Malfunction of Agglutination Test to Identify Methicillin-Resistant \textit{Staphylococcus aureus} Strains (MRSA)

Kumurya A. S.*

Department of Medical Laboratory Science, Faculty of Allied Health Sciences, Bayero University, Kano, Nigeria

Abstract

\textit{Background}: Most routine laboratory detection of \textit{Staphylococcus aureus} isolates is based on rapid agglutination test systems. Failure of agglutination assays to identify methicillin-resistant \textit{S. aureus} strains (MRSA) has been demonstrated. \textit{Objectives}: The aim of this study was to evaluate the sensitivity and specificity of MRSA detection by agglutination test system using Mastalex\textsuperscript{TM} MRSA kit (Mast diagnostics, UK). The test kits used in this study have been tested for this purpose before. \textit{Methods}: As determined by Polymerase chain reaction, 100 MRSA strains staphylococci were included. Species identification and determination of susceptibility patterns were performed using colony morphology, Gram stain, catalase testing, tube coagulase testing, DNAase testing, mannitol fermentation, susceptibility testing towards oxacillin, and PCR of the \textit{mecA} gene. \textit{Results}: Among the 100 methicillin resistant \textit{Staphylococcus aureus} isolates tested oxacillin disc diffusion and latex agglutination technique, PCR of the \textit{mecA} gene confirmed the identification of only 5 (0.5\%) MRSA strains. Sensitivity of the agglutination tests ranged from 82·7 to 100·0\% for MRSA strains and 92·8 to 100·0\% for MSSA strains, respectively. Specificity of the test systems ranged from 91·3 to 99·1\%. None of the six agglutination assays produced correct reactions for all staphylococci tested. For the other tests kits, sensitivity of MRSA detection was lower than for MSSA isolates. 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. All the 423 MRSA isolates grew on oxoid blood agar with or without oxacillin. \textit{Conclusion}: Depending upon the local MRSA prevalence and the parameter of interest (sensitivity or specificity), these test systems may be useful for routine diagnostic purposes.

Keywords

Agglutination, MRSA, Sensitivity, Specificity, PCR

1. Introduction

\textit{Staphylococcus aureus} strains possessing \textit{mecA} produce an altered penicillin binding protein 2 (PBP2) and this altered enzyme, with reduced affinity for beta lactam drugs, is referred to as PBP2a. Strains carrying the \textit{mecA} gene may express it homogenously with all cells in the population being resistant or other strains express it heterogeneously with only a few cells producing sufficient PBP2a to be resistant. The Clinical and Laboratory Standards Institute (CLSI) states that the oxacillin MIC and cefoxitin disk test are equivalent in sensitivity and specificity for detection of \textit{mecA} mediated resistance in \textit{Staphylococcus aureus} (Cavassini et al., 1999).

Unlike coagulase-negative staphylococci (CNS), \textit{Staphylococcus aureus} strains are able to secrete free plasma coagulase, which is an important virulence factor for these bacteria. \textit{S. aureus} is a common pathogen in nosocomial infections, so exact identification of \textit{S. aureus} isolates is
essential for microbiology laboratories (Emori and Gaynes, 1993). The proportion of clinical *S. aureus* isolates that are resistant to methicillin (MRSA) has increased over recent years (Tiemersma et al., 2004). Compared with methicillin-sensitive *S. aureus* (MSSA), infections by MRSA strains are associated with increased morbidity and mortality in affected patients (Cosgrove et al., 2003).

Today, the gold standard for *S. aureus* identification is proof of free plasma coagulase in the tube coagulase test (Bannerman, 2003). However, confirmation of *S. aureus* by this method may take as long as 24 h. Rapid *S. aureus* agglutination tests have been developed as an alternative for routine diagnosis (Essers & Radebold, 1980). In these test systems, particles precipitate with one or multiple *S. aureus* surface antigens, and allow *S. aureus* and CNS isolates to be distinguished within a few seconds. Bacteria identified as *S. aureus* by these tests are then further checked for meticillin resistance. Unfortunately, the accuracy of these test systems is limited. In particular, MRSA may be easily misclassified in agglutination tests because of false-negative results (Aldridge et al., 1984; Brakstad et al., 1993; Croize et al., 1993; Fournier et al., 1989; Lairscey & Buck, 1987; Piper et al., 1988; Ruane et al., 1986; Winblad & Ericson, 1973), which might be due to changes in various surface components, such as capsular polysaccharides, the clumping factor or protein A (Fournier et al., 1987, 1989; Kuusela et al., 1994). However, correct identification of MRSA is essential for appropriate treatment strategies and sufficient infection control measures for the prevention of nosocomial infections (Muto et al., 2003).

The aim of this study was to evaluate the sensitivity and specificity of MRSA detection by agglutination test system using Mastalex™MRSA kit (Mast diagnostics, UK). The test kits used in this study have been tested for this purpose before.

## 2. Methods

### 2.1. Source of Bacterial Strains

A total of 100 Methicillin resistant *Staphylococcus aureus* (MRSA) isolates were obtained from clinical samples in the health institutions (Microbiology laboratories) across Northwestern Nigeria. The isolates were collected for duration of four months from January to April 2010. In addition, a strain ATCC 25923 (MRSA) was used as controls.

### 2.2. Genus Determination for *S. aureus*

MSSA and MRSA strains were included in this study if all of the following four criteria had been fulfilled: (i) typical morphology of colonies (Bannerman, 2003) on 5% Columbia sheep blood agar, (ii) Gram stain showing Gram-positive cocci in clumps, (iii) at least one positive reaction in the tube coagulase test evaluated after 2, 4 and 24 h (bioMérieux) and (iv) detection of the DNase-positive *S. aureus* strains. As it is known that there are DNase-negative (Menzies, 1977) or catalase-negative (Tu and Palutke, 1976) *S. aureus* strains and some *S. aureus* strains fail to grow on mannitol salt agar (Kampf I et al., 1997), we did not exclude strains with these characteristics.

### 2.3. 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.

### 2.4. Determination of Methicillin Resistance

After species determination, all *S. aureus* isolates were differentiated by susceptibility towards oxacillin. For confirmation of the antibiotic susceptibility phenotype, all *S. aureus* isolates were then genotyped by PCR. A PCR for detection of the mecA gene that encodes the penicillin-binding protein-2 was performed, as it is considered to be the ‘gold standard’ for identification of meticillin resistance in staphylococci (Wallet et al., 1996).

### 2.5. 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.

### 2.6. Latex Agglutination Assay

Mastalex TMMRSA 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 mecA gene) extracted from tested colonies (Muhammad et al., 2006). The test kit was used according to manufacturer’s instruction. Auto agglutination reactions in the negative control were excluded from calculations.
2.7. 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. 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).

2.8. 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’- AAT CT TGT CGG TAC ACG ATA TTT TCC - 3’ and A30 nucleotide reverse primer, 5’-CGT AA T GAG ATT TCA GTA GAT ACA ACA-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 GGT TGG C - 3’ and A22 nucleotide reverse primer, 5’- AGT TCT GCA GTA CCG GA T 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).

2.9. PCR Detection of MecA Gene

Assays for the *Staphylococcus aureus* specific and mecA genes were performed simultaneously using a multiplex PCR. Until further processing in 2% agarose gel electrophoresis, all PCR products were stored at 4°C. PCR products were detected by ethidium bromide stain.

2.10. Statistical Analysis

Assuming a *P* value $\leq 0.05$ for statistical significance, it was calculated that 115 isolates in the *S. aureus*. Statistical analysis of results was performed using 2×2 tables (Epi Info 6). The McNemar $\chi^2$ test was used to assess significant differences between agglutination test kit and the results of oxacillin disc diffusion test and PCR for detection of the mecA gene.

3. Results

Among the 100 methicillin resistant Staphylococcus aureus isolates tested oxacillin disc diffusion and latex agglutination technique, PCR of the mecA gene confirmed the identification of only 5 (0.5%) MRSA strains as shown in Table 2. 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. All the 423 MRSA isolates grew on oxoid blood agar with or without oxacillin (Table 3). The difference was statistically significant (*P* < 0.001). All agglutination reactions with colonies from blood agar were positive within the 3 minutes specified by the manufacturer of the Mastlex TMMRSA kit used.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Position</th>
<th>Nucleotide Sequence</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>A30 fwd5-34</td>
<td></td>
<td>AATCTTTGTCGTAACAGATATTCTTCACACG</td>
<td>Sa</td>
</tr>
<tr>
<td>A30 rev112-83</td>
<td></td>
<td>CGTAATGAGATTTTCAGTATAATACAAAAC</td>
<td>Sa</td>
</tr>
<tr>
<td>A22 rev 1282-1301</td>
<td></td>
<td>AAAATCGATGGTAAAGTTGTCG</td>
<td>mecA</td>
</tr>
<tr>
<td>A22 fwd 1814-1793</td>
<td></td>
<td>AGTTTCTGACGTACCGGATTTCG</td>
<td>mecA</td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotide Primers used in the PCR assay.

<table>
<thead>
<tr>
<th>mecA PCR result</th>
<th>No. of isolates tested</th>
<th>Latex</th>
<th>Oxacillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>5</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>Negative</td>
<td>95</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Key:
*Pos*= Positive
*Neg*= Negative

Table 2. Phenotypic and genotypic methicillin susceptibility testing of *Staphylococcus aureus* (n=100). No. of strains with result indicated.
4. Discussion

Routine microbiology laboratories need to perform rapid and accurate pathogen identification. For this purpose, several S. aureus agglutination test systems have been developed and are widely used. In most routine laboratories, MRSA detection is based on prior S. aureus species determination. Further rapid differentiation between MSSA and MRSA may then be performed by MRSA-specific agglutination test systems (Cavassini et al., 1999). Reliable S. aureus species determination is therefore essential as a first step. Evaluation of S. aureus agglutination test kits has to take into account the phenomenon that species misclassification is more likely to occur for methicillin-resistant strains (Aldridge et al., 1984; Brakstad et al., 1993; Croize et al., 1993; Fournier et al., 1989; Lairscey & Buck, 1987; Piper et al., 1988; Ruane et al., 1986; Winblad & Ericson, 1973). MRSA prevalence has increased worldwide and great variation in MRSA prevalence is seen in different countries (Tiemersma et al., 2004), so the rate of non-detectable MRSA isolates in laboratories influences the PPV and NPV of S. aureus agglutination tests. This is very important, because MRSA-positive patients, in particular, require special infection-control measures according to official guidelines in order to prevent interpatient spread (Muto et al., 2003).

Wichelhaus et al. (1999) calculated sensitivity for MRSA agglutination of 99.4% using PFGE-typed MRSA strains in the Dry Spot Staphytect Plus® test kit. Macro restriction profiles revealed 90 different genotypes among the 181 MRSA strains that they used. Their results are confirmed by our findings. The data presented in this study showed an MRSA sensitivity of 100% in 52 non-identical MRSA isolates.

The Mastalex™MRSA kit (Mast diagnostics, UK) test identified 0.5% of MRSA correctly in our panel of strains. Several other studies have evaluated MRSA sensitivity of this test kit, and all these studies found a sensitivity of 95.1% or above (Fournier et al., 1993; Tveten, 1995; van Griethuysen et al., 2001; Wichelhaus et al., 1999). Three other studies (Davies, 1997; Louie et al., 2000; Luijendijk et al., 1996; Personne et al., 1997) documented 100% MRSA sensitivity in 40, 78 and 144 MRSA strains, respectively. However, the latter studies did not perform prior MRSA genotyping so the use of duplicate strains cannot be excluded.

MRSA sensitivity of the c (Mast diagnostics, UK), from our data was 97.9%. This result is not concordant with strains PFGE-typed by others (Wichelhaus et al., 1999) giving a sensitivity of 97.2%. Results of other MRSA evaluations of this test system ranged from 95.4 to 100% (Croize et al., 1993; Davies, 1997; Wilkerson et al., 1997). Personne et al. (1997) reported an MRSA sensitivity of no more than 93-0% for this test kit. Although only epidemiologically unrelated MRSA strains had been used, their panels of strains were not typed for verification of actual genetic diversity. All published reports have in common that MSSA sensitivity was shown to be greater than sensitivity for MRSA detection. To our knowledge, no other comparable MRSA sensitivity evaluation of this test system has been published in which only non-identical MRSA strains, as determined by genotyping, have been used. Again, one may speculate that the selection of the bacterial panel has had a strong impact on the variation of results.

Other publication has reported on the sensitivity of the Mastalex™MRSA kit (Mast diagnostics, UK), for MRSA identification as S. aureus species (Davies, 1997). In contrast to this result (5 of 100 strains), all MRSA isolates (n=40) were detected in their work. They had used recent clinical isolates and did not document any prior typing of strains to exclude possible duplicates. Of course, considering the small number of strains that were tested, this difference may alternatively be explained by chance.

5. Conclusion

To assess the quality of agglutination assays, characterization of test strains is very important and genetically diverse isolates should be tested. The test system used in this study for genus and species determination of panel strains is well established. Depending on the parameter that is within the scope of a survey (sensitivity or specificity), the evaluated agglutination test system may not be valuable instruments for routine use in diagnostic microbiology laboratories.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Total No. of isolates (%)</th>
<th>No of Isolates</th>
<th>Latex Agglutination</th>
<th>Oxoid MSA media without oxacillin</th>
<th>Mast MSA media without oxacillin</th>
<th>Oxoid blood agar without oxacillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA</td>
<td>1269 (75.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRSA</td>
<td>423 (25.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Effect of Growth Medium on Detection of Methicillin resistance in S. aureus based on Latex agglutination test.

MSA=Mannitol Salt Agar
Acknowledgments

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

References


Kumurya A. S.: Malfunction of Agglutination Test to Identify Methicillin-Resistant Staphylococcus aureus Strains (MRSA)


