

Evaluation of a Triplex PCR Assay To Discriminate *Staphylococcus aureus* from Coagulase-Negative Staphylococci and Determine Methicillin Resistance from Blood Cultures

N. Maes,^{1*} J. Magdalena,^{1†} S. Rottiers,¹ Y. De Gheldre,¹ and M. J. Struelens¹

Center for Molecular Diagnostics, Service de Microbiologie, Hôpital Erasme,
Université Libre de Bruxelles, Brussels, Belgium

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A triplex PCR targeting the 16S rRNA, *mecA*, and *nuc* genes was developed for identification of staphylococci and detection of methicillin resistance. After validation of the assay with a collection of strains of staphylococci and enterococci ($n = 169$), the assay was evaluated with cultures of blood with gram-positive cocci from 40 patients. Accurate results were obtained for 59 (98%) of 61 cultures within 6 h of growth detection.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) are important causes of nosocomial infections (12, 15, 20, 22). As methicillin resistance mediated by PBP 2a is often heterogeneously expressed in staphylococci (2, 7, 24), PCR detection of *mecA* gene is the “gold standard” for the detection of methicillin resistance (1, 2, 10, 16, 21). In the case of severe infections, it is clinically useful to provide rapid identification and antimicrobial susceptibility test results (5). Many multiplex PCR techniques have been described for the characterization of staphylococci (4, 13, 14, 25, 27). Most of these are performed with genomic DNA extracts from bacterial colonies or broth culture lysates. Evaluation of *mecA* detection by PCR with simulated positive blood cultures, with blood samples spiked with staphylococci (4, 27), or from blood cultures directly has also been reported (6, 11; S. M. Benson, G. W. Coombs, and I. D. Kay, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 877, 1999). This report describes a PCR assay that targets three genes: *mecA*, a determinant of methicillin resistance; *nuc*, which encodes the *S. aureus*-specific region of the thermonuclease gene; and a genus-specific 16S rRNA sequence used as an internal amplification control for staphylococcal DNA. The expected sizes of the amplified DNA fragments are 533, 279, and 750 bp, respectively (Fig. 1). The objectives of this study were to evaluate the accuracy of characterization of staphylococci by the 16S rRNA-*mecA*-*nuc* triplex PCR directly from blood cultures and to compare its diagnostic performance with that of conventional methods in terms of specificity, sensitivity, and detection time.

Bacterial strains were phenotypically characterized by determination of coagulase activity and an agglutination test with a Pastorex Staph Plus kit (Sanofi Diagnostics Pasteur, Marnes la Coquette, France), and coagulase-negative staphylococci (CoNS) were identified with the ID 32 Staph system (Bio-

mérieux, Marcy l’Etoile, France). The strains were tested for oxacillin susceptibility by use of the Oxacillin Agar Screen (Becton Dickinson, Sparks, Md.) and the rapid ATB Staph system (Biomérieux) for *S. aureus* and by a 1- μ g disk (Rosco, Taastrup, Denmark) diffusion test for CoNS. Oxacillin MICs were determined by either the E-test (AB Biodisk, Solna, Sweden) for the reference strains of CoNS or the agar dilution method according to the guidelines of NCCLS (19) for the clinical isolates of CoNS selected for assay validation. The sets of primers were previously reported by Murakami et al. (18), Brakstad et al. (3), and Jaffe et al. (11), respectively.

In the first part of the study, the amplification conditions were optimized and validated with 169 well-characterized clinical isolates and reference strains (Table 1). The clinical isolates were blood isolates of MRSA ($n = 88$) and methicillin-susceptible *S. aureus* (MSSA; $n = 24$) selected from a multicenter survey of *S. aureus* bacteremia in Belgian hospitals conducted in 1999 and 2000 and blood isolates of CoNS ($n = 25$) obtained from a prospective study of bacteremia in patients admitted to Erasme Hospital during 1998 and 1999 (9). The *Staphylococcus* and *Enterococcus* isolates were grown on Columbia agar with 5% sheep blood (Biomérieux) for 24 h at 37°C. DNA was extracted from the colonies as described by Unal et al. (26). The 50- μ l PCR mixture contained 1 \times PCR buffer (Perkin-Elmer Applied Biosystems, Foster City, Calif.), 2 mM MgCl₂, 16S rRNA-specific primers (0.6 μ M) and *mecA*- and *nuc*-specific primers (0.4 μ M each) (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), deoxynucleoside triphosphates (250 μ M each; Promega, Madison, Wis.), and AmpliTaq Gold DNA polymerase (2 U; Perkin-Elmer Applied Biosystems). A DNA sample of 5 μ l was used as the target in the PCR. Amplification conditions consisted of 10 min at 94°C, followed by 23 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C, with a final step of 5 min at 72°C. The DNA fragments were separated by electrophoresis on a 1.5% agarose (Invitrogen, Merelbeke, Belgium) gel stained with ethidium bromide.

The PCR results for *nuc* and *mecA* detection were in agreement with those of conventional methods for 100 and 98% of strains, respectively. The staphylococcal 16S rRNA sequence was amplified for 98% of the strains. Discrepancies between phenotypic resistance in the absence of an amplified *mecA*

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Hôpital Erasme, 808, Route de Lennik, 1070 Brussels, Belgium. Phone: 32 2 555 45 17. Fax: 32 2 555 3110. E-mail: nimaes@ulb.ac.be.

† Present address: Division of Molecular Cell Biology, School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom.

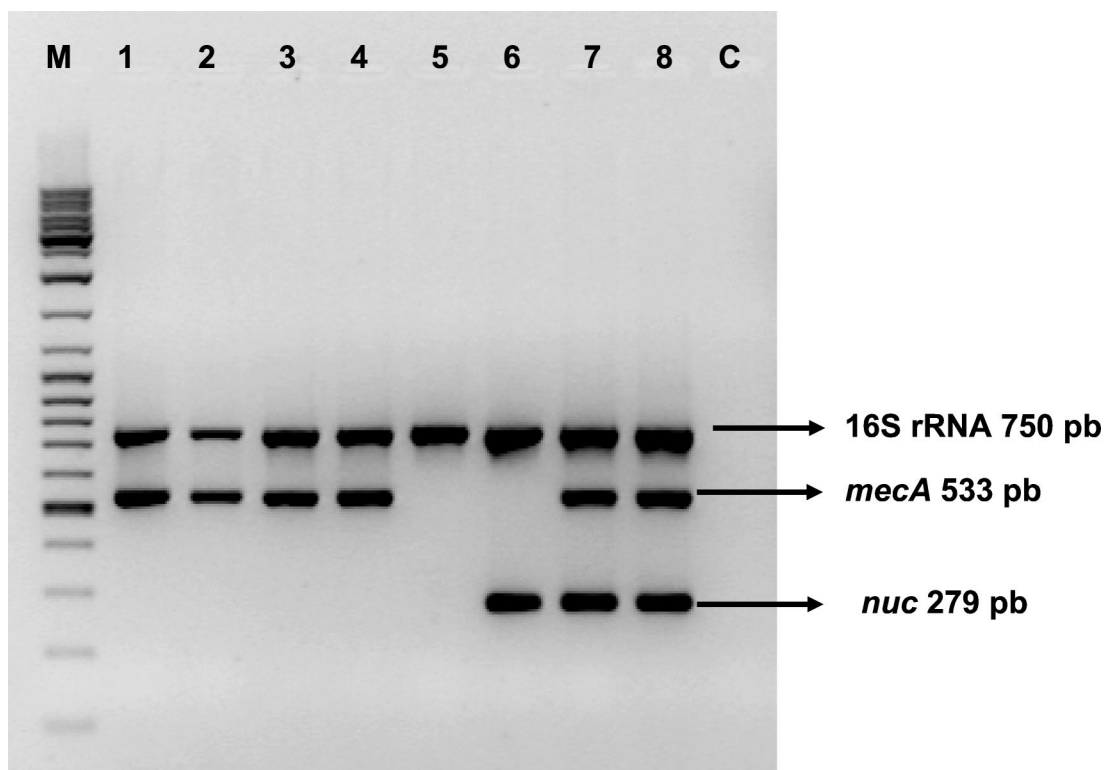


FIG. 1. Interpretation of PCR results after electrophoresis on an agarose gel. Lane M, molecular size marker (MBI Fermentas ladder mix); lanes 1 to 4, MRCoNS; lane 5, MSCoNS; lane 6, MSSA; lanes 7 and 8, MRSA; lane C, negative control; pb, base pairs.

gene fragment was observed with *Staphylococcus sciuri* subsp. *rodentium*, *Staphylococcus cohnii*, and *Staphylococcus lugdunensis* (Table 1). A weak *mecA* PCR signal was noted with *S. sciuri* subsp. *sciuri* and *S. sciuri carnaticus* strains, but no signal appeared with *S. sciuri* subsp. *rodentium*. Most of the *S. sciuri* strains carry a genetic element closely related to the *mecA* gene (8), and some *S. sciuri* isolates harbor a second copy of the gene identical to *mecA* (8). Therefore, our PCR results suggest that sequence variations in the *S. sciuri mecA* gene are probably localized in the region recognized by the *mecA*-specific primers. *mecA* failed to be amplified from isolates of *S. cohnii* and *S. lugdunensis* for which oxacillin MICs were 0.75 and 0.5 $\mu\text{g/ml}$, indicating that this borderline resistance is mediated by a mechanism other than *mecA*. The 16S rRNA signal was weak with *Staphylococcus simulans* and was absent with *S. sciuri* strains. The lack of a complete 16S rRNA sequence for these two species precluded a comparison of the region recognized by the primers.

In the second part of the study, the triplex PCR assay was prospectively evaluated directly with cultures of blood specimens from 40 patients for whom cultures of at least two blood samples showed the growth of gram-positive cocci in clusters. A total of 61 BACTEC-Plus (Becton Dickinson) blood culture broths were analyzed, as were colonies obtained by subculture of the broth cultures on Columbia agar with 5% sheep blood for 24 h at 37°C. Blood culture broths were processed for the PCR assay between 1 and 12 h after being flagged as positive by the instrument. A 100- μl aliquot was diluted 1:100 in brain heart infusion broth (Becton Dickinson) and incubated at 35°C for 45 min. After centrifugation at 2,000 $\times g$ for 5 min to

remove blood cells, followed by centrifugation at 13,000 $\times g$ for 5 min, the supernatant was discarded. The cell pellet was washed twice with 1 ml of sterile water to remove inhibitors. DNA extraction and amplification conditions were as described above, except that 5 μl of each of the DNA extracts was tested nondiluted and diluted 10-fold in distilled water. Isolates recovered from the subcultures of the blood cultures were identified by the conventional techniques described above as MRCoNS ($n = 22$), methicillin-susceptible coagulase-negative staphylococci (MSCoNS; $n = 4$), MSSA ($n = 10$), MRSA ($n = 4$), and a *Stomatococcus* sp. ($n = 1$). CoNS were identified as *Staphylococcus epidermidis* ($n = 22$), *Staphylococcus hominis* ($n = 2$), *Staphylococcus warneri* ($n = 1$), and *Staphylococcus capitis* ($n = 1$).

Staphylococcal bacteremia and methicillin susceptibility were accurately detected by PCR from blood culture broths for 59 of 61 samples within 6 h. In general, a bacterial cell pellet was visible after centrifugation of the brain heart infusion broth subculture. For two specimens, specific DNA fragments were correctly detected by PCR only when the test was repeated with a sample from the blood culture bottle. Identical results were obtained by the PCR performed with samples from the blood culture bottles and overnight colonies. In one mixed culture containing both MRSA and MRCoNS, the amplification signal for *nuc* with a sample of the blood culture broth was weak. The subculture showed a predominance of MRCoNS, which could explain the weak *nuc* signal in comparison with those for 16S rRNA and *mecA*. In one case, amplification occurred only with the diluted extract from the blood

TABLE 1. Bacterial strains used for preliminary validation of 16S rRNA-*mecA-nuc* triplex PCR

Strain(s)	No. of isolates	PCR results ^a			Oxacillin resistance	
		16S rRNA	<i>mecA</i>	<i>nuc</i>	Diffusion test ^b	MIC or MIC range (μg/ml)
MRSA clinical isolates	88	+	+	+	R	ND
MSSA clinical isolates	24	+	0	+	S	ND
MRSA reference strains ATCC 43300, ATCC 33592, CCM 885	3	+	+	+	R	ND
MSSA reference strain ATCC 29213	1	+	0	+	S	ND
<i>Staphylococcus epidermidis</i> clinical isolates	18	+	+	0	R	0.5->128
<i>Staphylococcus haemolyticus</i> clinical isolates	3	+	+	0	R	64-128
<i>Staphylococcus hominis</i> clinical isolate	1	+	+	0	R	>128
<i>Staphylococcus lugdunensis</i> clinical isolates	2	+	0	0	S	0.25
<i>S. lugdunensis</i> clinical isolate	1	+	0	0	R	0.5
<i>Staphylococcus auricularis</i> ATCC 33753	1	+	0	0	S	ND
<i>Staphylococcus capitis</i> CCM 2734	1	+	0	0	S	ND
<i>S. capitis ureolyticus</i> ATCC 49326	1	+	0	0	S	ND
<i>Staphylococcus caprae</i> CCM 3573	1	+	0	0	S	ND
<i>Staphylococcus cohnii cohnii</i> CCM 2736	1	+	0	0	R	0.75
<i>S. cohnii urealyticum</i> ATCC 49330	1	+	0	0	R	0.75
<i>S. epidermidis</i> CCM 2124	1	+	0	0	S	ND
<i>S. haemolyticus</i> CCM 2737	1	+	0	0	S	ND
<i>S. hominis hominis</i> DSM 20328	1	+	0	0	S	ND
<i>S. lugdunensis</i> ATCC 43809	1	+	0	0	S	ND
<i>Staphylococcus pasteurii</i> ATCC 51129	1	+	0	0	S	ND
<i>Staphylococcus saprophyticus</i> CCM 883	1	+	0	0	S	ND
<i>Staphylococcus schleiferi coagulans</i> GA 211	1	+	0	0	S	ND
<i>S. schleiferi schleiferi</i> ATCC 43808	1	+	0	0	S	ND
<i>Staphylococcus simulans</i> ATCC 27848	1	w	0	0	S	ND
<i>Staphylococcus wamerei</i> CCM 2730	1	+	0	0	S	ND
<i>Staphylococcus xylosus</i> ATCC 29971	1	+	0	0	S	ND
<i>Staphylococcus intermedius</i> CCM 5739	1	+	0	0	S	ND
<i>Staphylococcus sciuri sciuri</i> ATCC 29062	1	0	w	0	R	0.75
<i>S. sciuri rodentium</i> ATCC 700061	1	0	0	0	R	0.75
<i>S. sciuri camaticus</i> ATCC 700058	1	0	w	0	R	0.75
<i>S. hominis novobiosepticus</i> ATCC 700236	1	+	+	0	R	>256
<i>Enterococcus faecium</i> Iowa 1	1	0	0	0	ND	ND
<i>Enterococcus faecalis</i> ATCC 51299, 29212	2	0	0	0	ND	ND
<i>Enterococcus casseliflavus</i> ATCC 25788	1	0	0	0	ND	ND
<i>Enterococcus flavescens</i> CCM 439	1	0	0	0	ND	ND
<i>Enterococcus gallinarum</i> BM 4174	1	0	0	0	ND	ND

^a +, positive result; 0, negative result; w, weakly positive results; ND, not determined.

^b R, resistant; S, sensitive.

culture, suggesting the presence of inhibitors in the undiluted sample.

Our results are in agreement with those of recent studies of PCR detection of MRSA from blood. In a clinical evaluation of a *mecA* PCR assay with 181 BACTEC blood cultures, Carroll et al. (6) found that the results had a 99% correlation with the results of standard susceptibility tests. Their assay had a turnaround time of less than 3 h but could not differentiate *S. aureus* from CoNS. Benson et al. (39th ICAAC) reported *mecA-nuc* duplex PCR results in total concordance with phenotypic characterization for 184 BacTAlert blood cultures. However, no internal amplification control was included in the two assays described above. The region of 16S rRNA selected used as a positive control in the PCR assay described here was adequate for the detection of the most important species of staphylococci encountered in humans.

The PCR assay described here provided results, on average, 18 to 42 h faster than the conventional identification and susceptibility testing methods used in our laboratory, which required 24 to 48 h after detection of colonies on blood cultures, whereas the PCR required 6 h. Other investigators have evaluated rapid phenotypic methods directly with blood culture

specimens, such as the coagulase test for identification of staphylococci (23) and the BBL Crystal MRSA ID system (Becton Dickinson) for detection of methicillin resistance (17). The coagulase test was found to be fairly sensitive (92%), with results obtained within 2 h. Lower sensitivities (84 and 54% for *S. aureus* and CoNS, respectively) were found for the BBL Crystal MRSA ID test, with results obtained within 6 h.

In conclusion, the 16S rRNA-*mecA-nuc* triplex PCR is a good tool for rapid characterization of staphylococci in positive blood cultures. To confirm the specificity of this test, more samples from patients with *Enterococcus* and *Streptococcus* sp. bacteremias need to be studied. Further evaluations of the clinical impact and the cost-effectiveness of this rapid diagnostic test on the management of staphylococcal bacteremia are under way.

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