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 meca 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 meca 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 meca 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 meca 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), meca 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 meca 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 mecR1–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 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).

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 gene leads to derepression of mecA 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 mecA 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 at 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™ 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 (PBPa or PBP2') of S. aureus and PBPa (a product of mecA 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 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 CT TGT CGG TAC ACG ATC TTC ACG - 3’ and A30 nucleotide reverse primer, 5’-CGT AAT GAG ATT TCA GTA GAT AAT ACA A3’- (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).

MecA 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 x reaction buffer [16 mM (NH₄)₂SO₄,
Table 1. Methicillin resistance among *Staphylococcus aureus* isolates using conventional methods.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Strains isolated</th>
<th>Methods used for detection of MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDM</td>
<td>SAM</td>
</tr>
<tr>
<td>Number of isolates (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin Resistant <em>S. aureus</em> (MRSA)</td>
<td>445 (26.3)</td>
<td>415 (24.2)</td>
</tr>
<tr>
<td>Methicillin Susceptible <em>S. aureus</em> (MSSA)</td>
<td>1251 (73.9)</td>
<td>1283 (75.3)</td>
</tr>
</tbody>
</table>

\[\chi^2 = 3.14, \text{df} = 4, \text{P} = 0.534.\]

**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 *mecA* product, PBP2a (PBP')] using latex agglutination technique.

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 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 *mecA* product, PBP2a (PBP')] using latex agglutination technique.

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".
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been d-
uring storage at −80°C with the Microbank system has
mec
mec
al. (2005) where they found 14.4% MRSA isolates lost
S. aureus
loss of the
low level of
confirmed as MRSA by the detection of
out of the 100 isolates, 5 (5.0%) of the isolates were
(Cramton et al., 1999; Memmi et al., 2008). In this study,
addition to presence of
methicillin resistance (Hartman and Tomasz, 1984). In
influence of storage conditions on characteristics of the
microorganisms (Aulet et al., 2001). Studies mainly
concentrate on the viability of the microorganisms after a
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 heterogeneity of resistance
characterized of MRSA in contrast to almost all other
bacteria (Chambers, 1997).

DISCUSSION

The presence of the mecA 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
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bacteria (Chambers, 1997; Hiramatsu et al., 2001).

Though mecA gene is responsible for phenotypic
behaviour of methicillin resistance in this part of the
world, it s noteworthy that the loss of mecA gene there is
methicillin resistant S. aureus during storage. This result
indicates the acquisition of mecA gene is responsible for
methicillin resistance; concurring with the previous
studies on the role of mecA gene in developing high
methicillin resistance (Hartman and Tomasz, 1984). In
addition to presence of mecA 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 mecA gene detection can be explained due to
loss of the mecA 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
mecA gene during storage after two years. Loss of the
mecA 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 mecA gene at the time the isolates were
stored; therefore, it is not certain that all isolates carried
the mecA gene to start with (Hurlimann et al., 1992).

Loss of the mecA gene has also been observed in vivo
(Lawrence et al., 1996; Deplano et al., 2000). Katayama
et al. (2000) demonstrated that the SCCmec, which
contains the mecA gene, can be integrated to and
excised from the S. aureus chromosome. However,
spontaneous excision of the SCCmec did not occur
appreciably in the strain that was examined (Katayama et
al., 2000). The presence of the mecA 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 heterogeneity
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 mecA gene is related to the storage
method; this cannot be concluded that the mecA gene
was lost during storage at −80°C. This study can
therefore demonstrate that mecA 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

| Table 2. Measurement of specificity and sensitivity of different methods used. |
|--------------------------|-----------------|-----------------|-----------------|
| mecA                      | No. of isolates tested | No. of strains with result indicated |
|                          |                  | PBP Pos | PBP Neg | DDM Pos | DDM Neg | SAM Pos | SAM Neg |
| Positive                  | 5                 | 5      | 0      | 5       | 0       | 4       | 0       |
| Negative                  | 95                | 95     | 0      | 95      | 0       | 96      | 0       |
| Pos: Positive; Neg: negative. |                |        |        |         |         |         |         |
been previously stored at −80°C in any study, they have to be checked for the presence of the mecA 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 mecA 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 organisms such as antimicrobial susceptibility of the microorganisms after a certain storage period.

ACKNOWLEDGMENTS
Sincere gratitude to the management of the eight health institutions that participated in the study for their ethical permission to collect bacterial isolates from their facilities. Sincere 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 S. aureus isolates.

REFERENCES
Chambers HF (1987). Coagulase-negative staphylococci resistant to beta-lactam The study was aimed at determining whether loss of mecA gene in methicillin-resistant Staphylococcus aureus (MRSA) is related to the storage method.


