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Evaluation of Molecular Technique with Predictable Methods for Discrimination of Methicillin Resistant *Staphylococcus aureus* in Northwestern Nigeria

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Abstract:
***Background:** Accurate and rapid detection of methicillin-resistant Staphylococcus aureus is very important in a clinical laboratory setting to avoid treatment failure.*
***Methods:** A total of 100 non-duplicate 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 S. aureus specific sequence gene and 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 directlyfrom a single colony was employed. The result of the PCR was compared with conventional methods.*
***Results:** All the isolates (n=100) expressed S. aureus specific sequence gene in their PCR products. Only 5 isolates (5.0%) were confirmed as MRSA based on the detection of 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 mecA gene in S. aureus in Northwestern Nigeria is 5.0% and mecA gene detection is a good predictor of methicillin resistance in 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 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:** Staphylococcus aureus, MRSA, PCR, oxacillin, disc diffusion.*

I. Introduction

Methicillin-resistance in staphylococci constitutes resistance to all of the β-lactam antibiotics and their derivatives. The major mechanism is the acquisition of the 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 byculture condition such as temperature, medium pH and NaCl content in the medium (Sabath, 1982). The mecA gene is the gold standard for the detection of MRSA (Dominguez et al., 1997). Several PCR methods have been developed to detect the mecA gene (Murakami et al., 1991; Tokue et al., 1992); however, two pairs of PCR primers are most commonly used (Murakami et al., 1991; DelVechio et al., 1995) but no specific oligonucleotides have been compared.

The expression of the mecA gene and the resulting production of PBP2a is regulated by proteins encoded by the penicillinase-associated blaR1–blaI inducer–repressor system and the corresponding genomic mecR1–mecI elements (Tesch et al., 1990; Hackbarth and Chambers, 1993; Sharma et al., 1998). Hiramatsu et al.(1992) identified in Staphylococcus aureus N315 the mecR1–mecI regulator element, which is located upstream of the mecA gene and is divergently transcribed from mecA. The mecI gene codes for a repressor protein and the mecR1 gene for a 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 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). Theisolates were identified using standard microbiologicalprocedures. All isolates were identifiedas 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^{o}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^{o}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 35ºC for 24 hours.

Oxacillin screen agar (OSA)

All isolates were plates on Mueller-Hinton agar with 4% NaCl and 6 mg/l oxacillin. Bacteriawere inoculated at a final density of 5x105CFU/ml. Oxacillin resistance was confirmed by surface growth after incubation at 35ºC for24 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^{o}C. After 24 hours the viability of the organism was checked by thawing the suspension at 35^{o}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 30nucleotide forward primer 5'- AATCTTTGTCGGTACACG ATA TTCTTCACG -3' and A30 nucleotide reverse primer, 5'-CGTAAT GAG ATT TCAGTA GAT AATACAACA-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 22nucleotide forward primer 5'- AAA ATC GAT GGT AAA GGTTGG C - 3' and A22 nucleotide reverse primer, 5'-AGTTCTGCAGTACCG 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

Oligonucleotide position	Nucleotide Sequence	Target gene
A30fwd 5-34	AATCTTTGTCGGTACACGATATTCTTCACG	Sa
A30 rev 112-83	CGTAATGAGATTTCAGTAGATAATACAACA	Sa
A22fwd 1282-1301	AAAATCGATGGTAAAGGTTGGC	*mec*A
A22 rev 1814-1793	AGTTCTGCAGTACCGGATTTGC	*mec*A

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 µlof PCR mixture consisting of 1× reaction buffer [16 mM $(NH_4)_2SO_4$,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 4mMMgCl_2. For each sample, one reactionwas performed with the pair of primers to identify S. aureus specific sequence gene and with the mecA pairs of primers to detectboth resistance gene (mecA). In order to reduce the formation of nonspecificextension products, a hot-start PCR protocol was used; the tubeswere placed in the thermal cycler when the denaturing temperaturewas reached. All PCR assays were carried out with a negativecontrol containing all of the reagents without DNA template. DNAamplification 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 by1 cycle 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 afinal extension step at 72°C for 5 min. After PCR amplification,5 µl was removed and subjected to agarose gel electrophoresis(1.5% agarose, 1× Tris-borate-EDTA, 100 V, 40 min) to estimatethe 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 methodfrom 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' 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*.

mecAPCR result	No.ofisolates tested	No. of strains with result indicated							
		Methicillin Disc Diffusion result was:		Oxacillin Disc Diffusion was:		Methicillin Agar Screen was:		Oxacillin Agar Screen was:	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Positive	5	5	0	5	0	5	0	4	0
Negative	95	95	0	95	0	95	0	95	0

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

Specimen type	*Staphylococcus aureus* isolates		
	No. of *mecA*$^+$ (%)	No. of *mecA*$^-$ (%)	Total (%)
Wound swabs	3 (9.7)	28 (90.3)	31 (31.0)
Ear swabs	0 (0.0)	9 (100)	9 (9.0)
Blood culture	0 (0.0)	8 (100)	8 (8.0)
Urine	2 (7.7)	24 (92.3)	26 (26.0)
High vaginal swabs	0 (0.0)	12 (100)	12 (12.0)
Sputum	0 (0.0)	11 (100)	11 (11.0)
Semen	0 (0.0)	1 (100)	1 (100)
Urethral swabs	0 (0.0)	2 (100)	2 (100)
Total	**5 (5.0)**	**95 (95.0)**	**100 (100)**

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.

	***Staphylococcus aureus* isolates**		
Study Area	**No. of isolates (%)**	**No. of *mecA*$^+$ (%)**	**No. of *mecA*$^-$ (%)**
AKTH	17 (17.0)	0 (0.0)	17 (100)
ABUTH	12 (12.0)	1 (8.3)	11 (91.7)
MMSH	26 (2.0)	1 (3.8)	25 (96.2)
FMCB	13 (13.0)	1 (7.7)	12 (92.3)
FMCG	8 (8.0)	1 (12.5)	7 (87.5)
FMCK	6 (6.0)	0 (0.0)	6 (100)
IDH	14 (14.0)	1 (7.1)	13 (92.9)
SHS	4 (4.0)	0 (0.0)	4 (100)
Total	**100 (100)**	**5 (5.0)**	**95 (95.0)**

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 specificityof the conventional methods; however, it might beexplained by some other mechanism rather than the absence of the mecA gene. Three major mechanisms of resistance havebeen associated with the resistant phenotype:1) mec-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 usedhad a high correlation withthe 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 usedare more appropriate for the detection of the mecA gene. Phenotypic expression of methicillin resistanceis influenced by temperature, medium pH and NaCl content in the medium (Sabath, 1982) so these factors may also affect correspondencebetween 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 thepresence 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.

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