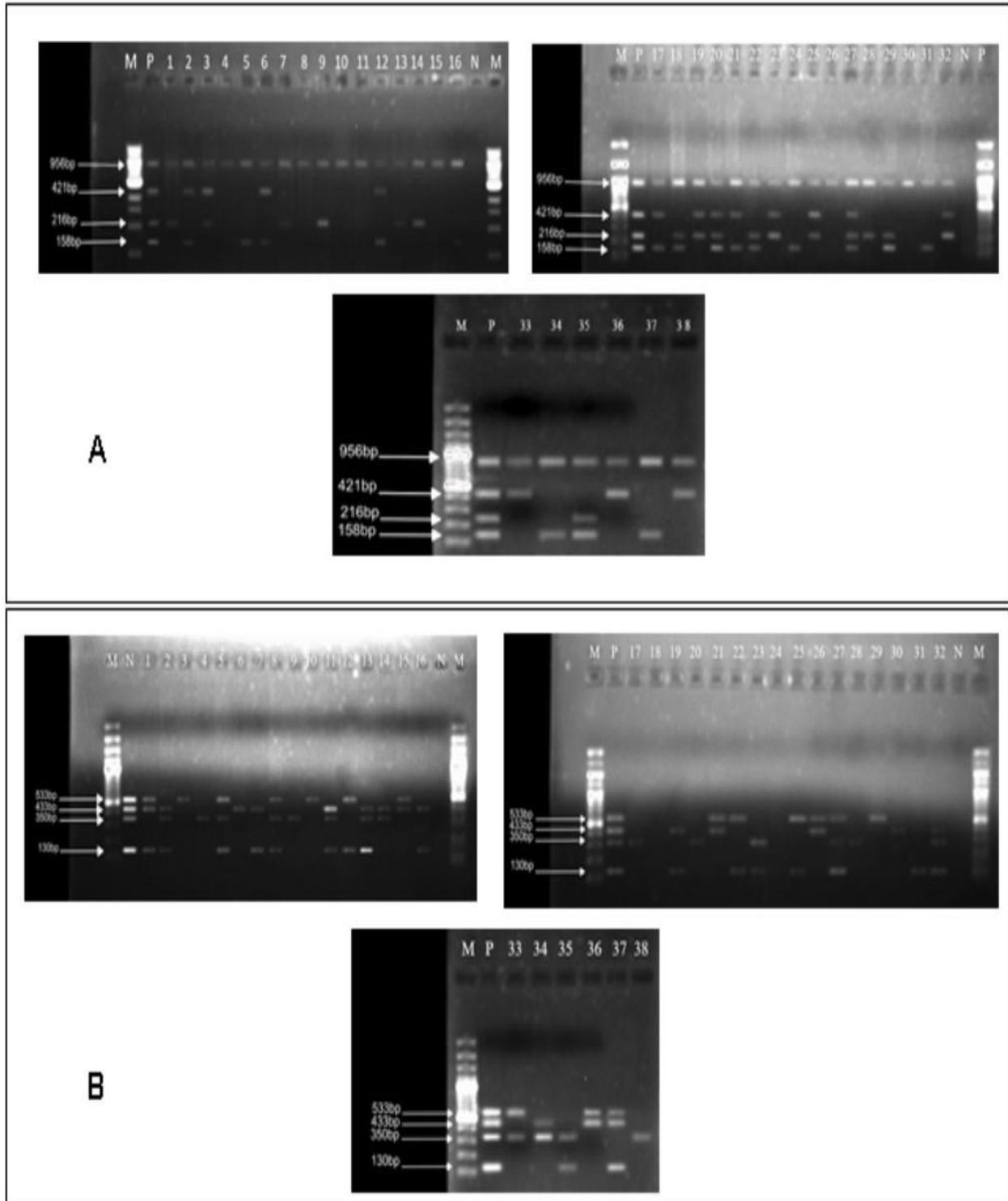
| Gene                                       | Primer         |   | Sequence (5'-->3')     | Product  | Accession |
|--|----------------|---|------------------------|----------|-----------|
| Housekeeping genetic marker                | <i>16SrRNA</i> | F | AGGCAGCAGTAGGGAATCTT   | 956 bp   | LC416382  |
|  |                | R | CCCGTCAATTCCTTTGAGTT   |          |           |
| Thermostable nuclease                      | <i>nuc</i>     | F | GGCATATGTATGGCAATTGTTT | 421 bp   | DQ507381  |
|  |                | R | ATACGCTAAGCCACGTCCAT   |          |           |
| $\beta$ -lactam resistance (penicillinase) | <i>blaZ</i>    | F | GCTTCAACTTCAAAAGCGATAA | 216 bp   | KY020077  |
|  |                | R | TTGATTCCACCGATTTTCGT   |          |           |
| Tetracycline resistance                    | <i>tetM</i>    | F | TCGAGGTCCGTCTGAACTTT   | 158 bp   | AY452663  |
|  |                | R | TGCAAATAACAATTTCTCCAG  |          |           |
| Methicillin resistance                     | <i>mecA</i>    | F | TGGTAAAGTTGGCAAAAAGA   | 533 bp   | KY788636  |
|  |                | R | TTGTCCGTAACCGGAATCA    |          |           |
| Panton Valentine leucocidin (Toxin)        | <i>pvl</i>     | F | TAAGGGCAAACACTTGTGGA   | 433 bp   | HQ020533  |
|  |                | R | CCATTTGATCAAGACGAGCA   |          |           |
| Staphylococcal Protein A (Toxin)           | <i>spa</i>     | F | CTCAAGCACCAAAGAGGAAG   | variable | EF203507  |
|  |                | R | ATGTACTCCGTTGCCGTCTT   |          |           |
| Staphylococcal complement inhibitor        | <i>scn</i>     | F | ACTTGCGGGAACCTTTAGCAA  | 130 bp   | LT992473  |
|  |                | R | GCATACATTGCTTTTTGACCTG |          |           |

Table 2. Distribution of amplified genes across 38 phenotypic MRSA Isolates

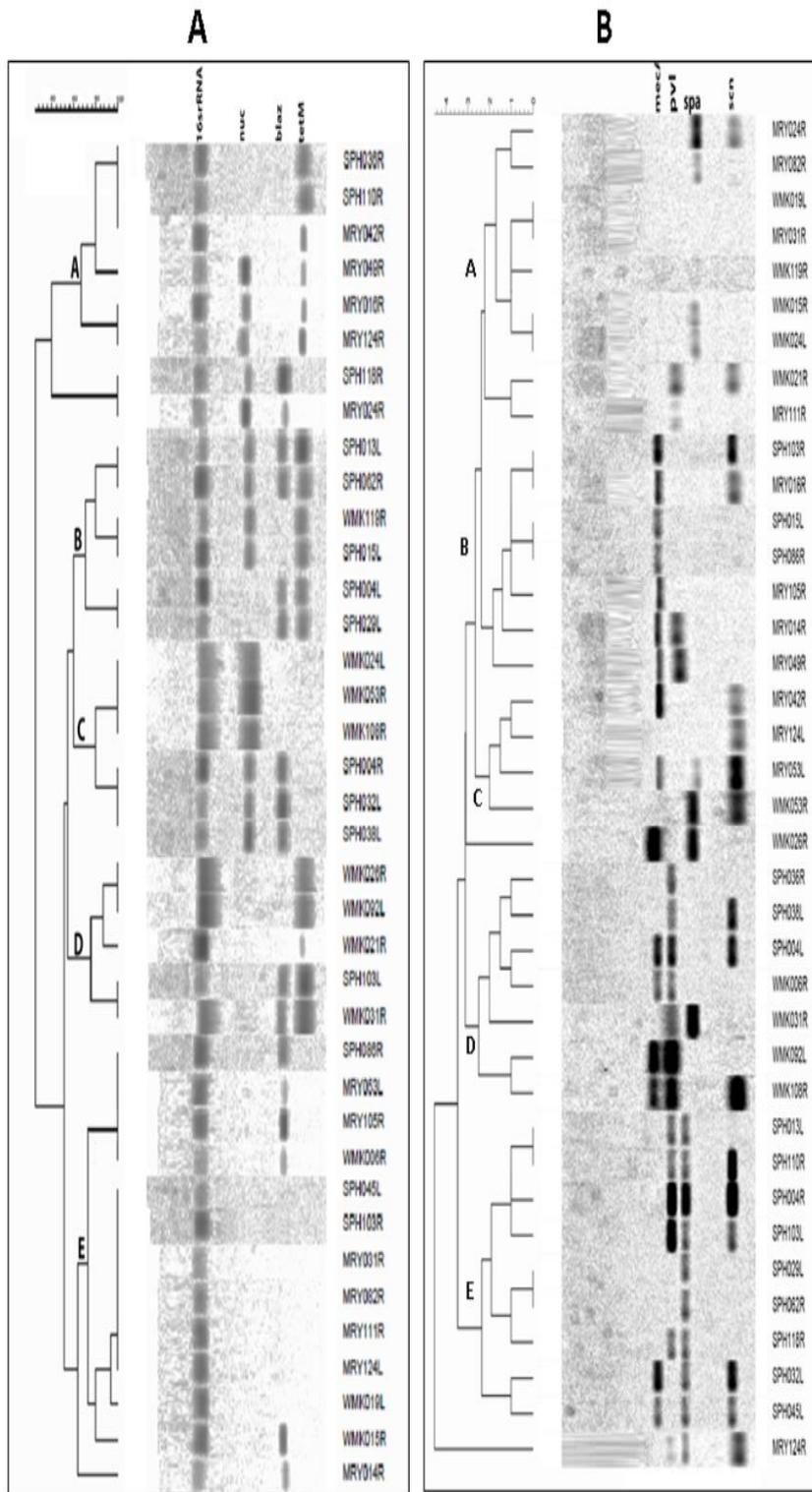
| Amplicons      |          | Frequency (n) | Percentage (%) |
|----------------|----------|---------------|----------------|
| <i>16SrRNA</i> | Positive | 38            | 100            |
| <i>nuc</i>     | Negative | 19            | 50             |
|                | Positive | 19            | 50             |
| <i>blaZ</i>    | Negative | 24            | 63.2           |
|                | Positive | 14            | 36.8           |
| <i>tetM</i>    | Negative | 20            | 52.6           |
|                | Positive | 18            | 47.4           |
| <i>mecA</i>    | Negative | 22            | 57.9           |
|                | Positive | 16            | 42.1           |
| <i>Pvl</i>     | Negative | 21            | 55.3           |
|                | Positive | 17            | 44.7           |
| <i>Spa</i>     | Negative | 20            | 52.6           |
|                | Positive | 18            | 47.4           |
| <i>Scn</i>     | Negative | 21            | 55.3           |
|                | Positive | 17            | 44.7           |

The difference carriage could probably be ascribed to strain diversity difference in laboratory methodologies. The dual presence *blaZ* and *mecA* genes among the strains agree with previous reports that the  $\beta$ -lactam resistance mechanism mediated by *mecA* keeps

the *bla* systems active (23). In this regard, the absence of *blaZ* gene among some MRSA positive strains in this study may be associated to the presence of other variants of the *bla* genes not investigated in this study.



**Figure 1. (A)** Electrophoretogram of ethidium bromide stained gel of 38 Nasal swab isolates showing amplification of *16SrRNA* (956 bp), *nuc* (421 bp), *blaZ* (216 bp), *tetM* (158 bp) Genes for identification and characterization of *Staphylococcus aureus*. In the figure, M is 100 bp+ DNA ladder, P and N is Positive and Negative control. **(B)** Amplification multiplex PCR products of *mecA* (533 bp), *pvl* (433 bp), *spa* (350 bp), *scn* (130 bp) gene for identification of MRSA and its virulence characteristics. In the figure M is 100 bp+ DNA ladder. P and N represents positive and negative controls.



**Figure 2.** Dendrogram representing the genetic relatedness of 38 methicillin resistant *S. aureus* in terms of presence of (A) *16srRNA*, *nuc*, *blaZ* and *tetM* genes (B) *mecA*, *pvl*, *spa*, *scn* amplicons. Strain designations are specified on the right end of the dendrogram. Cluster analyses was performed with dice comparison settings and an unweighted pair group method with arithmetic mean dendrogram type. The scale bar at the top of the dendrogram represents percentage similarity.

Tetracycline resistance genes when expressed, confer resistance by binding to the ribosome and clearing the drug from its binding site. The most studied and prevalent tetracycline resistance determinants are *tetM* and *tetO* [21]. We screened all MRSA strains for *tetM* because the ribosomal protection protein is the most widely distributed of the two and earlier studies have identified it in 80 different genera (39 gram-negative and 41 gram-positive)[22]. Of the screened strains, 47.4% (18/38) was *tetM* positive. This observed rate is consistent with 49% reported by [18] and lesser than 63.6% reported by [23]. The reported rates may be due to the difference in sample population, or increased acquisition of *tetM* in recent years [24]. Dual carriage of *mecA* and *tetM* genes among MRSA isolates reported in this study is less than 57.1% reported by [27]. This may have probably arisen because both genes might be located on the same or associated genetic element.

*scn* is a potent antigen that evokes high antibody titers in *S. aureus*-colonized individuals. It helps the *S. aureus* to invade complement systems of the human immune system. This molecular marker for livestock association was amplified in 44.7% of MRSA isolates in this study. Its absence in 55.3% suggests the possibility of zoonotic origin (Livestock associated MRSA) [26]. A previous study by [27] reported a higher rate of 95% carriage among MRSA isolates.

Panton Valentine leucocidin increases the pathogenicity of *S. aureus* by fast-tracking cell decay and death, thus contributing to morbidity and mortality [28]. *pvl* is used as a marker for community-acquired MRSA. i.e. it differentiates hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA). Reports from various countries show the rise prevalence of *pvl* among MRSA isolates. The high prevalence of the *pvl* (44.7%) gene detected in this study corroborates 48.7% carriage rate reported by a previous study [27]. The carriage of *pvl* gene varies greatly between geographical locations and populations. The reason for the observed difference is largely unknown. The variability in number of virulence genes in this study was not unexpected because

these genes are subjected to variable selective pressures.

In this study, 20 isolates of *Staphylococcus aureus* (52.6%) had no *spa* gene, similar result was observed in a study by [29], in which *spa* gene was absent in 58.3% of patients. The absence could be due to deletion or addition mutations by which nucleotides are inserted or deleted. These sorts of mutation may have hindered the primer used during the PCR reaction circles from binding to the modified nucleotide resulting in a non-*spa* typeable strain[30]. Most proteins are usually bound to the bacterial cell wall; however, during the stationary phase of bacteria growth some *S. aureus* strains have been known to release protein-A into the surrounding medium due to autolysis [30]. This phenomenon may have contributed to prevalent *spa*-negative strains found in this study.

A drawback of our study was the few number of *S. aureus* investigated which may not have given a true picture of various genetic distribution. However, our findings stress the need for improved MRSA surveillance in the study centers.

### Conclusion

We documented genetic methicillin resistance in this study by the successful detection of 16SrRNA and *mecA* gene among phenotypic MRSA isolates. The virulence genes reported here brings to light pathogenic propensities of MRSA. Existence of *tetM* genes confirms that besides being methicillin resistant, the isolates were tetracycline resistant. The results suggest the likelihood that infections caused by MRSA exists in our study center. We cannot overemphasise the need for improved vigilance to recognize MRSA strains. These strains will increase exponentially if we do not control MRSA spread.

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