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Malfunction of Agglutination Test to Identify Methicillin-Resistant *Staphylococcus aureus* Strains (MRSA)

Kumurya A. S.*

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

Abstract

Background: Most routine laboratory detection of *Staphylococcus aureus* isolates is based on rapid agglutination test systems. Failure of agglutination assays to identify methicillin-resistant *S. aureus* strains (MRSA) has been demonstrated. *Objectives:* 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. *Methods:* As determined by Polymerase chain reaction, 100MRSA 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 *mecA* gene. *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. 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. *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

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1. Introduction

Staphylococcus aureus strains possessing *mec*A 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 *mec*A 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 *mec*A mediated resistance in *Staphylococcus aureus* (Cavassini *et al*., 1999).

Unlike coagulase-negative staphylococci (CNS), *Staphylococcus aureus* strains are able to secrete free plasma coagulase, which is an important virulence factor for these bacteria. *S. aureus* is a common pathogen in nosocomial infections, so exact identification of *S. aureus* isolates is

* Corresponding author
E-mail address: askumurya.med@buk.edu.ng

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 ^{TM}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 100Methicillin 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^{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.

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 *mec*A gene that encodes the penicillin-binding protein-2 was performed, as it is considered to be the 'gold standard' for identification of methicillin 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^{o}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 30nucleotide forward primer 5'- AAT CTT TGT CGG TAC ACG ATA TTC 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, (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 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). *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 ⩽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 χ^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 *mec*A 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 1. Oligonucleotide Primers used in the PCR assay.

Oligonucleotide Position	Nucleotide Sequence	Target Gene
A30 fwd5-34	AATCTTTGTCGGTACACGATATTCTTCACG	Sa
A30 rev112-83	CGTAATGAGATTTCAGTAGATAATACAACA	Sa
A22 rev 1282-1301	AAAATCGATGGTAAAGGTTGGC	mecA
A22 fwd 1814-1793	AGTTCTGCAGTACCGGATTTGC	mecA

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

mecA PCR result	No. of isolates tested	Latex agglutination test result for PBP2a was:		Oxacillin Disc Diffusion was:	
		Pos	Neg	Pos	Neg
Positive	5	95	0	96	0
Negative	95	5	0	4	0

Key:
Pos= Positive
Neg= Negative

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

Strains	Total No. of isolates (%)	No of Isolates							
		Latex Agglutination		Oxoid MSA media without oxacillin		Mast MSA media without oxacillin		Oxoid blood agar without oxacillin	
		Positive	Negative	Growth	No growth	Growth	No growth	Growth	No growth
MSSA	1269 (75.0)	0 (0.0)	1269 (100)	-	-	-	-	-	-
MRSA	423 (25.0)	423 (100)	0(0.0)	328 (77.5)	95 (22.5)	366 (86.5)	57 (13.5)	423 (100)	-

MSA=Mannitol Salt Agar

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 upon 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.

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