EVALUATION OF POLYMERASE CHAIN REACTION, CONVENTIONAL AND MRSA SCREEN LATEX AGGLUTINATION METHODS FOR DETECTION OF METHICILLIN-RESISTANT, -BORDERLINE AND -SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS*

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA), is difficult and expensive to treat, therefore early screening is essential. Several phenotypic and genotypic methods are used to detect MRSA; however, the method of choice remains problematic. We have evaluated four phenotypic methods, broth microdilution (MIC), oxacillin disk agar diffusion (ODD), oxacillin screening salt agar (OSS), and a new rapid phenotypic (MRSA screen latex agglutination, MSLA) with the genotypic gold standard of PCR *mecA* detection to determine the most appropriate method for routine laboratory use. We randomly collected 203 *S. aureus* isolates from patients and carriers at two hospitals in Thailand. Using MIC method, three sub-groups were differentiated from among these isolates, namely MRSA (106 isolates), borderline-resistant *S. aureus* (BRSA) (65 isolates), and methicillin-susceptible *S. aureus* (MSSA)(32 isolates). A total of 10 methicillin-resistant *S. epidermidis* (MRSE) isolates were also included. The sensitivity and specificity of MIC, ODD, OSS, and MSLA were 99 and 96, 100 and 97, 100 and 97, and 100 and 100%, respectively. Our study indicated that ODD is still appropriate for routine laboratory. MSLA had the highest sensitivity and specificity and is rapid but expensive, so is the most appropriate method for emergency cases. MIC method was better for BRSA detection and OSS method was more appropriate for screening clinical specimens and carriers.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infection and is of serious concern in control measures. In most routine microbiological laboratory, the detection of MRSA, borderline-resistant *S.aureus* (BRSA) and methicillin-susceptible *S. aureus* (MSSA) are based on phenotypic methods, such as microdilution broth test for minimum inhibitory concentration (MIC), oxacillin disk agar diffusion (ODD), and oxacillin salt screening test (OSS).

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The phenotypic methods can be problematic with strains expressing low level of oxacillin resistance, since OSS and ODD may not distinguish BRSA from true MRSA strain (NCCLS, 2000). Detection by phenotypic methods is further complicated because phenotypic expression in many strains is heterogeneous (Hartman and Tomasz, 1986; de Lencastre *et al*, 1991) and is influenced by culture conditions such as temperature, medium, pH, and NaCl concentration (Chambers and Hackbarth, 1987; Huang *et al*, 1993; Petersson *et al*, 1999).

Genetic confirmation of MRSA strains, based on detection of the *mecA* gene encoding the penicillin binding protien2a (PBP2a), which has reduced affinity for β–lactam antibiotics, is the gold standard (Dominguez *et al*, 1997; Sakoulas *et al*, 2001). The *mec*A gene detection

test, however, is based on polymerase chain reaction (PCR) that is available only in specialized laboratories and is relatively expensive. A simple and rapid method, MRSA screen latex agglutination assay (MSLA) for the detection of methicillin resistance using a specific monoclonal antibody directed toward the PBP2a antigen has been developed (Cavassini *et al*, 1999). Although MSLA is a simple and rapid method, it is still expensive for developing countries.

Phenotypic detection of MRSA remains controversial (Cavassini *et al*, 1999; Prasad *et al*, 2000). Errors in detection can lead to adverse clinical consequences. False results of susceptible detection may lead to treatment failure and may cause the spread of MRSA due to a failure of appropriate control measures, whereas the false results in resistance detection may lead to increased health care costs and overuse of antimicrobial agents.

The purpose of this study was to compare the conventional methods (ODD, OSS, MIC) with the new phenotypic MSLA method to determine which is the most appropriate method for routine laboratory use. Detection of the *mecA* gene by PCR was used as the gold standard in this evaluation.

MATERIALS AND METHODS

Bacterial isolates

Two hundreds and three isolates of *S. aureus* and 10 methicillin-resistant *S. epidermidis* (MRSE) were collected from patients and carriers at two medical school hospitals in Khon Kaen and in Bangkok, Thailand. These isolates were identified using standard microbiological procedures (Kloos and Bennerman, 1999).

Oxacillin disk agar diffusion (ODD) technique

A disk diffusion test was performed following National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1993). A sterile swab was dipped in a suspension of *S. aureus* (McFarland standard 0.5) and plated onto Muller-Hinton agar supplemented with 4% NaCl. Oxacillin disks (1 μg; Becton Dickinson, Heidelberg, Germany) were applied using sterile forceps. The agar plates were incubated at 35ºC for 24 hours. For *S. aureus*, an inhibition zone ≤10 mm indicated oxacillin-resistance, ≥13 mm oxacillin-sensitivity and 11-12 mm intermediate resistance. For *S. epidermidis*, the inhibition zone ≤17 mm indicated oxacillin-resistance.

Broth microdilution (MIC) technique

Minimum inhibitory concentrations (MIC) were determined using the broth microdilution method at a two-fold dilution of oxacillin in Muller-Hinton broth (Difco Laboratories, Detroit, Mich) supplemented with 2% NaCl as recommended by the NCCLS. The bacterial suspension at a final density of $5x10^5$ CFU/ml was inoculated in Muller-Hinton broth and incubated at 35ºC for 24 hours before determining MIC. High MRSA (HMRSA), MRSA, BRSA and MSSA were indicated by MIC ≥256, between 16 and 128, between 2 and 8, and <2 mg/l, respectively. Methicillin-resistant *S. epidermidis* (MRSE) was indicated by MIC ≥0.5 mg/l (Cavassini *et al*, 1999; NCCLS, 1999; Jafri *et al*, 2000).

Oxacillin salt screening (OSS) method

All isolates were plated on Mueller-Hinton agar with 4% NaCl and 6 mg/l oxacillin. Bacteria were inoculated at a final density of $5x10^5$ CFU/ml. Oxacillin resistance was confirmed by surface growth after incubation at 35ºC for 24 hours (NCCLS, 1993).

MRSA-screen latex agglutination (MSLA) test

The MRSA- screen latex agglutination (MSLA) test (Denka Seiken, Tokyo, Japan) was performed according to the manufacturer's instruction. In brief, 10 to 20 Staphylococci colonies from a fresh blood agar plate were suspended in 200 μl of extraction reagent No.1 (0.1M NaOH). The suspension was boiled for 3 minutes, and then mixed with 50 μl of extraction reagent No.2 (0.5 M KH_2PO_4). After centrifugation at 1,500*g* for 5 minutes at room temperature, 50 μl of supernatant were placed on a slide with 25 μl (1 drop) of anti PBP2a monoclonal antibody sensitized latex, and then mixed for 3 minutes with a shaker. Agglutination occurring within 3 minutes was visually quantified on a scale between 1+ and 3+. Control, a 50 μl aliquot of supernatant, was placed on the slide and mixed with 1 drop (25 μl) of the control latex provided in the kit.

DNA preparation

DNA was prepared following the method of

Table 1
Comparison of *mecA*, OSS, and ODD methods in analysing 203 *S. aureus* and 10 MRSE isolates.

MIC		*mecA*		OSS		ODD		
Group	N	+	-	+	-	R	I	S
MRSA	106	102	4	103	3	103	0	3
BRSA	65	1	64	3	62	1	5	59
MSSA	32	0	32	0	32	0	0	32
MRSE	10	9	1	9	1	9	0	1
Total	213	112	101	115	98	113	5	95

N = Number of isolates; R = resistant, S = sensitive, I = intermediate sensitive

Weller (1999). A single colony of *Staphylococcus* was grown overnight at 37ºC in brain-heart infusion broth. A 500 ml aliquot of cell suspension was centrifuged and the pellet washed and resuspended in 400 ml of lysis solution (50 mM Tris pH 8, 5 mM EDTA, 50 mM NaCl). Lysostaphin (Sigma Chemical, St Louis, Mo) was added at a final concentration of 20 mg/l. The suspension was incubated at 37ºC with shaking for 1 hour. A 80 µl aliquot of proteinase K solution (50 mM Tris pH 8, 0.4 M EDTA, 0.5% sodium dodecyl sulfate containing 0.5 mg of proteinase K (Boehringer Mannheim, Lewes, UK)) was added and the cell suspension incubated at 50ºC for 2 hours. DNA was extracted with phenol-chloroform and precipitated in an ice-cold ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 25 µl of TE buffer (10 mM Tris pH 8, 1 mM EDTA) and stored at -20ºC.

PCR

PCR was performed using the primers *mecA*1 (5′-AAAATCGATGGTAAAGGTTGGC) and *mecA*2 (5′-AGTTCTGCAGTACCGG ATTTTGC) (Murakami *et al*, 1991). The reaction was conducted in 50 µl of a reaction mixture containing DNA (10-200 ng), 200 µM each of deoxynucleoside triphosphates (dNTP) (Gibco BRL), 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 nM each primer and 2.5 units of *Taq* polymerase (Gibco BRL). The thermal cycler (Perkin-Elmer, Gene Amp, PCR 2400) was programed for 30 amplification cycles consisting of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 1 minute. The amplification product was separated on 1.5 % agarose gel-electrophoresis and visualized by ethidium bromide staining. The 50 and 100 bp DNA ladders were used as DNA molecular weight standards. The positive result of PCR showed a 533 bp fragment of *mecA* gene.

Table 2
Comparison of MSLA and *mecA* methods in analysing 100 *S. aureus* and 10 MRSE isolates.

MIC		MSLA		*mecA*	
Group	N	+	-	+	-
MRSA	40	36	4	36	4
BRSA	40	1	39	1	39
MSSA	20	0	20	0	20
MRSE	10	9	1	9	1
Total	110	46	64	46	64

N = Number of isolates, R = resistant, S = sensitive, I = intermediate sensitive

RESULTS

Correlation between MIC, ODD, and OSS methods with detection of *mecA* gene by PCR

Of the 203 *S. aureus* isolates, the MIC method identified 106 MRSA, 65 BRSA and 32 MSSA (Table 1). Of the 106 MRSA isolates, 102 possessed the *mecA* gene while 103 were OSS and ODD positive. Of the 65 BRSA, one isolate had the *mecA* gene and was ODD and OSS positive and two lacked the *mecA* gene and were OSS positive and ODD of intermediate sensitivity. There was agreement of all methods for the 32 MSSA isolates identified. Any result that differed

Table 3
Correlation of the results of MIC, ODD, OSS, *mecA*, and MSLA methods in analysing 100 *S. aureus* and 10 MRSE isolates.

Characteristics	MIC (mg/l)	ODD	OSS	MSLA	*mecA*	N
HMRSA	≥256	R	+	+	+	30
MRSA	16 to 128	R	+	+	+	6
		R	+	-	-	1
		S	-	-	-	3
BRSA	2 to 8	R	+	+	+	1
		I	+	-	-	1
		I	-	-	-	2
		S	-	-	-	36
MSSA	<2	S	-	-	-	20
MRSE	≥0.5	R	+	+	+	9
		S	-	-	-	1

N = Number of isolates, R = resistant, S = sensitive, I = intermediate sensitive

from the PCR method was re-tested; however, the results remained unchanged.

Correlation between PCR and MSLA method

MSLA method was performed on 100 *S. aureus* isolates. A comparison of MSLA and the PCR gold standard showed 100% agreement for sensitivity and specificity (Tables 2, 3).

Efficiency of the conventional methods and MSLA compared to *mecA* gene

The sensitivity, specificity and positive and negative predictive values of ODD, OSS, MIC and MSLA methods indicated that all methods functioned satisfactorily (Table 4).

Table 4
Comparison of sensitivity, specificity, positive predictive value, and negative predictive value of ODD, MIC, OSS, and MSLA methods.

Test	Sensitivity %	Specificity %	PPV %	NPV %	*K*
ODD[a]	100	97	97	100	0.97
OSS[a]	100	97	97	100	0.97
MIC[a]	99	96	100	99	0.95
MSLA[b]	100	100	100	100	1

[a] = 213 samples; [b] = 110 samples; PPV = positive predictive value; NPV = negative predictive value; *K* = kappa value

DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the main contributor to the problem of hospital-acquired infection. An accurate and rapid detection technique is essential to reduce the morbidity and mortality caused by this infection. Several genotypic methods are in use but detection of *mecA* gene by PCR is currently considered as a gold standard (Hussain *et al*, 2000; Arbique *et al*, 2001; Sakoulas *et al*, 2001). However, PCR remains beyond the capacity of most developing countries, so more conventional methods were assessed and compared for reliability.

We classified 106 of 203 isolates as MRSA using the MIC method but only 102 isolates were positive for *mecA* (Table 1) indicating that not all resistance detected by MIC is the result of the *mecA* gene. In fact, several resistance mechanisms mediate methicillin resistance and phenotypic expression in many strains is heterogeneous (de Lencastre *et al*, 1991; Dominquez *et al*, 1997; Araj *et al*, 1999). In our study, the mechanism of methicillin resistance among MRSA via *mecA* was 96%. A study in Turkey found *mecA* accounted for 94% MRSA (Unal *et al*, 1994). Three isolates were classified as MRSA based on the MIC

method even though they lacked *mecA* and were negative for ODD and OSS suggesting the MIC method missed only 2.8% truly MRSA.

In agreement with previous studies, most BRSA isolates did not possess the *mecA* gene demonstrating that BRSA generally lacked the *mecA* gene (Louie *et al*, 2000; Udo *et al*, 2000). The overproduction of penicillinase may account for this result (Montanari *et al*, 1990). One isolate reported as BRSA was *mecA* positive perhaps because of low PBP2a expression or the lack of *mecA* repressor expression (*mecI* mutation or *mecI* deletion) (Kuwahara *et al*, 1996). Since *mecA* is the primary factor indicating methicillin resistance, it is not surprising that all the MSSA isolates were *mecA* negative (Krishnan *et al*, 2002; van Leeuwen *et al*, 2002).

Although other investigators reporting the reliability of susceptibility testing methods have ranked, without regards to genetic method OSS better than MIC which in here is better than ODD method (de Lencastre *et al*, 1991; Mackenzie *et al*, 1995; Chambers, 1997), our results suggested that the ranking should be OSS > ODD > MIC. There are conflicting reports on the reliability of these techniques (Huang *et al*, 1993; Frebourg *et al*, 1998; Cavassini *et al*, 1999; Prasad *et al*, 2000). These may be explained by the different culture conditions used (Chambers *et al*, 1987;Knapp *et al*, 1994; Resende and Figueiredo, 1997) and the nature of isolates in various geographic areas.

Some researchers have reported that management of BRSA infections is not different from that of MSSA infections since there are no significant differences in outcome of treatment with penicillinase-resistant penicillins (PRP) (Massanari *et al*, 1988). Therefore, ODD and OSS method should be sufficient for the identification of MRSA and MSSA, since the majority of BRSA lacking the *mecA* gene identified by MIC method was sensitive to ODD and OSS methods. Only one isolate with the *mecA* gene (0.5%) showed resistance when using ODD and OSS methods.

Correlation of *mecA* gene detected by PCR and MSLA was concordant in 110 *Staphylococci* isolates (Table 3), similar to results of previous reports (Cavassini *et al*, 1999; Jafri *et al*, 2000). No false negative was found in this study, indicating high expression of *mecA* gene in these isolates, a result similar to that previously reported (Gerberding *et al*,1991). Thus, there is no need to induce the expression of resistance gene with beta-lactam antimicrobial agent before testing as suggested by some investigators (Kuwahara-Arai *et al*, 1991).

For the application of MSLA method to detect MRSE, 10 MRSE isolates were included for detection of *mecA* in comparison with MSLA. Nine of ten MRSE isolates from MIC determination were *mecA* positive. Agreement between PCR and MSLA methods was found in all 10 MRSE isolates. This result suggested that MSLA method can be applied in MRSE detection as recommended by some investigators (Udo *et al*, 2000; Louie *et al*, 2000).

Among the methods used, OSS technique was the fastest since specimens could grow directly on the media without prior culture, indicating resistance or sensitivity. ODD and MIC methods gave more detailed results. The diameter of the clear zone can indicate intermediate resistance in ODD method and MIC can determine the specific concentration resulting in inhibition.

Techniques used in this study have their own advantages and disadvantages. The PCR technique for *mecA* gene detection is considered as a gold standard but is quite expensive and requires specialized laboratory setting and skilled persons. MSLA method is rapid, easy to perform and can detect expression of altered *mecA* gene, but remains expensive for developing countries, although it has the highest sensitivity and specificity compared to conventional methods. Broth microdilution for MIC determination gives specific information on drug response but may not be helpful in guiding antimicrobial therapy of BRSA infection. ODD and OSS methods can be performed in any microbiology laboratories and ODD is useful for multiple drug sensitivity testing. We found all of the tests reliable, highly specific and sensitive (Table 4). Microbiologist or laboratory technician may need to evaluate the method most suitable for detecting MRSA in their area. Based on our study, ODD method was a very accurate, reliable, and inexpensive. It is suitable for use in general routine clinical microbiology laboratory. For rapid result, MSLA is a good

choice especially in emergency cases.

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