EVALUATION OF DIFFERENT PRIMERS FOR DETECTING *MEC*A GENE BY PCR IN COMPARISON WITH PHENOTYPIC METHODS FOR DISCRIMINATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract. Detection of the *mec*A gene by polymerase chain reaction (PCR) is the gold standard for identifying methicillin-resistant *Staphylococcus aureus* (MRSA). PCR assays, employing MR1-MR2 primers (primer set 1) and MR3-MR4 primers (primer set 2) to generate 154 and 533 bp fragment, respectively, are most widely used for amplification of *mec*A gene. The purpose of this study was to evaluate the presence of *mec*A gene in 100 clinical isolates of *S. aureus* using PCR with the two pairs of primers. The results were compared to the broth dilution MIC method, oxacillin salt screening method (OSS) and oxacillin disk agar diffusion method (ODD). Fifteen of the 100 isolates showed a discrepancy between the *mec*A primer sets 1 and 2. Three isolates (3%) without the *mec*A gene showed discrepancies with phenotypic methods. The sensitivity, specificity and positive and negative predictive values for the 154 and 533 bp products of *mec*A were 79, 85, 83, 81 and 94, 100, 100, 94%, respectively. The results indicated that primer set 2 was more appropriate than primer set 1 for the detection of *mec*A gene in MRSA. There was a good correlation among the *mec*A gene detection, ODD and OSS methods. The discrepancy of three isolates between PCR and phenotypic methods should be clarified for other resistant mechanisms.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of the increasingly prevalent, difficult and expensive-to-treat nosocomial infections worldwide. Methicillin-resistance in staphylococci constitutes resistance to all of the β-lactam antibiotics and their derivatives. The major mechanism is the acquisition of the *mec*A gene that codes for additional penicillin-binding protein2a (PBP2a) (Murakami *et al,* 1991). The *mec*A gene is the gold standard for the detection of MRSA (Dominguez *et al,* 1997). The phenotypic methods such as broth microdilution test for minimal inhibiory concentration (MIC), oxacillin disk agar diffusion (ODD) and oxacillin salt screening test (OSS) are widely used in routine microbiological laboratory (NCCLS, 1993; Kampf *et al,* 1997; 1998). The problem with phenotypic methods is that they can be influenced by culture condition such as temperature, medium pH and NaCl content in the medium (Sabath, 1982).

Several PCR methods have been developed to detect the *mec*A gene (Murakami *et al,* 1991; Tokue *et al,* 1992); however, two pairs of PCR primers are most commonly used (Murakami *et al,* 1991; DelVechio *et al,* 1995) but no specific oligonucleotides have been compared. Our purpose was to determine the efficiency of the PCR method with different primers for dectecting the *mec*A gene in MRSA and to compare the sensitivity and specificity of PCR with conventional methods.

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MATERIALS AND METHODS

Bacterial isolates

One hundred isolates of *S. aureus* were collected from patients and carriers at Srinagarind Hospital, Khon Kaen and Ramathibodhi Hospital, Bangkok, Thailand. The isolates were identified using standard microbiological procedures. All isolates were identified as *S. aureus* based on morphology, positive catalase, positive coagulase and fermentation mannitol (Kloos and Bennerman, 1995).

Oxacillin disk agar diffusion (ODD)

A disk diffusion test was performed following the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS). A sterile swab was dipped in an *S. aureus* suspension (McFarland standard 0.5) and plated onto Muller-Hinton agar. Oxacillin disks (1 µg; Becton Dickinson, Heidelberg, Germany) were applied using sterile forceps. Agar plates were incubated at 35ºC for 24 hours. An inhibition zone <10 mm in diameter indicated oxacillin resistance.

Broth microdilution

MICs 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 had a final density of $5x10^5$ CFU/ml, which was inoculated in Muller-Hinton broth and incubated at 35ºC for 24 hours before determining the MICs.

MICs >256 mg/l indicated a high MRSA (HMRSA), those between 16 and 28 mg/l MRSA, those between 2 and 8 mg/l borderline resistance to *S. aureus* (BRSA) and those <2 mg/l MSSA (Cavassini *et al,* 1999; Jafri *et al,* 2000).

Oxacillin salt screening method (OSS)

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.

DNA preparation

The DNA preparation used in this study was performed following Weller (1999). A single colony of *S. aureus* was grown overnight at 37ºC in brain-heart infusion broth. An aliquot of 500 µl of suspension was centrifuged and the cell pellet was washed and suspended in 400 µl of lysis solution (50mM Tris, pH 8; 5mM EDTA, pH8; 50 mM NaCl). Lysostaphin (Sigma Chemical Co, St Louis, Mo) was added to a final concentration of 20 mg/l. The suspension was incubated at 37ºC and shaken for 1 hour. An aliquot of 80 µl of proteinase solution (50 mM Tris ; 0.4 M EDTA, pH 8 ; 0.5% sodium dodacyl sulfate (SDS) containing 0.5 mg proteinase K (Boehringer Mannheim, Lewes, UK) was added and incubated at 50ºC for 2 hours. The DNA was extracted with phenol-chloroform and precipitated by ice-cold ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 25 µl of TE buffer (10 mM Tris; 1 mM EDTA, pH8) and stored at -20ºC until used.

Primers

The *mec*A gene was amplified with primer set 1 (MR1 and MR2) and primer set 2 (MR3 and MR4) (Murakami *et al,* 1991; Del Vechio *et al,* 1995) (Table 1).

PCR

PCR was performed in 50 µl of a reaction mixture containing DNA (10-200ng), 200 µg each of deoxynucleoside triphosphates (dNTP) (Gibco BRL), 1.5 mM $MgCl_2$, 20mM Tris-HCl (pH 8.4), 50 mM KCl, 200 nM (each) primer and 2.5 units of *Taq* polymerase (Gibco BRL). Thermal cycling was conducted for 30 seconds at 94ºC; 30 seconds at 58ºC and 30 seconds at 72ºC, 30 amplification cycles using primer set 1. Amplification of the *mec*A with primer set 2 followed the same procedure except that the annealing temperature was reduced to 55ºC and the extension time was 1 minute. The amplification product was separated on 2% agarose gel electrophoresis and visualized by

ethidium bromide staining. The 50 bp and 100 bp DNA ladders were used as DNA molecular weight standards.

RESULTS

The results obtained from MIC determinations, ODD, OSS and PCR with primer set 1 and 2 assays are summarized in Table 2. The discrepancy test results obtained from these methods showed that for high level MRSA (MIC ≥ 256 mg/l), 2 (5%) of the 41 isolates was positive in the ODD and the OSS assays but negative for PCR using both primer sets. Thirty-two (78%) isolates were positive by all methods whereas 7 (17%) were negative only in the PCR using primer set 1. For MRSA (MIC 16 to 128 mg/l), 5 (62%) isolates were positive by all methods whereas 2 (25%) isolates were negative for all methods. There was 1 isolate (12%) positive in the ODD and the OSS assays but negative for PCR using both primer sets. For BRSA (MIC 2 to 8 mg/l), 25 (86%) of 29 isolates were negative by all methods whereas 3 (10%) isolates were positive with PCR using primer set 1. There was 1 isolate (3%) positive in all methods. For MSSA (< 2 mg/l), 17 (77%) isolates were negative for all methods whereas 5 (23%) isolates were positive with only PCR using primer set 1. Fifteen (15%) of 100 isolates showed a discrepancy for *mec*A primer sets 1 and 2.

The sensitivity, specificity and positive

Table 1
Oligonucleotide primers used for *mec*A PCR amplification for the identification of methicillin-resistance in *S. aureus.*

Primer-*mec*A	Primer sequence (5′-3′)	Amplicon Size (bp)	Nucleotide position
MR1	TAGAAATGACTGAACGTCCG	154	179 - 198
MR2	TTGCGATCAAATGTTACCGTAG		332 - 312
MR3	AAAATCGATGGTAAAGGTTGGC	533	1282 - 1303
MR4	AGTTCTGCAGTACCGGATTTGC		1814 - 1793

Table 2
Discrepancies between the results of PCR using two pairs of *mec*A primers, ODD, OSS and MICs assays in 100 isolates of *S. aureus.*

Identification [oxacillin MIC (μg/ml)]	Outcome				No.of isolates
	ODD	OSS	*mec*A PCR		
			Primer set 1	Primer set 2	
HMRSA (≥256)	+	+	-	-	2
	+	+	+	+	32
	+	+	-	+	7
MRSA (16-128)	+	+	+	+	5
	+	+	-	-	1
	-	-	-	-	2
BRSA (2-8)	-	-	-	-	25
	-	-	+	-	3
	+	+	+	+	1
MSSA (<2)	-	-	-	-	17
	-	-	+	-	5

Table 3
Comparison of the results for ODD and OSS assays and PCR using two pairs of *mec*A primers.

S. aureus strain	ODD and OSS		*mec*A Primer set 1		*mec*A Primer set 2	
	+	-	+	-	+	-
MRSA (49)	47	2	37	12	44	5
BRSA (29)	1	28	4	25	1	28
MSSA (22)	0	22	5	17	0	22
Total	48	52	46	54	45	55

Table 4
Comparison of sensitivity, specificity, positive and negative predictive values of *mec*A PCR primer sets 1 and 2.

*mec*A	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Primer set 1	79 (38/48)	85 (44/52)	83 (38/46)	81 (38/46)
Primer set 2	94 (45/48)	100 (52/52)	100 (45/45)	94 (52/55)

and negative predictive values of the *mec*A primer sets 1 and 2 are shown in Table 3. Our results indicate that the PCR with primer set 2 more accurately and sensitively detected oxacillin resistance than primer set 1.

Fig 1 shows representative data of the PCR assay for PCR primer sets 1 and 2 for 4 representative *S. aureus* strains. Most high level MRSA isolates carried *mec*A in their genome resulting in a 154 bp and a 533 bp fragment with the PCR primer set 1 and 2, respectively (Fig 1; lane 2, 8). Ten isolates (5 MSSA and 5 BRSA) were positive for primer set 1 with weak amplification, but negative for primer set 2 (Fig 1; lane 3, 9). Seven isolates (HMRSA) were negative for primer set1 but positive for primer set 2 (Fig 1; lane 4, 10). Most BRSA (25 isolates) and MSSA (17 isolates) presented neither the 154 bp nor the 533 bp fragments (Fig 1; lane 5, 11).

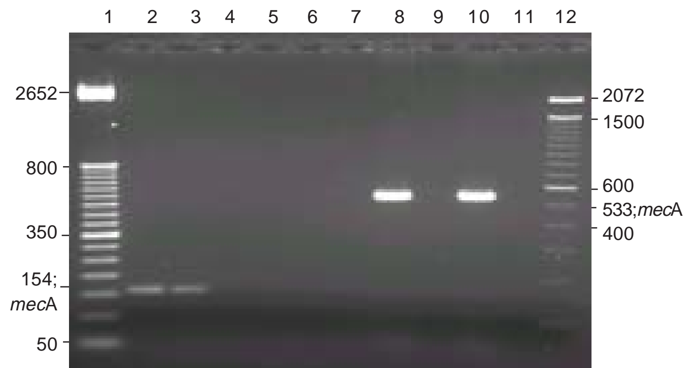

Fig 1–2% Agarose gel electrophoresis of the 154 bp and 533 bp DNA fragment corresponding to the *mec*A gene of PCR primer set 1 and PCR primer set 2. PCR primer set 1: lanes 2-5; PCR primer set 2 : lanes 8-11; lane 1, 12: DNA size markers, lane 1 : 50 bp DNA ladder, lane 12 : 100 bp DNA ladder; lane 2, 8: strain 5A (HMRSA); lane3,9 : strain 131A (MSSA); lane 4, 10: strain 41B (HMRSA); lane 5,11 : strain 31C (MSSA). Lane 6,7 was negative control for primer set 1 and primer set 2.

DISCUSSION

Our aim was to compare the efficiencies of the PCR method with the different primers used to detect the *mec*A gene for oxacillin resistance. Generally, PCR assays are used to detect the *mec*A gene of MRSA; however, no specific oligonucleotides have been suggested and compared (Frebourg *et al,* 1998; Louie *et al,* 2000). The oxacillin screen plate test is the gold standard for the phenotypic method (Frebourg *et al,* 1998; Kampf *et al,* 1998). Thus we compared both the OSS and the ODD methods with the *mec*A gene detection using PCR primer sets 1 and 2.

In several studies, most of the high methicillin resistant strains (MICs ≥ 256 mg/l) were *mec*A PCR positive (Tomasz *et al,* 1989; Dominguez *et al,* 1997). In this study, however, 3 PCR negative strains for both PCR primer sets 1 and 2 were present among the HMRSA and MRSA strains. This result caused a reduction in the sensitivity and specificity of the PCR method; however, it might be explained by some other mechanism rather than the absense of the *mec*A gene.

Three major mechanisms of resistance have been associated with the resistant phenotype: 1) *mec*-encoded resistance, 2) overproduction of penicillinase and 3) modifications of normal penicillin-binding proteins. BRSA is generally related to overproduction of penicillinase (Franciolli *et al*, 1991; Lorian, 1996; Santos *et al,* 1999). The absence of *mec*A gene in the 25 BRSA isolates may be explained by overproduction of penicillinase (Montanari *et al,* 1990); nevertheless, the mechanism of resistance for the three of HMRSA and MRSA isolates needs to be clarified.

The synthetic oligonucleotide primers of nucleotide 179 to 798 and 332 to 312 (MR1-MR2) have been used by some researchers (Del Vecchio *et al*. 1995; Santos *et al,* 1999) while those of nucleotides 1282 to 1303 and 1814 to 1793 (MR3-MR4) have been used by others (Murakami *et al,* 1991; Louie *et al,* 2000). Our results show that MR3-MR4 primers (primer set 2) had a high correlation with the presence or absence of the *mec*A gene, the level of bacterial resistance to oxacillin, and positive or negative ODD and OSS assays. The sensitivity, specificity and positive and negative predictive values for the MR3-MR4 primers were higher than those of the MR1-MR2 primers. This suggests that MR3-MR4 primers are more appropriate for the detection of the *mec*A gene.

Phenotypic expression of methicillin-resistance is influenced by temperature, medium pH and NaCl content in the medium (Sabath, 1982) so these factors may also affect correspondence between the presence of the *mec*A gene and phenotypic expression of MRSA as assessed by ODD and OSS methods. Our study, however, showed a good correlation of the presence or absence of the *mec*A gene with ODD and OSS assays.

Our results indicated the MIC test did not clearly differentiate between BRSA and MRSA when compared to ODD and OSS methods since two MRSA isolates which lacked *mec*A could not grow on OSS and were susceptible in ODD assay. One BRSA isolate could grow on OSS but was susceptible in ODD assay and also contained the *mec*A gene.

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