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Full Length Research Paper

Loss of the *meca* gene during storage of methicillin-resistant *Staphylococcus aureus* isolates in Northwestern Nigeria

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Preservation of strains in a microbiology laboratory is of great importance for quality control, teaching, and research. Freezing is a very common method of preservation and storage of microorganisms. The evaluation of new diagnostic or *in vitro* antimicrobial susceptibility tests for methicillin-resistant *Staphylococcus aureus* (MRSA) requires well-defined strain collections. The study was aimed at determining whether loss of *mec*A gene in MRSA is related to the storage method. A total of 1692 non-duplicate *S. aureus* isolates were collected from different human clinical specimens at 8 different health institutions in Northwestern Nigeria from February, 2008 to April, 2010. All the isolates were screened for methicillin resistance using disc diffusion method (DDM), screen agar method (SAM) and latex agglutination techniques (PBP). Thereafter, the isolates were stored in 16% v/v glycerol broth at -80°C. In December, 2011, the isolates were retested by polymerase chain reaction (PCR) which was used to amplify both the *S. aureus* specific sequence gene and *mec*A gene of 100 isolates, with the amplicon size of 107 and 532 bp. The prevalence rate of MRSA on DDM, SAM, and PBP were 26.3, 24.2 and 25.0%, respectively. Surprisingly, the *mec*A gene was lost in 95.0% of 100 MRSA isolates after 2 years of storage at −80°C with the Micro bank system (Pro-lab Diagnostics, Austin, Tex.). This study demonstrates that *mec*A can be lost from MRSA strains stored at −80°C with the Micro bank system. This finding has important implications for the management of strain collections and is of use for all future biobanking projects.

Key words: *Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA), *mec*A gene, storage, human, Northwestern Nigeria.

INTRODUCTION

Resistance of staphylococci to methicillin and all β-lactam antibiotics is associated with the low affinity of a penicillin-binding protein, PBP2a, which is not present in susceptible staphylococci. (Hartman and Tomasz, 1984; Pierre et al., 1990; Chambers, 1987, 1997, 1999; Muhammad et al., 2006). This protein is encoded by the *mec*A gene, which is located in the *mec* region and which is DNA of foreign origin (Matsuhashi et al., 1986).

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 *mec*R1–*mecI* elements (Tesch et al., 1990; Hackbarth and Chambers, 1993; Sharma et al., 1998; Muhammad et al., 2006). Hiramatsu et al. (1992) identified in *Staphylococcus aureus* N315 the *mec*R1–*mec*I regulator element, which is located upstream of the *mecA* gene and is divergently transcribed from *mec*A. The *mecI* gene

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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 *mec*A is fully repressed by *mec*I (Hiramatsu, 1995; Weller, 1999). The induction of *mec*A transcription is very slow and might be due to mutations of *mec*I (Weller, 1999).

The distribution of *mec* regulator genes among methicillin-resistant *Staphylococcus* strains from various countries has already been studied by hybridization and sequencing, which showed that the loss or inactivation of the *mec*I gene leads to derepression of *mec*A gene transcription (Suzuki et al., 1993; Shimaoka et al., 1994; Hiramatsu, 1995; Weller, 1999). *In vitro* amplification of DNA by polymerase chain reaction (PCR) is a rapid and sensitive method for the detection of specific DNA sequences and requires fewer cells than DNA hybridization protocols (Tokue et al., 1992; Unal et al., 1992). The method has already been applied for the identification of *mecA*-positive strains directly in clinical specimens or in DNA extracts (Tokue et al., 1992; Ubukata et al., 1992). The present study was aimed at determining whether loss of *mec*A gene in MRSA isolates can be related to the storage method.

MATERIALS AND METHODS

Bacterial isolates

A total of 1692 consecutive non-duplicated *S. aureus* isolates were obtained from clinical samples in 8 health institutions (Microbiology department) across Northwestern Nigeria. 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). *S. aureus* ATCC 25923 was used as a reference control organism. All confirmed *S. aureus* isolates were stored in 16% v/v glycerol broth at -80°C.

Disc diffusion method (DDM)

Methicillin disk susceptibility testing was performed according to National Clinical Laboratory Standards (NCCLS, 2003, 2008). Briefly, a bacterial suspension adjusted to 0.5 McFarland was inoculated onto Muller-Hinton agar. Filter paper disks containing 5 µg methicillin and 1 µg oxacillin (Becton Dickinson, Heidelberg, Germany) were placed on the inoculated Muller-Hinton agar using sterile forceps. All plates were incubated in 35°C for 24 h. The diameters of zone of inhibition were recorded.

Screen agar method (SAM)

All isolates were plated on Mueller-Hinton agar with 4% NaCl and 10 mg/l methicillin or 6 mg/l oxacillin. The isolates were inoculated at a final density of 5 × 105 CFU/ml. Methicillin resistance was confirmed by surface growth after incubation at 35°C for 24 h.

Latex agglutination assay (PBP)

Mastalex ^{TM}MRSA kit (Mast diagnostics, UK), a commercially available *S. aureus* agglutination test kit was used for the latex agglutination test. The MRSA screen test is a slide latex agglutination test based on the reaction of latex particles sensitized with monoclonal antibodies against penicillin binding protein 2a (PBP2a or PBP2') of *S. aureus* and PBP2a (a product of *mec*A gene) extracted from tested colonies (Muhammad et al., 2006). The test kit was used according to manufacturer's instruction. Autoagglutination reactions in the negative control were excluded from calculations.

Storage of the isolates

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

DNA extraction method

Pure culture of *S. 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; Perez et al., 2001; Anna-Kaarina et al., 2009).

Oligonucleotide primers

The oligonucleotide primers used in this study have been previously described (Martineauf 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 (chromosomal DNA) was amplified using A 30 nucleotide forward primer 5'- AAT CTT TGT CGG TAC ACG ATA TTC ACG -
3' and A30 nucleotide reverse primer, 5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3' (which hybridize to 5-34 and (112-83), respectively (Martineauf 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 GGT TGG C - 3' and A22 nucleotide reverse primer, 5'- AGT TCT GCA GTA CCG GAT TTG C-3' (which hybridize to sites 1282-1301 and 1814-1793) (Robert Koch institute, 2003) (Table 1). *S. aureus* specific gene and *mecA* gene have the amplicon size of 107 and 532 bp using primers described by Meshref et al. (2011).

*Mec*A gene detection by polymerase chain reaction

PCR assays were all directly performed from the bacterial suspension obtained after the rapid DNA extraction method earlier described. An aliquot of 5 µl of this suspension was added to 95 µl of PCR mixture consisting of 1× reaction buffer [16 mM $(NH_4)_2SO_4$,

Table 1. Methicillin resistance among *Staphylococcus aureus* isolates using conventional methods.

Parameter	Strains isolated Methods used for detection of MRSA		
	DDM	SAM	PBP
Number of isolates (%)			
Methicillin Resistant *S. aureus* (MRSA)	445 (26.3)	415 (24.2)	423 (25.0)
Methicillin Susceptible *S. aureus* (MSSA)	1251 (73.9)	1283 (75.3)	1269 (75.0)

$X^2 = 3.14$, df = 4, P = 0.534.

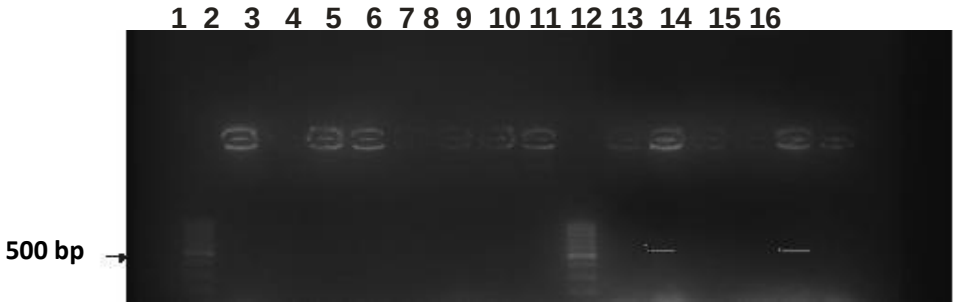

Figure 1. Representative of agarose gel electrophoresis of PCR products. Lanes 12, 14 are positive for *mec*A as indicated by 532 bp PCR product, lane 2 to 9,11,13, 15, are negative for *mec*A, Lane 16: negative control (methicillin susceptible *S. aureus,* ATCC 25923); lane 1,10: molecular weight size marker.

67 mM Tris-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), 1.0 µM of each primer, and *mecA* primer, and 1.25 U of The Dream Taq™ Green PCR Master Mix (2×) (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 4 mM $MgCl_2$. 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 resistance gene (*mec*A).

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 thermocycler (Bibby Scientific Ltd) with the following thermal cycling profile: an initial denaturation step at 94°C for 5 min was followed by 30 cycles of amplification. Each cycle consisted of the following steps; 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 agarose gel electrophoresis (1.5% agarose, 1× 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 ultra violet (UV) light box. 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 h.

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".

RESULTS

The prevalence of methicillin resistant among 1692 *S. aureus* isolates in disc diffusion method (DDM) and screen agar method were 26.3 and 24.2%, respectively as shown in Table 1. Four hundred and twenty three *S. aureus* isolates [(were found to be resistant to methicillin by detection of *mec*A product, PBP2a (PBP')] using latex agglutination technique. Ninety five (22.5%) and fifty seven (13.5%) of the 423 MRSA isolates did not grow on oxoid mannitol salt agar (MSA) and mast MSA media without oxacillin, respectively. The difference was statistically significant (P < 0.001).

Out of total 100 isolates of methicillin resistant strains recovered by the conventional techniques, the *mec*A gene was detected from only 5 (5.0%) of the 100 MRSA strains. The size of the amplicon for the mec*A* gene was expected to be 532 base pairs (bp). An amplicon of 532 bp was seen in only 5 of the 100 isolates tested. PCR amplification of *mec*A gene demonstrating amplicon of 532 bp products are given in Figure 1. Table 2 shows the

Table 2. Measurement of specificity and sensitivity of different methods used.

mecA	No. of isolates tested	No. of strains with result indicated					
		PBP		DDM		SAM	
		Pos	Neg	Pos	Neg	Pos	Neg
Positive	5	5	0	5	0	4	0
Negative	95	95	0	95	0	96	0

Pos: Positive; **Neg:** negative.

measurement of specificity and sensitivity of different methods used. Surprisingly, 95 (95.0%) of the 100 isolates did not harbor the *mec*A gene. The *mec*A gene was lost in 95.0% of 100 methicillin-resistant *S. aureus* isolates after 2 years of storage at −80°C with the Micro bank system (Pro-lab Diagnostics, Austin, Tex).

DISCUSSION

The presence of the *mec*A gene is considered the hallmark for identification of MRSA strains and can be difficult to distinguish using phenotypic methods because of the possibility of missing some resistant strains using standard phenotypic susceptibility testing methods due to heterogenous phenomena. This phenomenon was recognized soon after the discovery of MRSA; that in cultures of most strains only a proportion of cells (usually one 10^3 to 10^6) were highly resistant to methicillin while majority expressed resistant level at or near those of susceptible strains such as heterogenecity of resistance characterized of MRSA in contrast to almost all other bacteria (Chambers, 1997; Hiramatsu et al., 2001).

Though *mec*A gene is responsible for phenotypic behaviour of methicillin resistance in this part of the world, it s noteworthy that the loss of *mec*A gene there is methicillin resistant *S. aureus* during storage. This result indicates the acquisition of *mec*A gene is responsible for methicillin resistance; concurring with the previous studies on the role of *mec*A gene in developing high methicillin resistance (Hartman and Tomasz, 1984). In addition to presence of *mec*A gene in MRSA, PBP2a and *ica* gene cluster can also encode resistant in MRSA (Cramton et al., 1999; Memmi et al., 2008). In this study, out of the 100 isolates, 5 (5.0%) of the isolates were confirmed as MRSA by the detection of *mecA* gene. This low level of *mec*A gene detection can be explained due to loss of the *mec*A gene during storage methicillin-resistant *S. aureus* strains at −80°C as studied by Griethuysen et al. (2005) where they found 14.4% MRSA isolates lost *mec*A gene during storage after two years. Loss of the *mec*A gene in such a large percentage of MRSA isolates during storage at −80°C with the Microbank system has been described by Hurlimann et al. (1992) where they described the apparent loss of the *mecA* gene in methicillin-resistant *S. aureus* isolates stored as lyophilized cultures. However, they did not confirm the presence of the *mec*A gene at the time the isolates were stored; therefore, it is not certain that all isolates carried the *mec*A gene to start with (Hurlimann et al., 1992).

Loss of the *mec*A gene has also been observed *in vivo* (Lawrence et al., 1996; Deplano et al., 2000). Katayama et al. (2000) demonstrated that the SCC*mec*, which contains the *mec*A gene, can be integrated to and excised from the *S. aureus* chromosome. However, spontaneous excision of the SCC*mec* did not occur appreciably in the strain that was examined (Katayama et al., 2000). The presence of the *mec*A gene is considered the hallmark for identification of MRSA strains and can be difficult to distinguish using phenotypic methods since heterogenous phenomena may compound the possibility of missing some resistant strains using standard phenotypic susceptibility testing methods. This phenomenon was recognized soon after the discovery of MRSA where cultures of few strains were highly resistant to methicillin while majority expressed resistant level at or near those of susceptible strains such as heterogenecity of resistance which is a characteristic of MRSA in contrast to almost all other bacteria (Hiramatsu, 1995; Chambers, 1997).

Preservation of strains in a microbiology laboratory is of great importance for quality control, teaching, and research (Harbec and Turcotte, 1996). Freezing is a very common method of preservation and storage of microorganisms (Aulet et al., 2001). Studies mainly concentrate on the viability of the microorganisms after a certain storage period. Little attention is given to the influence of storage conditions on characteristics of the stored strain such as antimicrobial susceptibility. The Microbank bacterial preservation system (Pro-lab Diagnostics) is a well-known system for freezer storage of all kinds of microorganisms and is used in laboratories all over the world. One of the issues that remain is whether loss of the *mec*A gene is related to the storage method; this cannot be concluded that the *mec*A gene was lost during storage at −80°C. This study can therefore demonstrate that *mec*A can be lost from MRSA strains stored at −80°C with the Micro bank system. This has important implications for the management of strain collections. Prior to the use of MRSA isolates that have

been previously stored at −80°C in any study, they have to be checked for the presence of the *mec*A gene at that moment in time. Maybe storage of MRSA strains can be improved by altering the storage conditions by, for example, the addition of oxacillin to the cryopreservative. This needs to be evaluated in future studies.

Conclusion

This study demonstrates that *mec*A can be lost from MRSA strains stored at −80°C with the Micro bank system. This finding has important implications for the management of strain collections. On the basis of this finding, attention should also be given to the influence of storage conditions on characteristics of the stored strains such as antimicrobial susceptibility of the microorganisms after a certain storage period.

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