Detection of Methicillin Resistance in *Staphylococcus aureus* Isolated from Pediatric Patients: Is the Cefoxitin Disk Diffusion Test Accurate Enough?

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We evaluated the performance of several methods for the detection of methicillin resistance in *Staphylococcus aureus* using 101 clinical *S. aureus* isolates from pediatric patients in a tertiary hospital in Brazil; 50 isolates were *mecA*-positive and 51 were *mecA*-negative. The Etest and oxacillin agar screening plates were 100% sensitive and specific for *mecA* presence. Oxacillin and cefoxitin disks gave sensitivities of 96 and 92%, respectively, and 98% specificity. Alterations of CLSI cefoxitin breakpoints increased sensitivity to 98%, without decreasing specificity. Our results highlight the importance of a continuing evaluation of the recommended microbiological methods by different laboratories and in different settings. If necessary, laboratories should use a second test before reporting a strain as susceptible, especially when testing strains isolated from invasive or serious infections. With the new (2007) CLSI breakpoints, the cefoxitin-disk test appears to be a good option for the detection of methicillin resistance in *S. aureus*.

Key-Words: MRSA, disc diffusion, cefoxitin, *mecA*.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major epidemiological and clinical problem over the last decades. These strains have spread worldwide, causing nosocomial and, more recently, community-based infections [1]. This has led to the overuse of glycopeptides, and to the emergence of vancomycin-resistant *S. aureus* [2]. In this setting, rapid and accurate detection of methicillin resistance would help ensure correct use of antibiotics and appropriate epidemiological control of MRSA. Methicillin resistance in *S. aureus* is primarily mediated by overproduction of PBP2a protein, an altered penicillin-binding protein with lower affinity for beta-lactam antibiotics than PBP2, the main physiological methicillin target. PBP2a is encoded by the *mecA* gene, a component of a larger DNA fragment designated the *mec* region. Phenotypic expression of resistance may vary depending on culture conditions, such as temperature or osmolarity of the medium, despite genetic homogeneity [3].

This heterogeneous resistance phenotype may complicate the detection of MRSA by conventional susceptibility methods. Oxacillin-disk diffusion has been the traditional method for methicillin-resistance routine screening; but recently good accuracy of the cefoxitin disk for predicting methicillin resistance has been reported [4-11], and CLSI (Clinical and Laboratory Standards Institute) has recommended that cefoxitin should be preferred over oxacillin for the detection of *mecA*-mediated resistance [4]. However, the choice of the best phenotypic method for detecting methicillin resistance in *S. aureus* remains controversial. Good accuracy of other methods, such as Etest and oxacillin-agar screening plate, has also been demonstrated [12-15]. Detection of the gene is considered the reference method [3], but this is not feasible in most laboratories throughout the world.

Our main objective was compare oxacillin and cefoxitin-disk tests, Etest and oxacillin-agar screening plates for detection of methicillin resistance in *S. aureus*, using PCR for *mecA* as the "gold standard" comparison assay.

Materials and Methods

Strains

We studied 101 pediatric clinical isolates of *S. aureus* (isolated from different anatomical sites of different pediatric patients) that were collected from April 2004 to June 2005 and identified by biochemical procedures. *Staphylococcus aureus* ATCC 25923, ATCC 29213 and ATCC 33591 were used as quality-control strains.

Detection of the *mecA* Gene

A single bacterial colony was obtained from a fresh subculture and was resuspended in 25 μL of sterile water. The suspension was boiled at 95°C for DNA extraction. One microliter of the DNA samples was added to 19 μL of PCR mixture, consisting of 1U *Taq* polymerase, 1X polymerase buffer [50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$], 200 μM dNTPs mixture and 0.5 μM of each primer. Amplification was performed using a Perkin Elmer 2400 thermocycler (Applied Biosystems, California, USA). After an initial denaturation step (three minutes at 94°C), 30 cycles of amplification were performed: denaturation at 94°C for one minute, annealing at 56°C for one minute and DNA extension at 72°C for one minute. The reaction was finished with a final extension step at 72°C for seven

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minutes. The set of primers used (M1, 5'-TGGCTATCGTGTCACAATCG-3' and M2, 5'-CTGGAACTTGTT-GAGCAGAG-3') was described by Vannuffel [16]. The amplified product was a 310-bp DNA fragment that was detected by 1.5% agarose gel electrophoresis, with ethidium bromide staining visualized under UV light.

Susceptibility Tests

The isolates were tested with oxacillin (1 µg) and cefoxitin (30 µg) disks, using Mueller-Hinton agar plates inoculated with a suspension (equivalent to a 0.5 McFarland standard) of the *S. aureus* clinical isolates. The plates were incubated at 35ºC for 24 hours and inhibition zones were measured. The oxacillin MICs (minimum inhibitory concentration) were determined by Etest (AB Biodisk, Solna, Sweden) using Mueller-Hinton agar plates supplemented with 2% NaCl. The CLSI 2005 criteria [4] were used for interpretation (Table 1). Isolates were also tested with oxacillin agar screening, which was performed by inoculating a direct colony suspension (0.5 McFarland standard) with a swab, spotting an area 10 to 15 mm in diameter, on Mueller-Hinton agar supplemented with 4% NaCl and oxacillin at 6 mg/L. After incubation for 24 hours, any growth was interpreted as a positive result for MRSA.

Results and Discussion

Among the 101 strains included in our study, 50 were *mecA*-positive and 51 were *mecA*-negative. The results of the phenotypic tests are shown in Table 2. Etests and oxacilin plates were the most accurate methods. Both were 100% sensitive and specific.

In other studies that used the presence of *mecA* as the gold standard, the accuracy of these techniques was also very good [12-15].

The oxacillin and the cefoxitin disk tests showed sensitivities of 96% and 92%, respectively, and 98% specificity. Six strains had discrepant results between at least one of the disks and the *mecA* gene (Table 3). The accuracy of the cefoxitin disk test in our study was different from that previously reported. However, among the four *mecA*-positive strains giving false-negatives by the cefoxitin test, three had an inhibition zone of 20 mm, and none of the *mecA*-negative strains had such a large zone diameter. Thus, if there were changes in the cefoxitin breakpoints, the sensitivity of the method could be increased, perhaps without any decrease of specificity. Other authors have also suggested different breakpoints in the interpretative zone diameters of cefoxitin for better detection of methicillin resistance in *S. aureus* [7,10].

We evaluated our strains in 2005, using the former CLSI cefoxitin breakpoints (R: ≤ 19 mm; S: ≥ 20 mm). But, in 2007 the recommended CLSI breakpoints were changed [17], in order to increase the accuracy of the method. With the new breakpoints (R: ≤ 21 mm; S: ≥ 22 mm), the three strains that had a 20 mm zone would have been correctly diagnosed, increasing the sensitivity to 98%, again without any decrease in specificity, since we did not find any *mecA*-negative strains with inhibition zones smaller than 22 mm.

Although the number of isolates tested in our study was low and there was a chance of clonality, our results highlight the importance of a continuing evaluation of recommended microbiological methods by different laboratories and in different settings. If necessary and if the evidence supports it,

Table 1. CLSI 2005 Interpretative criteria for methicillin resistance in *Staphylococcus aureus* isolated from pediatric patients [4].

	Inhibition zone (mm)		
	R	I	S
Oxacillin disk	≤10	11-12	≥13
Cefoxitin disk	≤19	-	≥20
	Minimum inhibitory concentration (µg/mL)		
	R	-	S
Oxacillin	≥4	-	≤2

R: resistant; S: susceptible; I: intermediate.

Table 2. Results of the phenotypic tests of *Staphylococcus aureus* isolated from pediatric patients and correlation with *mecA*.

	Etest		**Agar screening**		**Oxacillin disk**		**Cefoxitin disk**	
	R	**S**	**R**	**S**	**R ou I**	**S**	**R**	**S**
mecA+	50/50	0/50	50/50	0/50	48/50	2/50	46/50	4/50
mecA-	0/51	51/51	0/51	51/51	1/51	50/51	1/51	50/51

R: resistant; S: susceptible.

Table 3. Isolates with discrepant results between at least one of the disks and the *mecA* gene in *Staphylococcus aureus* isolated from pediatric patients

Isolate	*mecA*	Oxacillin (mm)		Cefoxitin (mm)		Etest (µg/mL)	Agar screening
1	-	12	I	16	R	0.5	S
2	+	0	R	20	S	>256	R
3	+	14	S	18	R	>256	R
4	+	16	S	20	S	>256	R
5	+	0	R	20	S	>256	R
6	+	0	R	22	S	>256	R

R: resistant; S: susceptible.

the method should be withdrawn from the recommendations or its breakpoints changed. In addition, laboratories could use a second test before reporting a strain as susceptible, especially when testing strains from invasive or serious infections.

Conclusion

In our study the oxacillin agar screening plate appeared to be a good option for the detection of methicillin resistance in *S. aureus*, due to its great accuracy and low cost. With the new (2007) CLSI breakpoints, the cefoxitin-disk test may also be a reasonable alternative.

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