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Comparison of Real-Time PCR with Disk Diffusion, Agar Screen and E-test Methods for Detection of Methicillin-Resistant *Staphylococcus aureus*

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Abstract Methicillin-resistant *Staphylococcus aureus* (MRSA) is a nosocomial pathogen. Our main objective was to compare oxacillin disk test, oxacillin E-test, and oxacillin agar screen for detection of methicillin resistance in *S. aureus*, using real-time PCR for *mecA* as the "gold standard" comparison assay. 196 *S. aureus* isolates were identified out of 284 Staphylococcus isolates. These isolates were screened for MRSA with several methods: disk diffusion, agar screen (6.0 μg/ml), oxacillin E-test, and real-time PCR for detection of *mecA* gene. Of the 196 *S. aureus* isolates tested, 96 isolates (49%) were *mecA*-positive and 100 isolates (51%) *mecA*-negative. All methods tested had a statistically significant agreement with real-time PCR. E-test was 100% sensitive and specific for *mecA* presence. The sensitivity and specificity of oxacillin agar screen method were 98 and 99%, respectively and sensitivity and specificity of oxacillin disk diffusion method were 95 and 93%, respectively. In the present study, oxacillin E-test is proposed as the best phenotypic method. For economic reasons, the oxacillin agar screen method (6.0 μg/ml), which is suitable for the detection of MRSA, is recommended due to its accuracy and low cost.

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Introduction

Staphylococcus aureus is the causal agent of most of the staphylococcus-related diseases which has evolved resistance to all antibiotic classes [17]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a nosocomial pathogen leading to increased morbidity and mortality. Extending the hospitalization period, it considerably increases the costs of healthcare systems [8]. These strains are globally distributed [4]. The increase in MRSA strains and the resultant overuse of glycopeptide antibiotics, has brought about the emergence of vancomycin-resistant *S. aureus* [23]. Methicillin resistance is developed by production of an abnormal penicillin-binding protein (PBP), called PBP2′ or PBP2a, which is encoded by the *mecA* gene [38]. PBPs are enzymes bound to the cell membrane that catalyze transpeptidation reaction, a key step for cross-linkage of peptidoglycan chains [12]. Beta-lactam antibiotics, such as methicillin, are the structural analogues of D-alanyl D-alanine and can bind covalently to PBPs of *S.aureus*. This, in turn, prevents peptidoglycan synthesis and culminates in the destruction of the bacteria. However, since PBP2′ has a very low affinity to beta-lactam antibiotics, peptidoglycan synthesis continues to occur even in their presence, hence leading to the development of resistance to these antibiotics.

Routine methods such as disk diffusion are unable to accurately detect the so-called heterogeneous resistance occurring in oxacillin resistant staphylococci [3]. In heterogeneous resistance, susceptible and resistant sub

populations of *S. aureus* may coexist in the same culture because not all the bacterial cells having resistance genes in their genomic DNA have been able to express them in routine susceptibility testing performed in the laboratory.

Rapid and correct identification of the methicillin resistance gene would ensure proper administration of antibiotics and prompt adoption of epidemiological control measures for MRSA [5]. Laboratory testing to assess the antimicrobial susceptibility include agar dilution, micro-dilution, E-test, and disk diffusion. Disk diffusion is easy to handle and is the mostly used method for detecetion of MRSA in routine laboratories. Unfortunately, the oxacillin disk diffusion test has been shown to be the least reliable test for detection of MRSA [28]. In order to improve the identification problem of oxacillin resistant staphylococci, the oxacillin agar screen test has been used as a supplementary method to the disk assay [34]. Clinical and Laboratory Standards Institute (CLSI) has suggested the oxacillin agar screen to identify the resistant strains of *S.aureus* because of its cost-effectiveness and availability [6].

The gold standard has been MIC (Minimum Inhibitory Concentration) determination by the dilution or E-test methods [18], which have largely been replaced by the faster and more precise molecular methods including PCR and real-time PCR that detect *mecA* gene. Real-time PCR is even faster and more precise than the conventional PCR for determining methicillin resistance. Although more expensive than the conventional PCR, real-time PCR can specially be exploited for urgent cases when time is more critical [30]. Currently, these assays are available in reference and a growing number of routine diagnostic laboratories.

The main objective of this study was to compare disk diffusion, agar screen, E-test, and real-time PCR for detection of methicillin-resistant *S. aureus* in clinical isolates.

Materials and Methods

Bacterial Isolates and Bacteriologic Methods

Two hundred eighty-four Staphylococcus isolates were collected between November 2007 and November 2008. The Isolates were selected randomly from routine clinical specimens including deep and superficial wounds, blood, urine, CSF, and venous catheter etc. No two isolates were collected from the same patient. *Staphylococcus aureus* isolates were identified based on colonial morphology on blood agar (Merck-German) plates, Gram stain characteristics, mannitol fermentation, catalase test, coagulase test, and DNase test agar [14].

Antimicrobial Susceptibility Testing

Oxacillin Disk Diffusion Test

The CLSI (Clinical and Laboratory Standards Institute) reference method for disk diffusion was applied to test oxacillin (1 μg, Himedia-India) [6]. Mueller-Hinton agar plates were inoculated with a suspension (equivalent to a 0.5 McFarland standard) of the *S. aureus* clinical isolates. The plates were then incubated at 35°C, and zone diameters were read at 18 to 24 h. Following breakpoints were considered: Resistant: $\leq$10 mm, intermediate: 11–12 mm and susceptible $\geq$13 mm.

Oxacillin Agar Screen Test:

The suspension of bacteria (adjusted to match 0.5 MacFarland turbidity standard) was inoculated on Muller-Hinton agar (Himedia-India) supplemented with 4% NaCl and 6 μg/ml of oxacillin. Plates were incubated at 35°C for 24 h. Any growth on the plate containing oxacillin was regarded as resistant to methicillin [6].

Oxacillin E-test

Muller-Hinton agar plates supplemented with 0.85% NaCl (Merck) were inoculated by streaking the standardized inoculums with a sterile swab. Oxacillin E-test strips (AB Biodisk, Solna, Sweden) were placed on the plates, followed by an incubation at 37°C for 16–20 h in ambient air. MIC for each isolate was read at the intersection point of the zone of growth inhibition with the graduated strip (Resistant $\geq$ 4, susceptible $\leq$ 2) [10].

Note that in all the tests mentioned above, *S. aureus* (ATCC 29213) and *S. aureus* (ATCC 43300) were used as methicillin sensitive and resistant controls, respectively.

mecA Real-time PCR

Following DNA extraction from bacteria by the Promega Magnesil bead kit, the *mecA* gene was detected using a TaqMan real-time PCR technique in a Rotor Gene 3000 real-time PCR system (Corbett Research). The primers used in the PCR included:

Primer F:5′GGCAATATTACCGCACCTCA3′ and Primer R: ‘GTCTGCCACTTTCTCCTTGT3′ and the probe was: AGATCTTATGCAAACTTAATTGGCAAATCC (FAM was used as the reporter dye that was quenched with 3′ TAMRA) [25]. Each PCR reaction mixture (12.5 μl) contained 6.25 μl master mix (2x.Ampliqon-USA), 0.5 μl of each of the primers (10 PM. Methabion- Germany), 0.5 μl of *mecA* probe (5 PM. Methabion—Germany), 1 μl $MgCl_2$ (50 mM), 1.25 μl H_2O, and 2.5 μl DNA of bacteria. Thermal cycling was carried

out under the following conditions: 2 min at 95°C, followed by 30 cycles of 95°C for 30 s and 60°C for 1 min. The presence of the *mecA* gene was considered the "gold standard" for oxacillin resistance.

Staphylococcus aureus (ATCC 29213) was used as the *mecA* gene negative control and *S. aureus* (ATCC 43300) was used as the *mecA* gene positive control.

Statistical Analysis

Results were analyzed using SPSS statistical software version 13. Difference in susceptibility methods and significance of the results was calculated by the chi-square test or Fisher exact test. The P value of <0.05 was considered to be statistically significant. Validity tests including sensitivity, specificity, positive predictive value, and negative predictive value were calculated; sensitivity was defined as the percentage of *mecA*-positive isolates determined to be nonsusceptible by phenotypic testing, and specificity was defined as the percentage of *mecA*-negative isolates determined to be susceptible by phenotypic testing. The 2006 CLSI criteria were used to designate susceptible or nonsusceptible strains.

Results

One hundred ninety-six out of 284 Staphylococcus isolates were identified as *S. aureus.* Among the 196 strains included in our study, 96 were *mecA*-positive and 100 were *mecA*-negative (Fig. 1). The results of the phenotypic tests for detection of methicillin resistance are shown in Table 1. The results of the three phenotypic methods tested conformed significantly with real-time PCR.

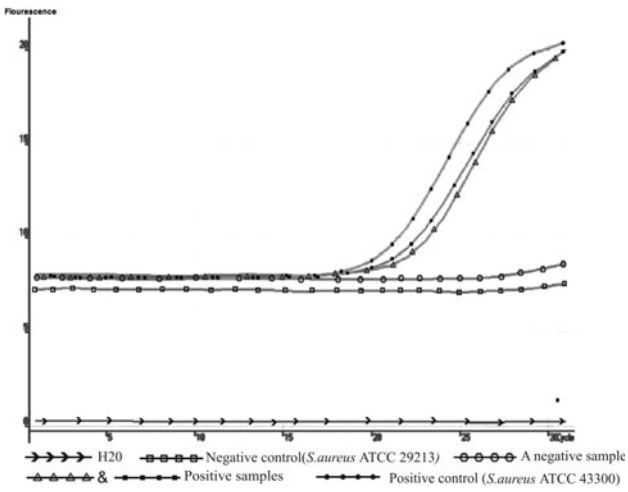

Fig. 1 Detection of the *mecA* gene by real-time PCR. Two positive samples are shown against negative and positive controls as well as H_2O and another negative sample

The validity of the results is shown for all the tested methods (Table 2). The best performance was found with oxacillin E-test with 100% sensitivity, specificity, negative predictive value, and positive predictive value. The sensitivity and specificity of oxacillin agar screen method were 98 and 99%, respectively, in comparison with *mecA* gene real-Time PCR. The sensitivity and specificity of oxacillin disk diffusion method were 95 and 93%, respectively. The result of agar screen test indicate one false positive and two false negatives for the detection of MRSA, while oxacillin disk diffusion indicates seven false positives and five false negatives.

Discussion

MRSA is a major human pathogen accounting for a wide spectrum of diseases [7]. Thus, its accurate diagnosis is vital for patient management. Given a variety of testing methods for detection of MRSA, the obtained data for different surveys are sometimes difficult to compare. To make it worse, laboratories often adopt different standards for operating procedure and interpretation of breakpoint values for the same test [21]. Identification of methicillin-resistant staphylococci in the laboratory is often problematic due to the heterogeneous nature of the resistance and the fact that a variety of variables may affect its expression (i.e., medium, pH, inoculum size, temperature, and salt concentration) [35, 36]. For example, an incubation temperature of 30°C rather than 35°C has been suggested to increase the heterogeneously resistant subpopulations [19]. Thus, we expect only a fraction of the bacterial cell population to be PBP2a positive based on the heterogeneity of *mecA* gene expression [16].

In the present study, we used real-time PCR for rapid and specific detection of *mec*A gene, which is specific for resistance to methicillin/oxacillin, to determine the prevalence of MRSA from the collected clinical samples, and to compare the performance of E-test, agar screen, and disk diffusion methods with real-time PCR in determination of MRSA strains. Several studies have demonstrated the PCR to be a sensitive method for the detection of methicillin resistance in Staphylococci [1, 9, 26, 27, 31]. Furthermore, the main advantage of PCR methods over phenotypic methods is their time saving that enables us to take more rapid infection-control measures.

In this study, the presence of the *mecA* gene correlated 100% with the Oxacillin E-test method. This is in agreement with the results of another study by Ercis et al. [11]. However, sensitivity values of less than 100% have been obtained in most studies. The discrepancy could partly be explained in the light of heterogeneity: it most probably indicates that our studied isolates were not heterogeneous.

Table 1 The results of the phenotypic tests for detection of MRSA and their correlation with the existence of *mecA* verified by real-time PCR

Real-time PCR mecA detection	No. of isolates	No. (%) isolates with the indicated results					
		Ox E-test MIC (μg/ml)		Ox agar screen		Ox disk diffusion	
		≤2	≥4	Growth	No growth	Resistant	Sensitive
Positive	96	0 (0)	96 (100)	94 (97.9)	2 (2.1)	91 (94.8)	5 (5.2)
Negative	100	100 (100)	0 (0)	1 (1)	99 (99)	7 (7)	93 (93)

Ox Oxacillin, *MIC* minimal inhibitory concentration

Table 2 Validity tests for the oxacillin susceptibility test (Real-time PCR as gold standard)

Methods	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Oxacillin E-test	100	100	100	100
Agar screen (6.0 μg/ml)	98	99	99	98
Oxacillin disk diffusion	95	93	93	95

PPV positive predictive value, *NPV* negative predictive value

Our study revealed that oxacillin agar screen is an alternative method with high sensitivity and specificity for detection of MRSA. Our results conform with those of other studies that used the presence of the *mecA* gene as the ''gold standard'' [3, 13, 15, 20, 22, 29, 33].

According to our results, the oxacillin disk diffusion test had slightly lower values of sensitivity and specificity, respectively, for detection of MRSA. Different values of sensitivity and particularly specificity have been reported by different studies [2, 3, 24, 37].

Although disk diffusion method had a statistically significant agreement with real-time PCR, as shown in this study as well as others [26, 28], disk diffusion method is not reliable enough for detection of MRSA. The performance of this test was less than optimal with seven false positives (Table 1). In case medical laboratories use the test for its cost-effectiveness, it must be carried out according to CLSI standards (2007). In addition, when testing strains from invasive or serious infections, laboratories should use a second confirming test before reporting a strain as susceptible.

The conventional methods used for identification of MRSA isolates are generally time consuming. In this study, their reliabilities were found to be between 93 and 99%. In contrast, PCR and real-time PCR for amplification of *mec*A gene, specific for methicillin resistance [32], have been reported to rapidly and specifically detect and characterize MRSA. Although more expensive, real-time PCR is even more efficient than the conventional PCR as far as time is concerned. Reliable and rapid detection of patients infected by MRSA strains is pre-requisite to the successful prevention and control of MRSA infection outbreak in hospitals.

According to the results of the study, E-test was the best phenotypic method that can be recommended for detection of MRSA strains. For economic reasons, however, the oxacillin agar screen method (6.0 μg/ml), may be advocated in diagnostic laboratories due to its accuracy, reproducibility, and cost-effectiveness.

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