American Journal of Infectious Diseases 4 (2): 156-161, 2008
ISSN 1553-6203

Identification of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Resistant Coagulase-Negative Staphylococcus (CoNS) in Clinical Settings

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Abstract: To evaluate different methods for the identification of methicillin-resistant Staphylococci and their reliability, 112 Staphylococcal isolates (32 *Staphylococcus aureus* isolates and 80 coagulase negative Staphylococci isolates "CoNS") were collected from 118 nasal swab cultures and were subjected to three methods to detect oxacillin susceptibility of the isolates. The three methods were oxacillin disk diffusion method; the Epsilometer- test (E-test) and polymerase chain reaction (PCR). For the *S. aureus* strains, the E-test and the PCR methods showed discrepant results in two isolates (6.25%); that showed susceptible patterns with the E-test, but were resistant by the disk diffusion method. Both isolates were negative for the presence of *mecA* gene. Seven (8.75%) out of the 80 CoNS isolates showed conflicting results where four isolates showed resistance with the disk diffusion and the E-test methods, and had negative *mecA* gene by PCR. Three of the 7 CoNS with the conflicting results showed a susceptible pattern to oxacillin by the E-test method, while the PCR method showed the presence of *mecA* gene. We concluded that combination of molecular and conventional methods should be used to assess methicillin resistance of Staphylococci in clinical practices.

Key words: Methicillin, Resistance; Clinical

INTRODUCTION

The incidence of infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) is increasing which require rapid and accurate detection[1, 2]. Diseases due to *Staphylococcus aureus* include infections affecting the skin, bone infections (osteomyelitis), prosthetic devices related infections, and even life threatening infections including bacteremia, endocarditis with remote complications[3]. Coagulase- negative *staphylococci* (CoNS) are considered among the most isolated bacteria in clinical microbiology laboratory. Their importance as pathogens is established as hospital acquired infections, especially in debilitated patients[4].

Methicillin-resistant *Staphylococcus aureus* (MRSA) are bacteria that are resistance to penicillinase-stable semisynthetic penicillins such as Methicillin, Nafcillin, Oxacillin and Cloxacillin. The *mecA* gene encodes this type of resistance and expression of this gene results in production of a penicillin binding protein (PBP2a). This binding protein has low affinity to methicillin making bacteria that produce it resistant to all β-lactam antibiotics, and even to other antibiotics from other classes, including tetracyclines, macrolides, flouroquinolones. Infections with MRSA are more serious than other organisms as the available treatment options are limited; and there is already described resistance to these treatment options such as the described cases of vancomycin resistant *Staphylococcus aureus*[5]. The MRSA infections were usually encountered in health-care settings and more cases of MRSA infections were described as being community acquired[8]. Therefore, proper and prompt diagnosis of MRSA isolates will result in proper treatement and control measures[9].

Resistance in CoNS is attributed to the same mechanisms as in *Staphylococcus aureus* and *mecA*

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gene when present is expressed at a lower level than MRSA making its detection more difficult.[10]

Several risk factors for acquisition of MRSA have been identified. Hospitalizations, admission to intensive care units, having surgical wounds or being intravenously catheterized were found to be correlated to acquiring MRSA infections. A prolonged hospital stay, and prior exposure to broad-spectrum antimicrobial therapy also appear to be an important predisposing factor for MRSA colonization[7, 11, 12- 13].

Our study was designed to assess the reliability of some of the different available methods used in clinical practice for identifying MRSA and resistant CoNS.

MATERIALS AND METHODS

Sample collection: This investigation was carried out at King Abdullah University Hospital (KAUH), during the period between February to July 2004. One-hundred-eighteen nasal swabs were collected from health care workers (77 males and 41 females) aged between 23-40 years. Nasal swabs were saturated with sterile normal saline, and then rotated in the anterior nares. Collected swabs were cultured on mannitol salt agar (Difco, Detroit, MI, USA) and then incubated at 35^{o}C for 48 hours. The cultures were then characterized, and sub-cultured for the mixed colonies, coagulase test was performed for all the isolates. Bacterial identification were performed following standard procedures.

Oxacillin disk diffusion: The isolated Staphylococcus strains were tested for their susceptibilities to oxacillin and other antibiotics following previously reported method[14]. Briefly, isolates were spread on Mueller-Hinton Agar (Difco, Detroit, MI, USA) plates supplemented with 4% NaCl. Plates were then inverted and incubated for 18 hours at 35 °C. To obtain the best results, the agar plates had no excess moisture and the antibiotic disks containing 1 μg (including oxacillin) (Oxoid Ltd, Basingstoke, England) were brought to room temperature before use, and the distance between the disks was at least two centimeters. The diameter of the inhibition zones was measured and results were then reported according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines[15].

E- test Method: The E-test (PDM- Epsilometer, AB Biodisk, Solona, Sweden) was used to measure the individual minimal inhibitory concentrations (MICs) according to the NCCLS guidelines. Mueller-Hinton Agar (Difco, Detroit, MI, USA) supplemented with 2% NaCl was used for this purpose. Samples for the E-test was prepared according to Novak et al[16].

Polymerase chain reaction (PCR) for Identification of *mecA* Gene: This method was done according to Smyth et al[17, 18]. Briefly, Nucleotides primers were purchased from Alpha DNA, Montereal, Canada. Primers *mecA*-1, 5$^{/}$-GCA ATC GCT AAA GAA CTA AG and *mecA*-2 5$^{/}$-GGG ACC AAC ATA ACC TAA TA were derived from the gene *mecA* BB270 (EMBL data base). From the agar plate, 5 colonies were picked and suspended in 100 μL sterile water. Bacterial suspensions were run for 15 minutes at 98ºC in a DNA thermocycler (Mycycler, Biorad, USA) and cell debris were removed by centrifugation (13,000 rpm for 30s). The supernatant was used as DNA template for the PCR amplification. Two μL of DNA was added to 18 μL of chilled PCR mixture containing 10 mM Tris (pH 8.3), 5mM $MgCL_2$, 0.2mM dNTP, 0.5 μM of each primer and 0.5U Ampli Taq DNA polymerase. The following amplification protocol was used: denaturation at 94 ºC for 3 min , annealing at 53ºC for 2 min, and extension at 72 ºC for 5 min for 30 cycles to completion. The final cycle conisited of extension at 72ºC for 7 min. PCR products were detected by electrophoresis on a 3% agarose-Tris-borate-EDTA gel (Nusieve Bioproducts, Maine). After electrophoresis the gel was stained with ethidium bromide and photographed under UV light (Fig. 1). The results were reported as negative or positive.

RESULTS

Disk Diffusion Method: The percentage of oxacillin resistance among all samples was 66.9% (75 isolates), and the resistance percentages for other antibiotics was: Lincomycin 61.6% (69 isolates), Erythromycin 66.9% (75 isolates), Gentamicin 73.2 % (82 isolates), Augmentin 72.3% (81 isolates), and Cephalothin 66.9% (75 isolates) (Table I).

The susceptibility patterns among the eighty-coagulase negative samples were as follow: 69 isolates (86.2%) were resistant to Oxacillin and the other 11 isolates (13.8%) were susceptible.

Table1: Antimicrobial Susceptibility patterns among coagulase positive and coagulase negative isolates as measured by disk diffusion method.

Antibiotics	Coagulase positive			Coagulase negative			General for all isolates		
	S No. (%)	I No. (%)	R No. (%)	S No. (%)	I No. (%)	R No. (%)	S No. (%)	I No. (%)	R No. (%)
Oxacillin	25 (78.1)	1 (3.1)	6 (18.8)	11 (13.8)	-	69 (86.2)	36 (32.2)	1 (0.9)	75 (66.9)
Cephalothin	27 (84.4)	-	5 (15.6)	10 (12.5)	-	70 (87.5)	37 (33.0)	-	75 (66.9)
Gentamycin	20 (62.5)	-	12 (37.5)	10 (12.5)	-	70 (87.5)	30 (26.8)	-	82 (73.2)
Augmentin	22 (68.8)	-	10 (31.2)	9 (11.2)	-	71 (88.8)	31 (27.7)	-	81 (72.3)
Erythromycin	17 (53.1)	-	15 (46.9)	11 (13.8)	-	69 (86.2)	37 (33.1)	-	75 (66.9)
Vancomycin	32 (100)	-	-	80 (100)	-	-	112 (100)	-	-
Lincomycin	16 (50)	1 (3.1)	15 (46.9)	25 (31.2)	1 (1.3)	54 (67.5)	41 (36.6)	2 (1.8)	69 (61.6)

S: sensitive; I: intermediate; C: resistant

For coagulase positive isolates, disk diffusion method showed only 6 isolates (18.8%) resistant to Oxacillin and to all other antibiotics, making these isolates MRSA. On the other hand, 25 isolates (78.1%) were susceptible to Oxacillin, while only one sample (3.1%) had intermediate resistance to Oxacillin. All one hundred and twelve staphylococcal isolates (100%) were susceptible to Vancomycin (Table I)

E test method: The E-test method was carried out for all the thirty-two coagulase positive Staphylococcal isolates, and for the 80-coagulase negative isolates. Only five (15.6%) of the coagulase positive isolates were resistant to Oxacillin (having an MIC value equal to, or more than 4 µg/ml), the remaining twenty-seven isolates (84.4%) were susceptible to Oxacillin with MIC value less or equal to 2 µg/ml.

For the coagulase negative isolates, 69 (86.2%) were resistant to Oxacillin with MIC value equal to or more than 0.5 µg/ml and 11 (13.8%) isolates were susceptible to Oxacillin with MIC value less or equal to 0.25 µg/ml.

Table 2: Coagulase negative strains for which the disk diffusion, E-test, and mecA PCR gave discrepant results.

No. Of Isolate	Disk diffusion		E- test		mecA PCR
	Zone (mm)	Susc.	MICs (µg/ml)	Susc.	
3	20	S	0.25	S	+
6	19	S	0.25	S	+
9	15	R	1.0	R	–
31	14	R	0.5	R	–
44	14	R	1.5	R	–
60	13	R	3.0	R	–
80	20	S	0.25	S	+

Susc.: Susceptibility; S; susceptible, R; resistant

PCR Method:

The results of PCR indicated that 5 isolates of the coagulase positive Staphylococcal isolates (15.6%) were positive for the presence of *mecA* gene, and the remaining 27 isolates (84.4%) were negative for *mecA* gene. For coagulase negative isolates, 68 (85.0%) were positive for the presence of *mecA* gene and the other 12 (15.0%) isolates were negative. Two

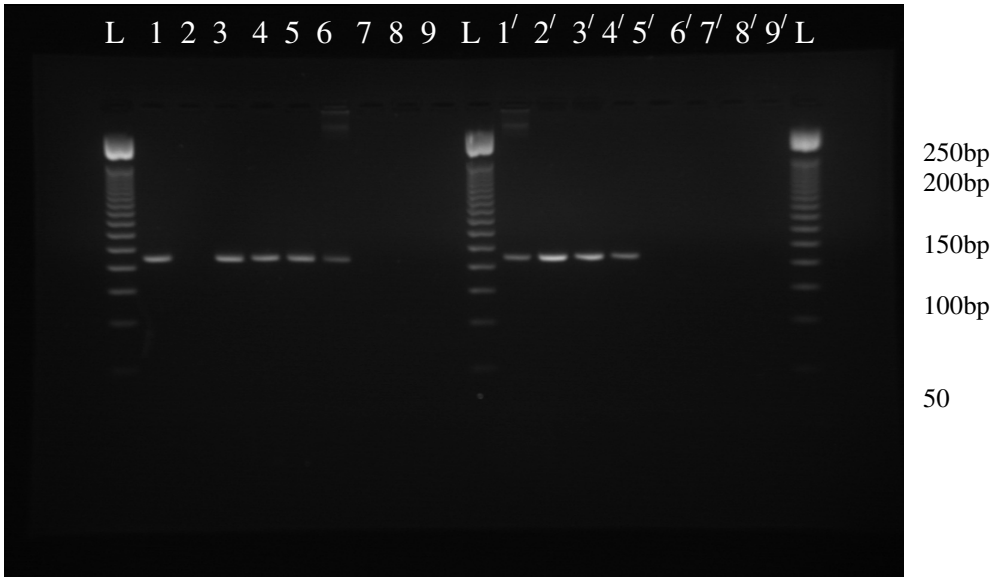

Fig. 1: Agarose Gel Electrophoresis of PCR for mecA Gene Presence. (L: 50bp DNA ladder, 1: MRSA positive control, 2: negative control, 3-6: coagulase positive MRSA (positive *mecA* gene), 7-9: coagulase positive MSSA (negative *mecA* gene). $1'$ - $4'$: coagulase negative oxacillin resistant (positive *mecA* gene), $5'$ - $9'$: coagulase negative oxacillin susceptible (negative *mecA* gene)).

coagulase positive isolates and seven coagulase negative isolates showed discrepant results between disk diffusion, E-test and *mecA* PCR methods. The discrepant results between the three methods are shown in Tables 2, 3.

Table 3: *Staphylococcus aureus* strains for which the disk diffusion, E-test, and mecA PCR gave discrepant results.

No. Of Isolate	Disk diffusion		E- test		mecA PCR
	Zone (mm)	Susc	MICs (µg/ml)	Susc.	
1	12	I	0.50	S	-
67	0	R	1.50	S	-

S: sensitive; I: intermediate; R: resistant

DISCUSSION

Staphylococcus aureus is one of the most important nosocomial pathogens, and now coagulase- negative *staphylococcus* (CoNS) is also recognized as an important cause of nosocomial infections. *Staphylococci* accounted for about fifty percent of blood stream infections in intensive care units according to the National Nosocomial Infections Surveillance System data between 1990-1999[19]. The major problem of resistance is increasing for all types of bacteria including the *Staphylococci*. Resistant *S. aureus* was seen in clinical practice as early as the 1950s, by acquiring a plasmid that encodes the production of β-lactamase enzymes causing resistance to penicillin. Then a synthetic type of penicillin (methicillin) became available for use in 1959. MRSA was described as early as the 1960, this was due to the acquisition of the *mecA gene*, which is responsible for the altered penicillin binding protein PBP2a accounting for MRSA resistance[20]. Now glycopeptide resistance has been described in *Staphylococci* making treatment options more limited and the prognosis worse [21].

Due to the major role of *Staphylococcus* as a major cause of nosocomial infections, this study was conducted to compare between three methods of Staphylococcal identification to evaluate the best method for rapid screening and detection of methicillin strains, so that health care workers can start their patients on the proper antibiotic therapy as soon as possible.

Among the 118 samples collected from nasal swabs in our study, one hundred and twelve *Staphylococcal* isolates were obtained, thirty-two *S. aureus*, and eighty isolates of coagulase negative

staphylococcus (CoNS). The coagulase positive strains 32 (27.1%) showed various susceptibility patterns for different antimicrobial agents with differences depending on the method used, as did the eighty CoNS isolates which showed more Oxacillin resistance. Huebner et al, found that 62-87% of coagulase negative strains were resistant to Oxacillin, Gentamycin and Trimethoprim[22].

Geary et al showed that most coagulase negative strains are resistant to β-lactam antibiotics and produce β-lactamase[23]. Wolfgang study showed that the incidence of Methicillin resistance *S. epidermidis* increased from 28% in 1983 to 77% in 1994 in Finland[24].

The discrepant results in our study cannot be related to technical problems as these results were confirmed by repeated testing. The discrepancies between the three methods can be in apart explained by the heterogeneous expression of resistance, for example Araj et al, study of thirty-two MRSA isolates initially identified by disk diffusion method, then the PCR method was done and only thirteen isolates were positive for the presence of *mecA* gene, seven isolates were found to be resistant by the E-test method[25]. Felten et al, calculated the sensitivities of different methods used for MRSA detection based on PCR as a standard, he showed that the E-test failed to detect resistance in seven isolates that were positive for the presence of *mecA* gene[26]. Ngui et al, evaluated E-test for detection the resistance in selected gram positive bacteria compared with standard reference methods and found that the accuracy of the Oxacillin E-test with *Staphylococci* was significantly improved by the use of salt-supplemented Mueller-Hinton agar[27].

There are other mechanisms of resistance in *Staphylococci,* for example methicillin resistance in *mecA* negative strains of *S. aureus* can be explained by hyper production of β-lactamase enzymes, also the production of a normal PBP but with altered binding capacity. The presence of a positive *mecA* gene doesn't always mean there is *Staphylococcal* resistance to methicillin as susceptible patterns can be seen with the E-test and disk diffusion methods, this could be explained by the presence of incomplete regulator genes (mecI and/or mecRI), also there are strains that posses *mecA* gene without being able to express it.[25] These mechanisms can help explain the discrepant findings among tests in our study.

In conclusion, this study suggest that the current available methods for detecting methicillin resistance for *Staphylococci* had limitations and that more accurate results may be obtained by combining conventional and molecular methicillin resistance detection methods

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