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Specific Detection of Methicillin-Resistant *Staphylococcus* Species by Multiplex PCR

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In *Staphylococcus aureus*, *mecA* and *femA* are the genetic determinants of methicillin resistance. By using a multiplex PCR strategy, 310- and 686-bp regions of the *mecA* and *femA* genes, respectively, were coamplified to identify susceptible (lacking *mecA*) and resistant (*mecA*$^+$) staphylococci and to differentiate *S. aureus* (*femA*$^+$) from coagulase-negative staphylococci (lacking *femA*). A third staphylococcal genomic sequence, corresponding to IS*431* and spanning 444 bp, was used as a PCR control. One hundred sixty-five staphylococcal strains were tested. All 72 methicillin-resistant strains were found to be *mecA*$^+$, and 92 of the 93 susceptible isolates lacked *mecA*. Only one coagulase-negative *Staphylococcus* isolate carrying the *mecA* gene was highly susceptible to oxacillin. The *femA* determinant was a unique feature of *S. aureus*; it was found in 100% of the *S. aureus* strains tested but was undetectable in all of the coagulase-negative staphylococci tested. The possibility of directly detecting the *mecA* and *femA* genes in blood samples was also investigated. After two amplification steps, a sensitivity of 50 microorganisms per ml of freshly collected spiked blood was achieved. In conclusion, coamplification of *mecA* and *femA* determinants proved to be very reliable both for rapid detection of methicillin resistance and differential diagnosis between *S. aureus* and other staphylococci. This technique, which can be successfully performed with blood samples, could be a useful tool in the diagnosis and treatment monitoring of staphylococcal infections.

Over the last 3 decades, methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* have emerged as significant pathogens in community-acquired and nosocomial infections (4, 20). The increasing resistance of staphylococci to β-lactam antibiotics has become a major clinical problem. It has led to the overuse of glycopeptides, creating a strong antibiotic pressure that has led to the emergence of vancomycin-resistant enterococci (6, 12). This and the risk of resistance transfer to staphylococci have highlighted the need for a drastic reduction of vancomycin use. In this setting, rapid identification of the *Staphylococcus* species and its resistance pattern may allow more judicious use of glycopeptides.

Although other mechanisms have been implicated, methicillin resistance in *S. aureus* and coagulase-negative species is primarily mediated by the overproduction of PBP 2a, an additional altered penicillin-binding protein with extremely low affinities for β-lactam antibiotics (9). The *mecA* gene, the structural determinant encoding PBP 2a, has very high levels of homology in MRSA and methicillin-resistant *S. epidermidis* (22), is absent from susceptible staphylococcal isolates (21, 24), and unlike PBP 2a, can be detected independently of growth conditions. The *mecA* gene is therefore considered a useful molecular marker of methicillin resistance in all staphylococci. This has prompted several investigators to test different approaches for the detection of *mecA*. In particular, PCR appears to be a rapid, sensitive, and specific assay for such detection compared with Southern blot hybridization, macrorestriction, fingerprinting, and the MIC of methicillin or oxacillin (7, 16).

Although it does not seem to be involved in intermediate resistance to methicillin, the quantitative contribution of the *mecA* gene to the resistance phenotype of MRSA is unknown (8, 14). Other chromosomally determined factors, such as the *femA-femB* operon, that act as regulator genes are essential for the expression of methicillin resistance in *S. aureus* (2, 10, 25). The cooperation of both *femA* and *mecA* determinants seems to be required, but the mechanism is not well understood. Interestingly, *femA* appears to be a unique feature of *S. aureus*; it is not found in other *Staphylococcus* species (27). Therefore, simultaneous detection of the *femA* and *mecA* genes in the same PCR tube has the advantage of identifying both the species and its phenotypic resistance. As a consequence, we have designed a multiplex PCR strategy that uses three sets of primers specific for *mecA*, *femA*, and the staphylococcal insertion element IS*431* (1), with the latter used as an internal control for potential false-negative results. This strategy was evaluated for rapid and specific differential diagnosis among methicillin-resistant and -susceptible strains of *S. aureus* and coagulase-negative staphylococci (CNS). The results were compared with those of susceptibility and typing methods. Finally, the possibility of detecting directly the *mecA* and *femA* determinants in whole-blood samples was investigated, and the results indicate that the multiplex PCR could be a useful tool for the treatment monitoring of staphylococcal infections.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. A total of 165 staphylococcal isolates were used in this study (Table 1). Included were 3 reference strains of methicillin-susceptible strains of *S. aureus* (ATCC 13565, ATCC 19095, and ATCC 25923), 62 clinical isolates of methicillin-susceptible *S. aureus* and MRSA (29 and 33 isolates, respectively), and 85 CNS (46 are methicillin susceptible and 39 are

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TABLE 1. Distribution of the staphylococcal isolates used in this study

Organism(s)	No. of isolates			
	Total	Oxacillin screen by disk diffusion test[a]		
		S	I	R
S. aureus	80	32	15	33
S. epidermidis	10	4		6
S. haemolyticus	5	2		3
S. cohnii	5	4		1
S. schleiferi	5	5		
S. capitis	4	4		
S. lugdunensis	4	4		
S. saprophyticus	3	3		
S. warneri	3	3		
S. sciuri	3	3		
S. simulans	2	2		
S. xylosus	2	2		
S. hominis	2			2
Unclassified CNS	37	10		27
Total	165	78	15	72

[a] S, susceptibility; R, resistance; I, intermediate resistance.

methicillin resistant), with 48 of them biochemically identified (36 are susceptible and 12 are resistant). Intermediately resistant *S. aureus* isolates ($n = 15$) and gram-negative isolates ($n = 20$) were also investigated. These strains were provided by the St. Luc University Hospital (Brussels, Belgium), the Queen Astrid Military Hospital (Brussels, Belgium), and the Princess Paola Hospital (Marcheen-Famenne, Belgium).

Susceptibility tests and identification of species. Screening for methicillin resistance was done by disk diffusion testing with 1 μg of oxacillin per disk (Becton Dickinson, Cockeysville, Md.) placed on Mueller-Hinton agar (Becton Dickinson) with 2% NaCl supplementation. The zones of inhibition were determined after an incubation of 24 h at 30°C. Methicillin resistance was defined according to the National Committee for Clinical Laboratory Standards breakpoints (18). Intermediate resistance (disk zone diameter of between 10 and 13 mm) was confirmed by the MIC determined with oxacillin E-test strips (AB Biodisk, Solna, Sweden) (3).

Biochemical identifications of staphylococci were performed according to standard laboratory criteria (11).

DNA purification from culture samples. Single colonies of isolates were cultured in Luria-Bertani media and incubated for 16 h at 37°C. An aliquot (0.1 ml) of overnight culture (10^8 CFU) was pelleted by centrifugation (5,000 × *g* for 5 min). The bacterial pellet, resuspended in 300 μl of lysis buffer (50 mM Tris HCl [pH 8.0], 100 mM EDTA, 150 mM NaCl, 1% [vol/vol] sodium dodecyl sulfate) containing 100 μg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) and 100 μg of RNase, was incubated at 37°C for 30 min. Lysis was achieved by incubation at 37°C for 30 min in the presence of 200 μg of proteinase K. Samples were treated with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then with 1 volume of chloroform-isoamyl alcohol (24:1) prior to precipitation of the aqueous phase in 2 volumes of 95% ethanol–0.2 M NaCl for 1 h at −20°C. DNA was pelleted by centrifugation (12,500 × *g* for 10 min), washed with 80% ethanol, air dried, and resuspended in 200 μl of distilled water.

DNA purification from blood samples. To determine the sensitivity of the PCR amplification, serial 10-fold dilutions of bacteria (10^8 to 10 CFU/ml) were added to blood samples (whole blood collected in EDTA-containing tubes [Monovette SE, Sartsted, Germany]). Blood cells were separated by centrifugation at 750 × *g* for 20 min, and bacteria were sedimented from 1 ml of supernatant at 12,500 × *g* for 10 min. Cell pellets were then lysed as described above prior to the submission of DNA extracts to one or two amplification steps with primer sets M1 and M2, F1 and F2, and C1 and C2.

Oligonucleotides. On the basis of the DNA sequences of the *mecA* (22) and *femA* (2) genes, the following four oligonucleotides were designed to amplify staphylococcal DNA: primers M1 (885-[5′]TGGCTATCGTGTCACAATCG-904) and M2 (1194-[5′]CTGGAACTTGTTGAGCAGAG-1175) and primers F1 (217-[5′]CTTACTTACTGGCTGTACCTG-237) and F2 (902-[5′]ATGTCGCTTGTTATGTGC-884), which amplified a 310-bp fragment of the *mecA* gene and a 686-bp fragment of the *femA* gene, respectively. Primers C1 (32-[5′]AGGATGTTATCACTGTAGCC-51) and C2 (476-[5′]GATGTACAATGACAGTCAGG-457), overlapping sequences of staphylococcal insertion element IS*431* (1), were used to give a 444-bp fragment as a positive control for the PCR. The combination of primer F1 and primer F3 (430-[5′]CCTGTAATCTCGCCATCATG-411) produced the $F_{1\text{-}3}$ probe, complementary to 213 bases at the 3′ end of the 686-bp *femA* fragment, which was used for Southern blot analysis of PCR products.

PCR amplification. Ten microliters of DNA samples was added to 90 μl of PCR mixture consisting of 10 mM Tris HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.25 mM (each) deoxynucleoside triphosphates, 100 pmol of each primer, and 1.25 U of DyNAzyme DNA polymerase (Finnzymes, Inc., Espoo, Finland). After an initial denaturation step (3 min at 92°C), 30 cycles of amplification were performed as follows: denaturation at 92°C for 1 min, annealing at 56°C for 1 min, and DNA extension at 72°C for 1 min with an increment of 2 s per cycle. The reaction was achieved with a final extension at 72°C for 3 min. Amplification was carried out in a GeneE thermal cycler (Techne, Cambridge, United Kingdom). After amplification, 15 μl of PCR samples was loaded on a 2% (wt/vol) agarose gel and horizontal electrophoresis was performed in 0.1 M Tris HCl (pH 8.6)–80 mM boric acid–1 mM EDTA containing 0.5 μg of ethidium bromide per ml. Amplified, ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator at 300 nm. *Bgl*I- and *Hin*fI-cleaved pBR328 DNA fragments were used as molecular weight markers.

In a first step, the specificity of the M1 and M2 primer set was assessed by using methicillin-susceptible and -resistant staphylococcal isolates from clinical sources. We then evaluated in parallel the value of *femA* as a marker of *S. aureus* and the expression of IS*431* as a control of *Staphylococcus* species, regardless of the susceptibility pattern. Three reference strains of *S. aureus* and one strain of each CNS species listed in Table 1 were used for this validation. The expression of the *femA* and IS*431* genes was investigated by PCR with primer sets F1 and F2 and C1 and C2, respectively.

Multiplex PCR assay. All staphylococcal isolates, collected from three centers, were submitted to multiplex PCR with the three sets of primers, M1 and M2, F1 and F2, and C1 and C2. The results of the multiplex PCR approach were compared with those of conventional biochemical and microbiological methods, and any discrepancies between microbiological data and PCR results were doubly cross-checked.

RESULTS

Primary validation of *mecA*, *femA*, and IS*431* amplification. The 310-bp *mecA* fragment was obtained from all of the resistant *Staphylococcus* isolates after DNA amplification, while no amplification product was detected for any methicillin-susceptible strain.

Production of the 686-bp *femA* fragment occurred in all *S. aureus* strains, whereas such amplification did not occur in any of the CNS or gram-negative species.

A probe corresponding to the 3′ end of the amplified fragment of the *femA* determinant hybridized to the PCR products of DNAs of *S. aureus* isolates, confirming the *femA* gene origins of these amplified products. The absence of signal with DNAs from CNS sources demonstrated the high-level specificity of the PCR strategy (data not shown).

The 444-bp control fragment from insertion element IS*431* was present after amplification of DNAs from all staphylococcal isolates, regardless of patterns of β-lactam susceptibility or coagulase production.

Multiplex PCR for diagnosis of staphylococcal infections. On the basis of specific amplifications of the *mecA*, *femA*, and IS*431* genes (Fig. 1), the multiplex PCR procedure allowed the specific identification of the staphylococcal species (*S. aureus* or CNS) and the determination of its susceptibility to β-lactam antibiotics (Fig. 2).

The results of the multiplex PCR amplification strategy are reported in Table 2. All the methicillin-resistant isolates (MRSA as well as CNS strains) displayed positive amplifications of the *mecA* determinant, while 92 of the 93 methicillin-susceptible isolates, including the 15 intermediately resistant isolates, were *mecA* negative. Only one *S. hominis* isolate, which displayed a high level of susceptibility to β-lactam antibiotics (oxacillin MIC, <0.016 μg/ml), was repeatedly found to be positive for the presence of the *mecA* gene. As far as the *femA* determinant is concerned, 100% of the *S. aureus* strains were characterized by positive amplification of this gene, which was lacking in all the CNS isolates.

Moreover, this multiplex PCR procedure was rapid, com-

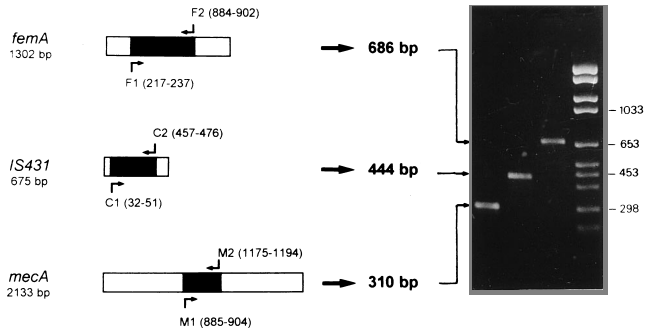

FIG. 1. Multiplex PCR strategy for the amplification of *femA*, IS*431*, and *mecA* genes. DNA fragments resulting from the specific amplification of each gene were separated by electrophoresis on an agarose gel. The lengths of amplified fragments and molecular size markers (in base pairs) are indicated on the left and right, respectively.

pared with the speed of conventional tests. This procedure, including the rapid lysis step and analysis of the amplified products on an agarose gel, was performed in less than 8 h.

Sensitivity of the multiplex PCR with blood samples. After 30 cycles of amplification, the DNA fragments corresponding to the *mecA* and *femA* genes could be visualized on gels for samples inoculated with at least 5×10^5 CFU of an MRSA isolate per ml. After a second amplification step, the level of sensitivity increased to 50 bacteria per ml of blood (Fig. 3).

DISCUSSION

Previous studies have assessed the feasibility of the PCR approach either for the identification of *S. aureus* strains or for intrinsic methicillin resistance on the basis of *femA* (27) and

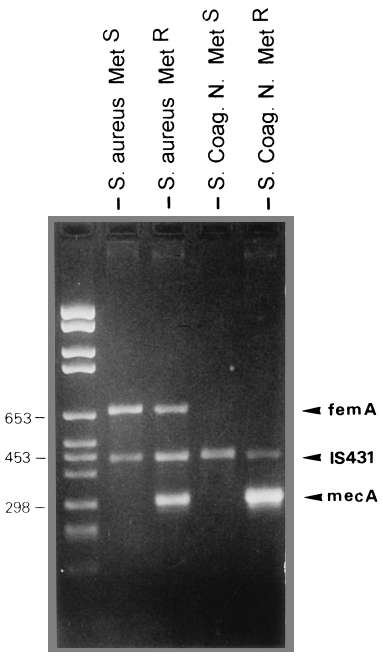

FIG. 2. Gel electrophoresis of DNA fragments generated by multiplex PCR amplification, showing the typical patterns of methicillin-susceptible *S. aureus* and MRSA as well as those of methicillin-susceptible and -resistant CNS (S. Coag. N.). The positions of the genes corresponding to the amplified fragments and of the molecular size markers (in base pairs) on the right and left, respectively.

TABLE 2. Results of multiplex PCR amplification with clinical samples

Organism(s)	Oxacillin screen by disk diffusion test[a]	No. of isolates tested	No. of isolates with result			
			mecA PCR		*femA* PCR	
			Positive	Negative	Positive	Negative
S. aureus	S	32		32	32	
	R	33	33		33	
	I	15		15	15	
CNS	S	46	1	45		46
	R	39	39			39
Gram-negative species	ND	20		20		20

[a] S, susceptibility; R, resistance; I, intermediate resistance; ND, not done.

mecA gene amplification, respectively (7, 16). In regard to methicillin resistance, all of them have pointed out the advantages of the PCR strategy over laboratory methods which can be hampered by the great variability of growth conditions for bacteria. A multiplex PCR approach associating amplifications of the *mecA* and 16S rRNA genes has also been reported as a valuable tool for the identification of MRSA, with the latter gene used as an internal control for sample DNA extraction and PCR failure (7).

In our study, we used a more comprehensive multiplex PCR assay which combines the advantages of the previous strategies by simultaneously identifying three genetic markers that characterize the species, the antibiotic resistance mechanism, and a consensus sequence used as the control. Amplification of the *mecA*, *femA*, and IS*431* genes in the same PCR tube allows a straightforward detection of *S. aureus* together with detection of the methicillin-resistant phenotype. The IS*431* gene was chosen in preference to the classical universal 16S rRNA control because it appears to be very widespread in various copy numbers and locations among staphylococci (1). The results prove the feasibility of this method, which also provides advantages over the former strategies in terms of cost and speed.

The specificity of each set of primers was first confirmed by testing ATCC reference strains and clinical strains isolated from intensive care and burn units and identified by conventional microbiological methods. To vary the sources of strains as much as possible, pathogens were collected from three separate (and distant) institutions. The feasibility and specificity of the multiplex PCR assay were then assessed for the whole

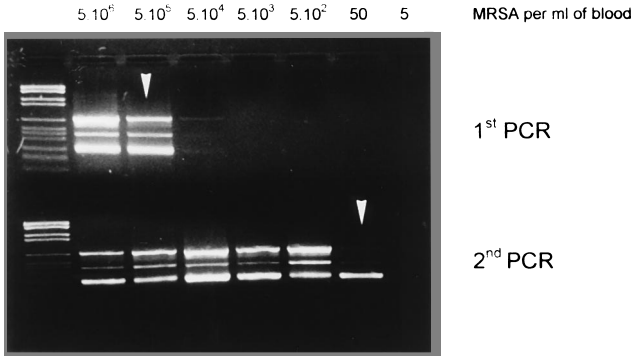

FIG. 3. Gel electrophoresis of PCR products amplified directly from blood samples inoculated with the following serial dilutions of an overnight culture of *S. aureus*: 5×10^6, 5×10^5, 5×10^4, 5×10^3, 5×10^2, 50, and 5 CFU of an MRSA isolate per ml of blood. The limits of detection after the first and second PCR runs are indicated by arrowheads.

population of bacterial isolates. The multiplex PCR strategy was endowed with high-level sensitivity (100% for both *mecA* and *femA* determinants) and specificity (98% for the *mecA* gene and 100% for *femA*). A perfect correlation between the expression of *femA* and the coagulase-positive pattern was found. These data confirm that *femA* expression is a unique feature of *S. aureus*, allowing its specific detection. Compared with standard susceptibility methods, resistance to methicillin was also predicted by the PCR assay; only one oxacillin-susceptible (MIC, <0.016 μg/ml) CNS strain expressed *mecA*. Unlike the observation of Geha et al. (7), the MIC for this particular strain remained consistently low over time. Similar results have been reported by others (16). A mutation or deletion in the *mecA* gene or in its regulatory sequences may explain such a discrepancy, but confirmation requires further investigation. According to the National Committee for Clinical Laboratory Standards standards, 15 intermediately resistant *S. aureus* strains did not express the *mecA* determinant; conversely, none of the *mecA*-negative strains expressed methicillin resistance. This observation is in accordance with the role of β-lactamase overexpression in intermediate resistance to penicillins.

On the basis of these results, the multiplex PCR strategy could give rapid and reliable information to clinicians not only for the identification of pathogenic bacteria but also for therapeutic management. Indeed, MRSA has become a major nosocomial pathogen not only in tertiary care hospitals but also in chronic care facilities (15, 20). The frequent resistance of MRSA to several antibacterial agents has prompted the overuse of vancomycin in first-line empirical therapy and even in prophylaxis therapy (13, 26). However, both the selection of vancomycin resistance (6, 23) and the potential transmission of such resistance between species (5, 19) encourage restricted use of glycopeptides. An early and specific diagnosis might help clinicians face this problem (17), and this prompted us to test the detection of staphylococcal sepsis. A sensitivity of close to 50 organisms per ml of whole blood was achieved when samples were submitted to two consecutive PCR amplifications. Whether this sensitivity has clinical relevance for the diagnosis of staphylococcal sepsis and for the monitoring of infection during antibiotic therapy remains to be determined.

In conclusion, the multiplex PCR approach can be a beneficial adjunct to standard microbiological methods for rapid and specific identifications of pathogens and resistance patterns. It could also be used as a tool to guide and reduce the use of glycopeptides in the clinical setting.

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