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ABSENCE OF *mecA* GENE IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES

[1*]B.O. Olayinka, [2]A.T. Olayinka, [1]A.F. Obajuluwa, [1]J.A. Onaolapo and [1]P.F. Olurinola

[1]Department of Pharmaceutics & Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences,
Ahmadu Bello University, Zaria, Nigeria, [2]Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria
***E-mail:** busayoolayinka@yahoo.com ; +234 803 703 3156

Abstract

Methicillin-resistant *Staphylococcus aureus* has emerged as a serious threat to public health, causing both hospital and community-associated infections. The gold standard for MRSA detection is the amplification of the *mecA* gene that codes for the production of the altered penicillin-binding protein (PBP2a) responsible for classical methicillin resistance. This work determined the nature of methicillin-resistance observed in staphylococcal isolates. *Staphylococcus aureus* isolates with phenotypic resistance to methicillin (oxacillin) were tested for the carriage of the *mecA* gene by multiplex PCR to detect and type the *SCCmec*. The isolates were tested for the production of the altered PBP2a by latex agglutination test and β-lactamase production/hyper production by microplate Nitrocefin assay. None of the isolates hybridized with any of the 16 sets of primers representing the five major *SCCmec* types, nor contained the *mecA* gene; and none was positive for the gene product PBP2a determined by the MRSA screen latex agglutination test. Majority of the isolates 72.2 % (26/36) tested positive for β-lactamase production while 11/26 (42.3%) were β-lactamase hyper producers. The MRSA phenotype observed in the isolates was not the classical *mecA*-mediated resistance, but most probably due to hyper-production of β-lactamase. Reports of loss of the *mecA gene* (believed to be stable) during storage and the fact that all PCR detection of *mec*A gene reported in Nigeria were done outside the country calls for attention on building local capacity for prospective molecular screening for MRSA in clinical and environmental isolates to adequately document their prevalence and monitor the increase. Appropriate guidelines should also be drawn up for the proper screening and reporting of MRSA isolates with the establishment of regional Reference Laboratories.

Key words: MRSA, *mecA*, *Staphylococcus aureus,* penicillin-binding protein, PBP2a.

Introduction

Staphylococcus aureus is a major pathogen both within hospitals and in the community (Stapleton and Taylor, 2002). In the pre-antibiotic era, the rate of mortality from invasive *Staphylococcus aureus* disease was high and the introduction of penicillin had a dramatic impact on treatment (Grubb, 1998). The semi-synthetic penicillin, methicillin was introduced in 1959 to overcome the problems that arose from the increasing prevalence of penicillinase-producing isolates of *Staphylococcus aureus* resistant to penicillin G and penicillin V. (Enright et al., 2002). Today, the whole world contends with methicillin-resistant *Staphylococcus aureus* (MRSA) as a major clinical problem (Benner, and Kaysser, 1968; Lowy, 1998) with many strains resistant to most other classes of antimicrobial agents (Panlilio et al., 1992; Speller et al., 1997).

Strains of *Staphylococcus aureus* resistant to methicillin have been isolated as a frequent pathogen in hospital settings in patients with well-described risk factors (Lowy, 1998). The acronym MRSA is somewhat misleading because the semi-synthetic β-lactam, methicillin is no longer used to treat *S. aureus* infections; MRSA has come to traditionally represent *S. aureus* strains that are resistant to multiple antibiotics and associated with nosocomial infections. (Hiramatsu et al., 2001, Chambers, 1997, Foster, 2004).

The mechanism of methicillin resistance is the possession of an altered penicillin binding protein (PBP2a), that is encoded by the *mecA* gene carried on a mobile DNA element, the Staphylococcal cassette chromosome mec (SCCmec) (Lowy, 2003; Katayama et al., 2000). The *mecA* gene-product, PBP2a is an inducible, 76-78 kDa penicillin binding protein which in MRSA strains substitutes the other PBPs and because of its low affinity for all β-lactam antibiotics enables the organism to survive exposure to high concentrations of this class of antimicrobial agents (Chambers, 1997; Chambers, 2001; Lowy, 2003).

Detection of methicillin-resistance is complicated by the fact that *mecA* expression can either be heterogeneous or homogenous (Chambers, 1997). MRSA strains expressing heterogeneous resistance characterized by a predominantly low-level resistant population co-existing with a small proportion of highly resistant cells, are often misclassified as methicillin-susceptible *Staphylococcus aureus* (MSSA) by most conventional culture methods and thus represent a hidden reservoir in hospitals (Foster, 2004). It has been observed that the heterogeneous expression of methicillin resistance and the slow growth of the resistant bacteria can make detection difficult. Detection using methicillin and oxacillin is therefore aided by carrying out the test at neutral pH; reduced incubation temperature of 33^0C–35^0C; supplementing the agar/broth with 2%-4% NaCl and 24 hr incubation time.

Methodology

Collection of isolates

Suspected Staphylococcal isolates were collected from clinical specimens submitted to the diagnostic Medical Microbiology laboratory of the Ahmadu Bello University Teaching Hospital, Zaria over a cumulative period of 18 months.

Identification of *S. aureus* isolates.

Isolates were inoculated unto Mannitol Salt agar plates and incubated at 37^0C for 24 to 48hrs. Plates were examined for growth of colonies with the characteristic golden colouration. The isolates were characterized by their Gram stain characteristics, catalase test, slide and tube coagulase tests to confirm the *S. aureus* isolates (Baird, 1996).

Determination of methicillin (oxacillin) susceptibility.

Nutrient agar medium containing 5% of sodium chloride (Cookson and Phillips, 1990) was prepared, distributed in 20ml aliquots into bottles and sterilized at I21^{0}C for I5 mins. Overnight cultures of the confirmed *S. aureus* isolates were emulsified in 3ml of Phosphate buffered solution (PBS) and turbidity adjusted to 0.5 McFarland were inoculated unto the NA plates by swabbing. Oxacillin discs, 1µg (Oxoid, UK) were placed on the dried agar plates and incubated for 18 hrs at 35^0C. The diameter of the zones of inhibition were measured and isolates classified as susceptible, intermediate and resistant based on CLIS interpretative chart of zone sizes (Cheesbrough, 2002; CLSI, 2006). Isolates with growth within the zone of growth inhibition were regarded as hetero-resistant strains.

Molecular detection of methicillin-resistance gene

A total of 36 isolates of *Staphylococcus aureus* with phenotypic resistance to methicillin/ (oxacillin1µg) and showing multiple antibiotic resistance (resistance to three antibiotic classes, apart from β-lactams) determined by DAD were selected for analysis and molecular detection of *mecA* gene. The isolates were part of those from specimens submitted to the diagnostic Medical Microbiology laboratory of Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria.

Multiplex PCR for detecting mecA gene and SCCmec type element assignment

Chromosomal DNA was extracted from the isolates by a rapid boiling extraction method (Caddick et al., 2005). Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element assignment was determined for all MRSA isolates using primers and cycle conditions previously described, Table 1 (Oliveira and de Lencastre, 2002). Multiplex PCR was performed using Gene Amp PCR system 9700 (Applied Biosystems, UK) in a 25µl reaction volume comprising of 17.7µl SDW, 2.5µl of 10x primer mix (Table3.1) 0.2µl of 25mM *dNTP*s (Promega, UK), 0.1µl of 1.25 units/µl *Taq* DNA polymerase (Promega, UK) and 2µl template DNA. SCC*mec* band pattern validation was carried out using the positive control strains COL, PER34, N315, ANS46, HU25 and MW2 representing SCC*mec* element types I, Ia, II, III, IIIa and IV respectively. A 2% agarose gel containing 1µg/ml of ethidium bromide was used to separate amplified fragments. Electrophoresis was performed in 1x TAE (40mM Tris, 1mM EDTA and 0.1% ($^{v}/_{v}$) glacial acetic acid) buffer at 100 volts for 1 hr.

Table1: Primers and stock concentrations used in SCC*mec* multiplex PCR (Oliveira and de Lencastre, 2002).

Primer	Locus	Primer sequence (5'-3')	10x primer mix (µM)	Amplicon size (bp)	Specificity (SCC*mec*)
A1	A	TTCGAGTTGCTGATGAAGAAGG	4	495	I, Ia
A2		ATTTACCACAAGGACTACCAGC	4		
B1	B	AATCATCTGCCATTGGTGATGC	2	284	II
B2		CGAATGAAGTGAAAGAAAGTGG	2		
C1	C	ATCAAGACTTGCATTCAGGC	4	209	II, III, IIIa
C2		GCGGTTTCAATTCACTTGTC	4		
D1	D	CATCCTATGATAGCTTGGTC	8	342	I, II, IV, Ia
D2		CTAAATCATAGCCATGACCG	8		
E1	E	GTGATTGTTCGAGATATGTGG	2	243	III, IIIa
E2		CGCTTTATCTGTATCTATCGC	2		
F1	F	TTCTTAAGTACACGCTGAATCG	4	414	III, IIIa
F2		GTCACAGTAATTCCATCAATGC	4		
G1	G	CAGGTCTCTTCAGATCTACG	8	381	II, Ia
G2		GAGCCATAAACACCAATAGCC	4		
H1	H	CAGGTCTCTTCAGATCTACG	8	303	III
H2		GAAGAATGGGGAAAGCTTCAC	4		
MEC1	*mecA*	TCCAGATTACAACTTCACCAGG	8	162	Internal
MEC2		CCACTTCATATCTTGTAACG	8		control

Detection of PBP2a by MRSA Screen Latex agglutination test

Detection of PBP2a was carried out using a slide agglutination kit (MRSA-Screen test Denka Seiken Co Ltd. Japan) and performed according to the manufacturer's instructions. EMRSA-15 was used as a *mec*-positive control strain and Oxford *Staphylococcus aureus* NCTC 8325 as a *mec*-negative control strain. The sample colonies were taken from a fresh BHI agar plate and re-suspended in 4 drops (approximately 200µl) of extraction reagent no.1 (0.1M NaOH) and boiled for 3 min. Following boiling 1 drop (approximately 50 µl) of extraction reagent no.2 (0.5M KH_2PO_4) was aliquoted into the suspension mix and vortexed for 30 seconds. The sample was then centrifuged at 1,500 × g for 5 min at room temperature. A 50 µl sample of the test isolate supernatant, the positive-control and the negative-control was placed on the provided slides. One drop (approximately 25 µl) of anti-PBP2a monoclonal antibody-sensitized latex was added to each sample for the positive control and one drop (approximately 25 µl) of negative-control latex was added to each sample for a negative control. The slides were rotated by hand for 3 mins and characteristic agglutination indicated a positive result for PBP2a production.

Determination of β-lactamase hyper production by Microplate Nitrocefin assay

The 1mg lyophilized Nitrocefin powder (Oxoid, UK) was reconstituted in 1.9ml of 0.1M phosphate buffer, pH7 supplied by the manufacturer. The reconstituted Nitrocefin was further diluted 1in 10 with PBS to give 50µg/ml solution. The disrupted cell preparations were used immediately by dispensing 50µL of preparation into separate wells of a 96 well plate. 50µL of diluted Nitrocefin solution was added into each of the wells and incubated at 37^0C for 10mins. In the presence of β-

lactamase, the chromogenic nitrocefin substrate changes colour from yellow to pink/red. A positive control was set up with MRSA-15.

Results

The staphylococcal isolates from clinical and environmental samples were made up of 254 (75.8%) *Staphylococcus aureus*, out of which a total of 139(54.7%) were resistant to methicillin as determined by DAD method on Nutrient agar medium containing 5% of sodium chloride. Twenty-seven isolates, representing 8.0% yielded hetero-resistant strains that were able to grow within the zone of growth inhibition. The distribution of the MRSA isolates by source is shown in Figure 1. Most of the isolates were from wound (42%), urine (15%) and urethral swab (11%).

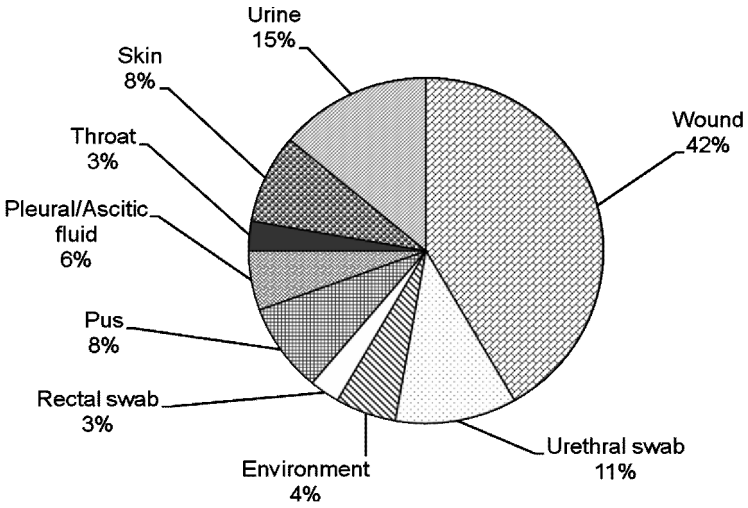

Figure1: Distribution of MRSA isolates by source.

Multiplex PCR for detecting mecA gene and SCCmec type element assignment

All the isolates were *mecA*-negative since there was no amplification of target DNA primers in the PCR run (Plate 1).

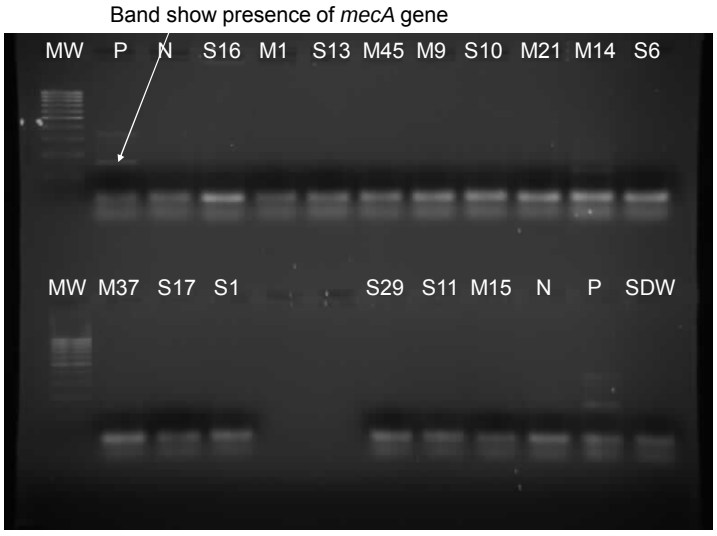

Plate 1: Multiplex PCR on isolates for *SCCmec* type assignment. P=Positive control, N=Negative control, MW=Molecular weight marker, SDW=Sterile distilled water.

Detection of PBP2a by MRSA Screen Latex agglutination test

The presence of the *mecA* gene product PBP2a was determined in isolates by the MRSA Screen Latex agglutination test kit (Denka Seiken Co Ltd. Japan).
None of the isolates were PBP2a-positive, since there was no characteristic agglutination except in the positive control, EMRSA-15 (Plate 2).

Detection of β-lactamase hyper-production.

Hyper-production of β-lactamase in isolates was detected by the intensity of the colour change in the Microplate Nitrocefin assay compared with the positive control (MRSA-15). A total of 26 out of the 36(72.2%) isolates tested produced β-lactamase giving the positive colour change from yellow to pink within the 10mins of incubation time. Intense colour change greater than control was observed in 11/36 (30.6%) of isolates.

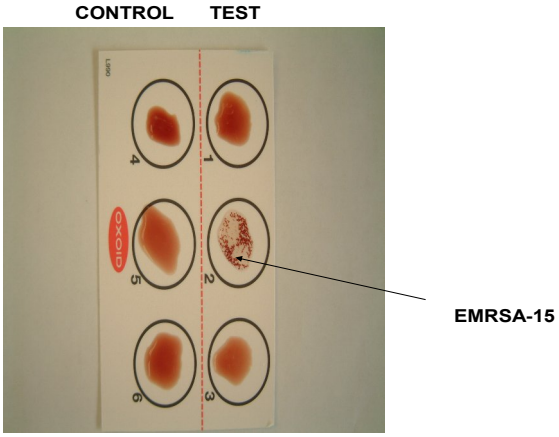

Plate 2: MRSA Screen Latex agglutination test result shows agglutination in the *mecA*-positive control (MRSA-15).

Discussion

The distribution of MRSA by source in this study is consistent with various studies that identified wounds (surgical/burns), urine and pus/abscess as important sources of MRSA isolation (Daxboeck et al., 2004; Orrett and Land, 2006).

Determination of methicillin (oxacillin) susceptibility of isolates by the DAD method revealed a worrisome 54.7% resistance rate. The rate of MRSA in this study is higher than those reported by other investigators in the country. In a survey on the prevalence of MRSA in eight African countries and Malta, Kesah et al. (2003) reported the highest rate of 29.6% for Lagos University Teaching Hospital, Nigeria. Taiwo et al. (2004) reported a rate of 34.7% for Ilorin; Olonitola et al (2007) reported a rate of 33.3% from a Federal Medical Centre and Ikeh (2003) had a rate of 43% for Jos.
Onanuga et al. (2006) reported a higher rate of 71.1% from urine of healthy women while Ikeh and Yakeu (2006) reported an alarming 92.6% MRSA out of the *S. aureus* isolated from bacteria flora on the hands of nursing service workers.

A common factor in all these studies is the method of detecting MRSA; the DAD susceptibility test against 5µg methicillin or 1µg oxacillin, which is generally regarded as less specific in detecting classical methicillin resistance mediated by *mecA* gene that codes for the production of an additional penicillin binding protein, PBP2a or PBP2' which confers resistance to all β-lactam antibiotics (Brown et al., 2005).

Methicillin resistance isolates with alterations to the existing PBPs have been described (Chambers et al, 1989; Montanari et al., 1990; de Lencastre et al., 1991) and they are termed moderately resistant *S. aureus* (MODSA). Under some test conditions it has also been reported that isolates which produce large amounts of penicilinase (β-lacatmase hyper producers) show low level

resistance to oxacillin and have been referred to as Borderline oxacillin-resistant *S. aureus* (BORSA) (Chambers et al., 1989). The clinical importance of MODSA and BORSA strains had been doubtful, since there had been no treatment failures with penicillinase resistant penicillins (Thauvin-Eliopoulos et al., 1990; Hirano and Bayer, 1991).

Although the disc diffusion method remains the most widely used in routine clinical laboratories, it has been suggested that oxacillin (methicillin) be replaced by cefoxitin, a more potent inducer of *mecA* expression, that is less affected by test conditions and hyperproduction of penicillinase (Brown et al., 2005). With the cefoxitin disc becoming increasingly available, it is hoped that this will eventually replace oxacillin (methicillin) in clinical laboratories and research work on MRSA prevalence.

The gold standard for detection of MRSA is the polymerase chain reaction, PCR that detects the *mecA* gene (Berger Bächi and Roher, 2002) or alternatively detecting the *mecA* gene product PBP2a by latex agglutination tests.

All the 36 isolates selected for molecular characterization by the amplification of the genetic determinant of methicillin resistance, *mecA* gene by the multiplex-PCR typing of the *SCCmec* element that carries the gene revealed that none of the isolates carried the *mecA* gene.

Most reports of MRSA in Nigeria have been based on the DAD test results (Taiwo et al., 2004; Ikeh, 2003; Onanuga et al., 2006; Ikeh and Yakeu, 2006). The reports of molecular detection of MRSA have been limited and had different rates for MRSA detected by DAD test and amplification of the *mecA* gene.

Adesida et al. (2005) reported that of the 276 *S. aureus* isolates tested, 26/276 (9.4%) seemed to be MRSA based on zone of inhibition diameter but only 4/26 (1.4%) carried the *mecA* gene and contained the *mecA* gene product, PBP2a determined by MRSA-Screen latex agglutination test. Shittu et al. (2006) found 3% of isolates resistant to oxacillin but only 3/200 (1.5%) were confirmed to be MRSA by PCR detection of the *mecA* gene. None of the isolates in this study produced the altered PBP2a. The question is what then is the nature of the methicillin resistance observed in these and other isolates in this study? Various other mechanisms have been adduced for observed methicillin resistance in *S. aureus* (Chambers, 1997; Brown et al., 2005). Strains of *S. aureus* with reduced susceptibility to penicillinase-resistant-penicillins (PRP) have been categorized as follows;

- 'Classical' MRSA which produce the altered, low-affinity PBP2a, encoded by the *mecA* gene
- The borderline (low-level)-methicillin resistant strains of *S. aureus* (BORSA) generally considered as being due to hyper-production of type-A-β-lactamase. These BORSA strains are *mecA*-negative, PBP2a-negative
- Strains with modified 'normal' PBPs due to altered binding capacity or over-production of PBPs. These strains are termed MODSA.

A total of 26/36 (72%) of isolates tested by the microplate Nitrocefin assay produced β-lactamase while 11/36 (30.6%) hyper-produced β-lactamase. The methicillin-resistance observed in the *mecA*-negative, PBP2a-negative *S. aureus* strains in this study may therefore be attributable to the hyper-production of β-lactamase in only 30.6% of the isolates tested. This can definitely not explain the high rate of phenotypic MRSA detected in this study.

The *SCCmec* element containing the *mecA* gene was generally believed to be stable after incorporation into the strain. However, Griethysen et al. *(*2005) reported the loss of the *mecA* gene during storage of 36/250 (14.4%) confirmed MRSA strains at -80^{0}C with the MicroBank system (Pro-Lab Diagnostics, Canada). The molecular characterization of isolates in this study as well as the two known reports (Adesida et al., 2005; Shittu et al., 2006) were all carried out abroad which would have meant collecting the isolates over a period of time, preliminary characterization, storing and sub-culturing over a considerable length of time before final transportation for molecular characterization. It is therefore not impossible given our power situation that some classical *mecA*-containing isolates might have lost the gene on prolonged storage at temperature much higher than -80^{0}C.

Conclusion

The phenotypic methicillin-resistance observed in staphylococcal isolates by the DAD test was not due to the production of the altered PBP2a encoded by the *mecA*-gene. The β-lactamase hyper-production observed in some of the isolates might have been responsible for their 'masquerading' as MRSA. However, the multiply resistance nature of these MSSA strains and the ability of *Staphylococcus aureus* to take up the *mecA*-gene, calls for continual surveillance for MRSA and the drawing up of guidelines for the prompt, effective and reliable detection of MRSA in resource limited setting like ours. MRSA has emerged as an important nosocomial and community-associated pathogen with constantly changing epidemiology worldwide. There is nothing more international as

infection and there are no barriers to a multidrug resistant pathogen carried across borders by man. Though classical *mecA*-mediated MRSA seems not to be a problem yet in our hospitals, now is the time to build local capacity in understanding and dealing with this clinically important pathogen with worldwide public health importance.

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