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Antimicrobial Susceptibility Studies

Agar diffusion, agar dilution, Etest®, and agar screening test in the detection of methicillin resistance in staphylococci☆

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Abstract

Methicillin resistant Staphylococcus is an important worldwide problem. Resistance is verified in strains harboring the *mecA* gene and laboratory methods used to detect resistance are object of constant investigation. In the present study, 99 clinical isolates of staphylococci (41 *S. aureus,* 33 *S. epidermidis,* 12 *S. saprophyticus* and 13 members of other species) were submitted to different phenotypic methods and conditions. Detection of the *mecA* gene by PCR was used as the reference method and detected 14/41, 10/33, and 10/25 isolates of *S. aureus, S. epidermidis* and other species, respectively. Results showed that, for *S. aureus* and *S. epidermidis,* agar diffusion, agar dilution, and the E test incubated during 24h at 35°C correctly discriminated *mecA* positive from *mecA* negative isolates. For other species, all methods and conditions presented low specificity (ranging from 20% to 66.7%) and, particularly *S. saprophyticus,* may need molecular methods to correctly assess methicillin resistance. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Members of the genus *Staphyloccoccus* are among the most important human pathogens, causing both community and nosocomial infections. In the United States of America, such organisms are responsible for half of all bloodstream infections of patients in Intensive Care Units and *Staphylococcus aureus* is one of the leading causes of pneumonia in this setting (NNIS, 1999). The emergence of methicillin resistance in staphylococci is a major global problem. In Latin America, 50.4% isolates of *S. aureus* implicated in pneumonia present resistance to methicillin (Sader et al., 1998). A multicentric study carried out in Brazil showed that methicillin resistance was observed in 29% of *S. aureus* isolates and in 87.7% of coagulase-negative staphylococci (CNS) isolated from bloodstream infections (Sader et al., 1999).

Methicillin resistance in staphylococci results from to the production of a modified Penicillin Binding Protein (PBP2a), encoded by the *mecA* gene. Molecular detection of the *mecA* gene is the reference method to assess methicillin resistance. Phenotypic methods for detection of methicillin resistance are object of continuous investigation, particularly experimental conditions as temperature and period of incubation, NaCl concentration, and inoculum density (Huang et al., 1993; Mulder et al., 1996; York et al., 1996; Weller et al., 1997; Frebourg et al., 1998; Kampf et al., 1998; Hussain et al., 1998; Kohner et al., 1999; Tenover et al., 1999; Hussain et al., 2000). The National Committee for Clinical Laboratory Standards (NCCLS) has recently introduced two important modifications. In 1998, new breakpoints for CNS were defined for both diffusion and dilution methods (NCCLS, 1998) and, since 2001, susceptibility tests are no longer recommended for *S. saprophyticus* (NCCLS, 2001).

The objective of this study was to evaluate different laboratory methods for detecting methicillin resistance in both *S. aureus* and CNS.

2. Materials and methods

Bacterial isolates: 100 consecutive clinical isolates of staphylococcal infections from patients at Hospital Mãe de

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Table 1
Comparison of susceptibility testing methods to *mecA* gene detection for 99 staphylococci

Method	NaCl[a]	T (°C)	Incubation period (hours)	*S. aureus* (n = 41)				*S. epidermidis* (n = 33)	
				R[b]/mecA pos	S[c]/mecA neg	Sensitivity (%)	Specificity (%)	R[b]/mecA pos	S[c]/mecA neg
Agar diffusion	0%	35	24	14/14	27/27	100	100	10/10	23/23
			48	14/14	27/27	100	100	10/10	23/23
E test	0%	35	24	14/14	27/27	100	100	10/10	23/23
			48	14/14	27/27	100	100	10/10	23/23
Agar dilution	0%	35	24	14/14	27/27	100	100	10/10	23/23
			48	14/14	27/27	100	100	10/10	22/23
		30	24	14/14	27/27	100	100	10/10	22/23
			48	14/14	27/27	100	100	10/10	22/23
	4%	35	24	14/14	24/27	100	88.9	10/10	22/23
			48	14/14	19/27	100	70.4	10/10	22/23
		30	24	14/14	24/27	100	88.9	10/10	23/23
			48	14/14	18/27	100	66.7	10/10	22/23
ASO[e]	4%	35	24	14/14	27/27	100	100	10/10	23/23
			48	14/14	23/27	100	85.2	10/10	15/23
		30	24	14/14	27/27	100	100	10/10	18/23
			48	14/14	21/27	100	81.5	10/10	16/23

[a] NaCl supplementation.
[b] R, resistant.
[c] S, susceptible.
[d] CNS, coagulase-negative staphylococci.
[e] ASO, agar-screening with 4% NaCl and oxacillin (6.0 μg/ml for *S. aureus* or 0.6 μg/ml for other species).

Deus, Porto Alegre, Brazil, were included. ATCC strains 25923 and 29213 were used for quality control (NCCLS, 2001). Tube coagulase test and biochemical tests by use of Positive Combo Panel Type 14 (Dade Behring Inc, West Sacramento, CA) were employed for identification of the isolates. Strains were kept frozen at -20°C in skim milk with 10% glycerol. One *S. aureus* isolate was not viable for susceptibility studies after storage.

Susceptibility tests: Isolates were inoculated on 5% sheep blood agar (35°C, 18–24 h). A Mc Farland 0.5 suspension was prepared for each isolate. Except for agar dilution, in which a 1:10 dilution was made prior to inoculation, other tests were directly inoculated with this suspension. Agar diffusion tests (NCCLS, 2000a) were incubated at 35°C, 24 and 48 h of incubation. The E test® (AB Biodisk, Solna, Sweden) was carried out according to manufacturer's instructions on Mueller-Hinton agar and incubated at 35°C, 24–48 h of incubation. For agar dilution tests, Mueller-Hinton agar plates with oxacillin concentrations ranging from 0.12 to 8 μg/ml were inoculated by Steers' replicator. Plates were supplemented with 0% or 4% NaCl and independently incubated at 30°C and 35°C. Results were observed and registered at 24 and 48 h of incubation. The agar screening procedure for *S. aureus* was performed according to previous recommendations (NCCLS, 2000b), i.e., Mueller-Hinton agar supplemented with 4% NaCl and 6 μg/ml of oxacillin. Plates were inoculated with a swab (spot) and incubated at two temperatures, 30°C and 35°C, for 24 and 48 h. The same screening procedure was followed for coagulase negative species, except that oxacillin concentration was 0.6 μg/ml (Kohner et al., 1999).

Mueller-Hinton agar plates used in diffusion tests, in the E-test, and 1μg oxacillin disks were purchased from Laborclin (Pinhais, PR, Brazil). Plates used in the agar dilution tests and in the screening tests were prepared in house using oxacillin from Sigma Chemical CO.(St. Louis, Mo) and Mueller-Hinton agar from Oxoid (Hampshire, England).

Amplification of *mecA* gene: The presence of the *mecA* gene was detected following a PCR procedure described elsewhere (Vannuffel et al., 1998). Primers used were *mecA*1: 5′-TGG CTA TCG TGT CAC AAT CG and *mecA*2: 5′- CTG GAA CTT GTT GAG CAG AG that amplify a 310-bp segment of the gene, visualized after electrophoresis on 2% agarose gels containing ethidium bromide. Reagents used in amplification were from Life Technologies (Gaithersburg, MD, USA).

3. Results

S. aureus was the species most frequently found (42 isolates), followed by *S. epidermidis* (33 isolates). Other coagulase negative species included *S. saprophyticus* (12 isolates), *S haemolyticus* (5 isolates), *S. hominis* (2 isolates), *S. lugdunensis* (2 isolates) and *S. cohni, S. schleiferi, S. simulans* and *S. warneri* (1 isolate each). The presence of *mecA* gene was observed in 14/41 (34.1%) *S. aureus* isolates, 10/33 (30.3%) *S. epidermidis,* in 2/12 (16.7%) *S. saprophyticus,* and in 8/13 (61.5%) isolates belonging to other coagulase negative species. Results of the different phenotypic tests are shown in the table.

		S. saprophyticus (n = 12)				CNS[d] other than *S. epidermidis* and *S. saprophyticus* (n = 13)			
Sensitivity (%)	Specificity (%)	R[b]/mecA pos	S[c]/mecA neg	Sensitivity (%)	Specificity (%)	R[b]/mecA pos	S[c]/mecA neg	Sensitivity (%)	Specificity (%)
100	100	1/2	5/10	50	50	8/8	5/5	100	100
100	100	1/2	5/10	50	50	8/8	5/5	100	100
100	100	2/2	1/10	100	10	8/8	3/5	100	60
100	100	2/2	1/10	100	10	8/8	3/5	100	60
100	100	2/2	1/10	100	10	7/8	3/5	87.5	60
100	95.6	2/2	0/10	100	0	8/8	3/5	100	60
100	95.6	2/2	0/10	100	0	8/8	3/5	100	60
100	95.6	2/2	0/10	100	0	8/8	3/5	100	60
100	95.6	2/2	0/10	100	0	8/8	4/5	100	80
100	95.6	2/2	0/10	100	0	8/8	4/5	100	80
100	100	2/2	0/10	100	0	7/8	4/5	87.5	80
100	95.6	2/2	0/10	100	0	8/8	5/5	100	100
100	100	1/2	6/10	50	60	8/8	4/5	100	80
100	65.2	1/2	2/10	50	20	8/8	3/5	100	60
100	78.3	1/2	2/10	50	20	8/8	4/5	100	80
100	69.6	1/2	1/10	50	10	8/8	4/5	100	80

4. Discussion

The detection of *mecA* gene is used as the reference method for methicillin resistance in staphylococci. Strains carrying this gene are resistant to all β-lactamic antimicrobial agents, representing an enormous impact in the treatment of staphylococcal infections. Laboratory methods for detecting resistance are influenced by variables as temperature and period of incubation, inoculum density, and salt concentration used in culture media. The NCCLS recommendations were recently modified as an attempt to adjust results of phenotypic tests with those of *mecA* gene detection for CNS (NCCLS, 1998; NCCLS, 2001).

The present study verified the performance of different phenotypic methods (agar diffusion and dilution, the E test and agar screening) in the definition of methicillin resistance in clinical isolates of staphylococci. Conditions as period and temperature of incubation, and NaCl concentration were also evaluated.

Overall, the performance of agar diffusion test was convenient to all species investigated, except *S. saprophyticus,* when NCCLS recommendations were followed (100% sensitive and specific). Studies accomplished in the past showed a lower sensitivity of diffusion tests in the detection of *mecA* among CNS, however, breakpoints used for definition of resistance were those used before 1998 (zone diameter 10 ≤mm for resistance) (York et al., 1996, Wallet et al., 1996; Mulder et al., 1996; Kohner et al., 1999).

The E test results were also 100% sensitive and specific for *S. aureus* and *S. epidermidis* under standard conditions. However, this test was not satisfactory to assess methicillin resistance of *S. saprophyticus* and, due to low specificity, requires further evaluation to other CNS species.

Agar dilution tests were accurate to discriminate between strains carrying the *mecA* gene from others in *S. aureus* and *S. epidermidis,* when agar without NaCl supplementation was used under standard conditions (35°C –24 h of incubation). The NCCLS, based on previous studies (Huang et al., 1993; Baker et al., 1994), recommends 2% NaCl supplementation for dilution based methods. In our study, the use of 4% NaCl supplementation generated false resistant results for both *S. aureus* and *S. epidermidis.* Modifications in the agar dilution tests as prolonged period of incubation (48 h), and lower temperature of incubation (30°C), did not improve the detection of strains carrying the *mecA* gene. In general, such modifications produced false resistant results, impairing the specificity of the test. The use of agar dilution in CNS other than *S. epidermidis* is limited due to false-resistant results. Our results are consistent with those of a multicentric study in which the major problem was the remarkable number of false-resistant results, mainly with CNS other than *S. epidermidis* (Tenover et al., 1999). Besides this, it was recently demonstrated that although the current breakpoint correctly classified strains of *S. epidermidis, S. haemolyticus* and *S. hominis* without *mecA* as susceptible, it was not specific for *S. cohnii, S. lugdunensis, S. saprophyticus, S. warneri* and *S. xylosus* (Hussain et al., 2000).

The screening agar used for *S. aureus* showed 100% sensitivity and specificity when the incubation period was limited to 24 h. This screening procedure is no longer recommended by the NCCLS (1998) for CNS. The use of 0.6 μg/ml, based on the tenfold decrease in the resistance breakpoint, was proposed for CNS in a recent study (Kohner et al., 1999). Our results using this concentration showed a 100% correlation with *mecA* gene detection for *S. epidermidis* when 35°C and 24 h of incubation were employed. For other species of CNS, however, and particularly to *S. saprophyticus,* the screening using 0.6 μg/ml can not be recommended.

In summary, our results indicated that for the two most clinically relevant species, *S. aureus* and *S. epidermidis,* the following methods and conditions correctly discriminated *mecA*-positive from *mecA*-negative isolates: agar diffusion

(24h, 35°C), E test (24h, 35°C), agar dilution (24h, 35°C, 0% NaCl supplementation). For *S. aureus,* the agar screening with 4%NaCl, 6.0 μg/ml oxacillin (24h, 30–35°C) was also adequate, as well as, for *S. epidermidis,* the agar screening with 0.6 μg/ml oxacillin (24h, 35°C). Modifications produced false resistant results in *mecA*-negative isolates, lowering specificity, especially when used in combinations. The generic identification "coagulase-negative staphylococci" may be inappropriate once its put together species that respond differently to the same experimental conditions with respect to *mecA* gene detection. Further studies may better define breakpoints for methicillin resistance in CNS other than *S. epidermidis.* The present study used only 25 of such isolates and a greater number would be required for validation of the interpretation.

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References

Baker, C. N., Huang, M. B., & Tenover, F. C. (1994). Optimizing testing of methicillin-resistant *Staphylococcus* species. *Diagnostic Microbiology and Infectious Disease, 19,* 167–170.

Frebourg, N. B., Nouet, D., Lemée, L., Martin, E., & Lemeland, J. F. (1998). Comparison of ATB Staph, Rapid ATB Staph, Vitek, and E-Test methods for detection of oxacillin heteroresistance in staphylococci possessing *mecA. Journal of Clinical Microbiology, 36*, 52–57.

Huang, M. B., Gay, T. E., Baker, C. N., Banerjee, S. N., & Tenover, F. C. (1993). Two percent sodium chloride is required for susceptibility testing of staphylococci of oxacillin when using agar-based dilution mehtods. *Journal of Clinical Microbiology, 25,* 2683–2688.

Hussain, Z., Stoakes, L., Lannigan, R., Longo, S., & Nancekivell, B. (1998). Evaluation of screening and commercial methods for detection of methicillin resistance in coagulase-negative staphylococci. *Journal of Clinical Microbiology, 36*, 273–74.

Hussain, Z., Stoakes, L., Massey, V., Diagre, D., Fitzgerald, V., El Sayed, S., & Lannigan, R. (2000). Correlation of oxacillin MIC with *mecA* gene carriage in coagulase-negative staphylococci. *Journal of Clinical Microbiology, 38*, 752–54.

Kampf, G., Lecke, C., Cimbal, A. K., Weist, K., & Rüden, H. (1998). Evaluation of mannitol salt agar for detection of oxacillin resistance in *Staphylococcus aureus* by disk diffusion and agar screening. *Journal of Clinical Microbiology, 36*, 2254–57.

Kohner, P., Uhl, J., Kolbert, C., Persing, D., & Cockerill, III F. (1999). Comparison of susceptibility testing methods with *mecA* gene analysis for determining oxacillin (methicillin) resistance in clinical isolates of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. *Journal of Clinical Microbiology, 37*, 2952–61.

Mulder, J. G. (1996). Comparison of disk diffusion, the E-test, and detection of *mecA* for determination of methicillin resistance in coagulase-negative staphylococci. *European Journal of Clinical Microbiology and Infectious Disease, 15*, 567–73.

National Committee for Clinical Laboratory Standards. (1998). Performance standards for antimicrobial susceptibility testing. M100–S8. Wayne, PA: National Committee for Clinical Laboratory Standards.

National Committee for Clinical Laboratory Standards. (2000a). Performance standards for antimicrobial disk susceptibility tests. M2–A7. Wayne, PA: National Committee for Clinical Laboratory Standards.

National Committee for Clinical Laboratory Standards. (2000b). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standards. M7–A5. Wayne, PA: National Committee for Clinical Laboratory Standards.

National Committee for Clinical Laboratory Standards. (2001). Performance standards for antimicrobial susceptibility testing. M100–S11. Wayne, PA: National Committee for Clinical Laboratory Standards.

National Nosocomial Infections Surveillance (NNIS) System. (1999). National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1990-May 1999, issued June 1999. *American Journal of Infection Control, 27:520–532.*

Sader, H., Jones, R. N., Gales, A. C., Winokur, P., Kugler, K. C., Pfaller, M. A., Doern, G., & SENTRY Latin America Study Group. (1998). Antimicrobial susceptibility patterns for pathogens isolated from patients in Latin America Medical Centers with a diagnosis of pneumonia: analysis of results from the SENTRY Antimicrobial Surveillance Program (1997). *Diagnostic Microbiology and Infecitous Disease, 32,* 289–301.

Sader, H., Sampaio, J. M. L., Zoccol C., & Jones, R. N. (1999). Results of the 1997 SENTRY Antimicrobial Surveillance Program in three Brazilian medical centers. *Brazillian Journal of Infectious Disease, 3*, 63–79.

Tenover, F. C., Jones, R. N., Swenson, J. M., Zimmer, B., McAllister, & S., Jorgensen, J. H. (1999). Methods for improved detection of oxacillin resistance in coagulase-negative staphylococci: Results of a multicenter study. *Journal of Clinical Microbiology, 37*, 4051–58.

Vannuffel, P., Laterre, P. F., Bouyer, M., Gigi, J., Vandercam, B., Reynaert, M., & Gala, J. L. (1998). Rapid and specific molecular identification of methicillin-resistant *Staphylococcus aureus* in endotracheal aspirates from mechanically ventilated patients. *Journal of Clinical Microbiology, 36*, 2366–2368.

Wallet, F., Roussel-Delvallez M., & Courcol, R. J. (1996). Choice of routine method for detecting methicillin-resistence in staphylococci. *Journal of Antimicrobial Chemotherapy, 37,* 901–909.

Weller, T. M. A., Cook, D. W., Crow, M. R., Ibrahim, W., Pennington, T. H., & Selkon, J. B. (1997). Methicillin susceptibility testing of staphylococci by E-test and comparison with agar dilution and *mecA* detection. *Journal of Antimicrobial Chemotherapy, 39*, 251–253.

York, M. K., Gibbs, L., Chehab, F., & Brooks, G. F. (1996). Comparison of PCR detection of *mecA* with standard susceptibility testing methods to determine methicillin resistance in coagulase-negative staphylococci. *Journal of Clinical Microbiology, 34*, 249–53.