Evaluation of Molecular Technique with Predictable Methods for Discrimination of Methicillin Resistant \textit{Staphylococcus aureus} in Northwestern Nigeria

*1Kumurya, A. S; 1Adamu, S.H; 2Auta, S.A and 3Saidu A.Y

1Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Bayero University, Kano, Nigeria.  
2Department of Biochemistry, Faculty of Basic Medical Sciences, Bayero University, P.M.B. 3011, Kano- Nigeria.  
3Department of Biological Sciences, Faculty of Science, Federal University Dutse, Jigawa State-Nigeria.

*Corresponding Author: askumurya.med@buk.edu.ng  
Phone: +2347032153561

Abstract:  
Background: Accurate and rapid detection of methicillin-resistant \textit{Staphylococcus aureus} is very important in a clinical laboratory setting to avoid treatment failure.  
Methods: A total of 100 non-duplicate \textit{Staphylococcus aureus} isolates were collected from different human clinical specimens at 8 different health institutions in Northwestern Nigeria. Polymerase chain reaction was used to amplify both the \textit{S. aureus} specific sequence gene and \textit{mecA} gene of the isolates with the amplicon size of 107 and 532 bp. To accelerate the procedure of identification in clinical microbiology laboratories, simple and rapid method for DNA extraction directly from a single colony was employed. The result of the PCR was compared with conventional methods.  
Results: All the isolates (n=100) expressed \textit{S. aureus} specific sequence gene in their PCR products. Only 5 isolates (5.0%) were confirmed as MRSA based on the detection of \textit{mecA} gene. There was a significant different between MRSA identified on conventional and molecular techniques (P < 0.05). The study reports that the prevalence of \textit{mecA} gene in \textit{S. aureus} in Northwestern Nigeria is 5.0% and \textit{mecA} gene detection is a good predictor of methicillin resistance in \textit{S. aureus}.  
Conclusion: On the basis of this finding; establishment of molecular diagnostic laboratory in secondary and tertiary health units is urgently required. The identification of multi-resistant methicillin susceptible \textit{Staphylococcus aureus} (mMSSA) indicates that strict antibiotic and infection control policies are important factors to be considered in health institutions during policy management decision. Control of MRSA infection is essential, and it can be achieved by proper implementation of hospital control measures.  
Key words: \textit{Staphylococcus aureus}, MRSA, PCR, oxacillin, disc diffusion.

I. Introduction

Methicillin-resistance in staphylococci constitutes resistance to all of the \textit{β}-lactam antibiotics and their derivatives. The major mechanism is the acquisition of the \textit{mecA} gene that codes for additional penicillin-binding protein 2a (PBP2a) (Murakami et al., 1991). The phenotypic methods such asbroth micro dilution test for minimal inhibitory concentration (MIC), oxacillin disk agar diffusion (ODD) and oxacillin salt screening test (OSS) are widely used in routine microbiological laboratory (NCCLS, 1993; Kampf et al, 1997; 1998). The problem with phenotypic methods is that they can be influenced by culture condition such as temperature, medium pH and NaCl content in the medium (Sabath, 1982). The \textit{mecA} gene is the gold standard for the detection of MRSA (Dominguez et al., 1997). Several PCR methods have been developed to detect the \textit{mecA} gene (Murakami et al., 1991; Tokue et al., 1992); however, two pairs of PCR primers are most commonly used (Murakami et al., 1991; DelVecchio et al., 1995) but no specific oligonucleotides have been compared.

The expression of the \textit{mecA} gene and the resulting production of PBP2a is regulated by proteins encoded by the penicillinase-associated \textit{blaR1}–\textit{βl} inducer–repressor system and the corresponding genomic \textit{mecR1}–\textit{mec} elements (Tesch et al., 1990; Hackbarth and Chambers, 1993; Sharma et al., 1998). Hiramatsu et al.(1992) identified in \textit{Staphylococcus aureus} N315 the \textit{mecR1}–\textit{mec} regulator element, which is located upstream of the \textit{mecA} gene and is divergently transcribed from \textit{mecA}. The \textit{mecI} gene codes for a repressor protein and the \textit{mecR1} gene for a \textit{β}-lactam-sensing transmembrane signalling protein. Methicillin and oxacillin are, however, not good inducers for this system, often resulting in slow induction of methicillin resistance. Phenotypically susceptible strains, known as pre-methicillin-resistant \textit{S. aureus} (pre-MRSA) and pre-
methicillin-resistant coagulase-negative staphylococci (pre-MRCNS), have been discovered, which do not express methicillin resistance, as mecA is fully repressed by mecI (Hiramatsu, 1995; Weller, 1999). The induction of mecA transcription is very slow and might be due to mutations of mecI (Weller, 1999).

Our purpose was to determine the efficiency of the PCR method with different primers for detecting the mecA gene in MRSA and to compare the sensitivity and specificity of PCR with conventional methods.

II. Materials And Methods

Bacterial isolates

A total of 1692 Staphylococcus aureus isolates (from various clinical samples) was obtained from eight health institutions (Microbiology department) across Northwestern Nigeria. The hospitals were two teaching hospitals [Aminu Kano Teaching Hospital (AKTH) and Ahmadu Bello University Teaching Hospital (ABUTH)], three Federal Medical Centres [Federal Medical Centre Birnin Kudu (FMCB), Federal Medical Centre Gusau (FMCG) and Federal Medical Centre Katsina (FMCK) located in Jigawa, Zamfara and Katsina state in Northwestern Nigeria respectively], two Specialist Hospitals [Murtala Muhammad Specialist Hospital (MMSH) and Specialist Hospital Sokoto (SHS) located in Kano and Sokoto states of Northwestern Nigeria respectively] and the Infectious Diseases Hospital (IDH) located in the city of Kano, Kano State. The isolates were collected for duration of two years from February 2008 to April 2010. The quality control and rejection criteria of specimen were followed (Isenberg, 1998). The isolates were identified using standard microbiological procedures. All isolates were identified as S. aureus based on morphology, positive catalase, positive coagulase and fermentation of mannitol (Kloos and Bennerman, 1995).

Methicillin Disc diffusion (MDD)

Methicillin disk susceptibility testing was performed according to National Clinical Laboratory Standards (NCCLS, 2008; NCCLS, 2003). Briefly a bacterial suspension adjusted to 0.5 McFarland was inoculated onto Muller–Hinton agar. Filter paper disks containing methicillin (5 μg; Becton Dickinson, Heidelberg, Germany) were placed on the inoculated Muller–Hinton agar. All plates were incubated in 35°C for 24 hours. The diameters of zone of inhibition were recorded.

Oxacillin Disc diffusion (ODD)

Oxacillin disk susceptibility testing was performed according to National Clinical Laboratory Standards (NCCLS, 2008; NCCLS, 2003). Briefly a bacterial suspension adjusted to 0.5 McFarland was inoculated onto Muller–Hinton agar. Filter paper disks containing oxacillin (1 μg; Becton Dickinson, Heidelberg, Germany) were placed on the inoculated Muller–Hinton agar. All plates were incubated in 35°C for 24 hours. The diameters of zone of inhibition were recorded.

Methicillin screen agar (MSA)

All isolates were plated on Mueller-Hinton agar with 4% NaCl and 10 mg/l methicillin. Bacteria were inoculated at a final density of 5x105CFU/ml. Oxacillin resistance was confirmed by surface growth after incubation at 35°C for 24 hours.

Oxacillin screen agar (OSA)

All isolates were plates on Mueller-Hinton agar with 4% NaCl and 6 mg/l oxacillin. Bacteria were inoculated at a final density of 5x105CFU/ml. Oxacillin resistance was confirmed by surface growth after incubation at 35°C for 24 hours.

Storage of the isolates

Using sterile swab, the entire growth of an overnight pure culture was sub-cultured in 5ml of sterile glycerol broth and immediately stored in freezer [Micro bank (Diagnostic pro-lab)] at –80°C. After 24 hours the viability of the organism was checked by thawing the suspension at 35°C and inoculated on blood agar plates.

Isolation of Template DNA

Pure culture of Staphylococcus aureus on agar slant was required for molecular analysis of the isolates. Nonviable and mixed cultures were not processed for the molecular analysis. Of the 423 MRSA isolates detected by latex agglutination technique, 100 isolates were randomly selected and used in the molecular analysis with representative from each of the study area. After overnight culture on brain heart infusion (Difco Laboratories) agar plates, one colony of each sample was resuspended in 25 μl of sterile distilled water and the suspension was then placed in a 100°C heat block for 15 min. From this suspension, a 5-μl volume was directly used as a template for PCR amplification (Bignardi et al., 1996; Cavassini et al., 1999; Perez et al., 2001; Anna-Kaarina et al., 2009).
Oligonucleotide primers

The oligonucleotide primers used in this study have been previously described (Martineau et al., 1998; Meshref et al., 2011) and were obtained from a commercial source (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). The 3-end region of the S. aureus specific gene was amplified using a 30 nucleotide forward primer 5'- AATCTTTGTGGTACAGC ATA TTCTTCCAG -3' and A30 nucleotide reverse primer, 5'- CGTAAT GAG ATT TCGAGTA GAT AATAACAACA-3' (which hybridize to 5-34 and (112-83), respectively, (Martineau et al., 1998). While The 3-end region of the mecA gene was amplified using A 22 nucleotide forward primer 5'- AAA ATC GAT GGT AAA GGTT GG C -3' and A22 nucleotide reverse primer, 5'- AGTTCTGGATACCG GAT TTG C -3' (which hybridize to sites 1282-1301 and 1814-1793) (Robert Koch institute, 2003) (Table 1). Staphylococcus aureus specific gene and mecA gene have the amplicon size of 107 and 532 bp using primers described by (Meshref et al., 2011).

Table 1: Oligonucleotide Primers used in the PCR assay

<table>
<thead>
<tr>
<th>Oligonucleotide Position</th>
<th>Nucleotide Sequence</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A30fwd 5:34</td>
<td>AATCTTTGTGGGATACAGCATAATTCTTCCAG</td>
<td>Sa</td>
</tr>
<tr>
<td>A30 rev 112-83</td>
<td>CGTAATGAGATTTCAGTAGATAATACAACA</td>
<td>Sa</td>
</tr>
<tr>
<td>A22fwd 1282-1301</td>
<td>AAAATCGATGTTAAAGCGTTGCC</td>
<td>mecA</td>
</tr>
<tr>
<td>A22 rev 1814-1793</td>
<td>AGTCTCGAAGTACGCGATTTGC</td>
<td>mecA</td>
</tr>
</tbody>
</table>

MecA gene detection by polymerase chain reaction

PCR assays were all directly performed from the bacterial suspension obtained after the rapid DNA extraction method described. An aliquot of 5 μl of this suspension was added to 95 μl of PCR mixture consisting of 1× reaction buffer [16 mM (NH₄)₂SO₄, 67 mMTris-HCl (pH 8.8)], a 0.5 mM concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), 1.0μM of each primer, and mecA primer, and 1.25 U of The Dream Taq™ Green PCR Master Mix (2x) (Fermentas Life Sciences, supplied by Inqaba Biotechnical Industries (Pty) Ltd., South Africa) is a ready-to-use solution containing Dream Taq™ DNA polymerase, optimized Dream Taq™ Green buffer and 4mM MgCl₂. For each sample, one reaction was performed with the pair of primers to identify S. aureus specific sequence gene and with the mecA pairs of primers to detect both resistance gene (mecA). In order to reduce the formation of nonspecific extension products, a hot-start PCR protocol was used; the tubes were placed in the thermal cycler when the denaturing temperature was reached. All PCR assays were carried out with a negative control containing all of the reagents without DNA template. DNA amplification was carried out in a Techne PCR system TC-5000 thermo cycler (Bibby Scientific Ltd.) with the following thermal cycling profile: initial denaturation step at 94°C for 5 min was followed by 30 cycles of amplification this was followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s ending with a final extension step at 72°C for 5 min. After PCR amplification, 5 μl was removed and subjected to electrophoresis (10% agarose, 1x Tris-borate-EDTA, 100 V, 40 min) to estimate the sizes of the amplification products by comparison with a 100-bp O’GeneRuler™ 100 bp molecular size standard DNA Ladder, ready-to-use designed by Fermentas Life sciences (supplied by Inqaba Biotechnical Industries (Pty) Ltd., South Africa) The gel was stained with ethidium bromide, and the amplicons were visualized using a UV lightbox. This protocol, including the rapid DNA extraction method from a single colony and electrophoretic analysis of the amplified products on an agarose gel, was performed in less than 4 hours.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) for windows version 11.0 was used for statistical analysis and data interpretation. The statistical analysis was done using median, averages, ranges, ± standard deviation, chi square, Student’s test and Pearson correlation were applied. The p value ≤ 0.05 was considered as “Statistically Significant".
III. Results

Out of total 423 cases of methicillin resistant strains recovered by conventional techniques, 100 strains were randomly selected and further studied for the detection of mecA gene by PCR technique. The mecA gene was detected from only 5 (5.0%) cases and considered as true MRSA strains. Table 2 shows methicillin resistance among Staphylococcus aureus isolates identified by five techniques. Accuracy of the MRSA detection was based upon mecA detection by PCR technique as a gold standard. The number of false positive MRSA cases was found to be 95(95.0%) isolates for all the remaining five conventional techniques. The difference was statistically significant (P < 0.001).

Table 2: Phenotypic and genotypic methicillin susceptibility testing of Staphylococcus aureus.

<table>
<thead>
<tr>
<th>mecPCR result</th>
<th>No. of isolates tested</th>
<th>Methicillin Diffusion result was:</th>
<th>Oxacillin Diffusion result was:</th>
<th>Methicillin Agar Screen was:</th>
<th>Oxacillin Agar Screen was:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>5</td>
<td>Pos (5)</td>
<td>Neg (0)</td>
<td>Pos (5)</td>
<td>Neg (0)</td>
</tr>
<tr>
<td>Negative</td>
<td>95</td>
<td>Pos (95)</td>
<td>Neg (0)</td>
<td>Pos (95)</td>
<td>Neg (0)</td>
</tr>
</tbody>
</table>

Key: Pos, Positive; Neg, Negative

The isolates of Staphylococcus aureus that carry the mecA gene were reported as methicillin resistant while the isolates that did not carry mecA gene were reported as methicillin susceptible. Only 5 of the 100 Staphylococcus aureus isolates were found to have the PCR amplification of mecA gene demonstrating the expected 532 bp product. Therefore the prevalence rate of MRSA in current study was 5.0 %. The distribution of mecA-positive Staphylococcus aureus according to type of specimen is shown in Table 3. The statistical analysis of the result shows significant difference (p< 0.05) between mecA positive Staphylococcus aureus and mecA negative Staphylococcus aureus isolates.

Among the MRSA strains, three MRSA isolate was detected in wound samples; two from in-patients at the Murtala Muhammad Specialist Hospital (MMSH) and Federal Medical Centre Gusau (FMCG) while the remaining 1 MRSA was isolated from out-patient at Ahmadu Bello University Teaching Hospital (ABUTH). The remaining 2 MRSA isolates were detected in urine samples from out-patients at the Federal Medical Centre Birnin-kudu and Infectious Diseases Hospital (IDH). The overall distribution of Staphylococcus aureus used in the PCR assay according to the study area is given in Table 4.

Table 3: Distribution of Staphylococcus aureus isolates according to the type of specimen

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Staphylococcus aureus isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mecA⁺</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
</tr>
<tr>
<td>Wound swabs</td>
<td>3 (9.7)</td>
</tr>
<tr>
<td>Ear swabs</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Blood culture</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Urine</td>
<td>2 (7.7)</td>
</tr>
<tr>
<td>High vaginal swabs</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Sputum</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Semen</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Urethral swabs</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>5 (5.0)</td>
</tr>
</tbody>
</table>
Mean = 0.285714, SE = 0.285714, SD = 0.755929, CL (95.0%) = 0.699118
Key: meca⁺, meca positive; meca⁻, meca Negative

Table 4: Distribution of Staphylococcus aureus isolates used in the PCR assay according to the study area.

<table>
<thead>
<tr>
<th>Study Area</th>
<th>No. of isolates (%)</th>
<th>No. of meca⁺ (%)</th>
<th>No. of meca⁻ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKTH</td>
<td>17 (17.0)</td>
<td>0 (0.0)</td>
<td>17 (100)</td>
</tr>
<tr>
<td>ABUTH</td>
<td>12 (12.0)</td>
<td>1 (8.3)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>MM2H</td>
<td>26 (2.0)</td>
<td>1 (3.8)</td>
<td>25 (96.2)</td>
</tr>
<tr>
<td>FM2B</td>
<td>13 (13.0)</td>
<td>1 (7.7)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td>FM2G</td>
<td>8 (8.0)</td>
<td>1 (12.5)</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>FM2K</td>
<td>6 (6.0)</td>
<td>0 (0.0)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>ID2H</td>
<td>14 (14.0)</td>
<td>1 (7.1)</td>
<td>13 (92.9)</td>
</tr>
<tr>
<td>SH2S</td>
<td>4 (4.0)</td>
<td>0 (0.0)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>100 (100)</td>
<td>5 (5.0)</td>
<td>95 (95.0)</td>
</tr>
</tbody>
</table>

Mean = 0.714286, SE = 0.184428, SD = 0.48795, CL (95.0%) = 0.451279
Key: Meca⁺, meca positive; Meca⁻, meca Negative

IV. Discussion

Generally, PCR assays are used to detect the meca gene of MRSA. Our aim was to compare the efficiencies of the PCR method with the oligonucleotide primers previously described by Martineau et al., (1998), Meshref et al., (2011) and Mathews et al., (2012) to detect the meca gene for methicillin resistance. The oxacillin screen plate test is the gold standard for the phenotypic method (Frebourg et al., 1998; Kampf et al., 1998). Thus we compared the MDD, the ODD, the MSA and the OSA methods with the meca gene detection. In several studies, most of the highmethicillin resistant strains in the phenotypic methods were meca PCR positive (Tomasz et al., 1989; Dominguez et al., 1997). In this study, however, 95 PCR negative strains for the PCR primer were present among the MRSA strains. This result caused a reduction in the sensitivity and specificity of the conventional methods; however, it might be explained by some other mechanism rather than the absence of the meca gene. Three major mechanisms of resistance have been associated with the resistant phenotype: 1) meca-encoded resistance, 2) over production of penicillinase and 3) modifications of normal penicillin binding proteins. BRSA is generally related to over production of Penicillinase (Franciolli et al., 1991; Lorian, 1996; Santos et al., 1999). The absence of meca gene in the 95 BRSA isolates may be explained by overproduction of penicillinase (Montanari et al., 1990); nevertheless, the mechanism of resistance for the MRSA isolates needs to be clarified. The synthetic oligonucleotide primers of nucleotide 1282 to 1301 and 1814 to 1793 have been used by some researchers (Murakami et al., 1991; Martineau et al., 1998; Louie et al., 2000; Robert Koch institute, 2003; Meshref et al., 2011; Obeseyi O. 2013). Our results show that the primers used had a high correlation with the presence or absence of the meca gene, the level of bacterial resistance to oxacillin, and positive or negative MDD, ODD, MSA and OSA assays. This suggests that the primers used are more appropriate for the detection of the meca gene. Phenotypic expression of methicillin resistance is influenced by temperature, medium pH and NaCl content in the medium (Sabath, 1982) so these factors may also affect correspondence between the presence of the meca gene and phenotypic expression of MRSA as assessed by MDD, ODD, MSA and OSA methods. Our study, however, showed a poor correlation of the presence or absence of the meca gene with ODD, OSS, OSA and MSA assays.

V. Conclusion

On the basis of this finding; establishment of molecular diagnostic laboratory in secondary and tertiary health units is urgently required. The identification of multi-resistant methicillin susceptible Staphylococcus aureus (mMSSA) indicates that strict antibiotic and infection control policies are important factors to be
considered in health institutions during policy management decision. Control of MRSA infection is essential, and it can be achieved by proper implementation of hospital control measures.

Acknowledgements
We thank the management of the eight health institutions for their ethical permission to collect bacterial isolates from their facilities. Our appreciation also goes to the entire staff of Medical Microbiology Laboratories of the various health institutions for their valuable contributions and assistance in the collection of the Staphylococcus aureus isolates.

References