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MOLECULAR IDENTIFICATION AND TYPING

Specific information concerning taxonomy, pathogenicity and methicillin resistance of staphylococci obtained by a multiplex PCR

F.-J. SCHMITZ*‡, C. R. MACKENZIE*, B. HOFMANN*, J. VERHOEF‡, M. FINKEN-EIGEN†, H.-P. HEINZ* and K. KÖHRER†

*Institut für Medizinische Mikrobiologie und Virologie and †Molekularbiologisches Zentrallabor, Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-Universität Düsseldorf, Germany and ‡Eijkman-Winkler Institute for Medical Microbiology, University of Utrecht, the Netherlands

The use of DNA amplification techniques such as the polymerase chain reaction (PCR) in modern diagnostic microbiology not only allows the sensitive and specific identification of micro-organisms but also the detection of specific antibiotic resistance genes. This study describes a multiplex PCR on bacterial colonies picked directly from agar plates without preceding DNA preparation. Eubacteria and staphylococci were identified by 16S rRNA specific PCR products. In parallel, specific primers were used for the detection of staphylococcal *coa* and *mecA* genes. This 4-h multiplex PCR, consisting of four sets of primers, was evaluated for rapid and specific differential diagnosis of methicillin-resistant and methicillin-susceptible strains of *Staphylococcus aureus* and coagulase-negative staphylococci. To analyse specificity of the amplification products, 100 non-staphylococcal, eubacterial isolates and 20 *Candida albicans* strains were tested. In a first step, specificity of all four single sets of primers was evaluated before the co-amplification within the multiplex PCR procedure was performed. The results were compared with those of conventional susceptibility and typing methods. The specific 16S rRNA PCR product for eubacterial isolates (n = 786) and staphylococci (686) was found in all strains tested. The *coa* gene was detected only in *S. aureus* (488) strains with a specificity of 100%, and was not detected in any of the coagulase-negative staphylococci (198). The *mecA* gene was detected in 98% of methicillin-resistant staphylococci (393) and in 2% of all methicillin-susceptible staphylococci (293). The multiplex PCR with co-amplification of different determinants provides rapid reliable information on staphylococcal identification and methicillin susceptibility supporting the diagnosis, treatment and control of staphylococcal infections.

Introduction

Methods of DNA amplification such as the polymerase chain reaction (PCR) are becoming increasingly useful in clinical microbiology laboratories, not only for the specific and sensitive detection of organisms in specimens, but also, by identifying resistance genes, as an important clue towards antimicrobial resistance [1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. epidermidis* (MRSE) are important causes of nosocomial infection [2, 3], and the rapid and reliable detection of methicillin resistance assists in the implementation of appropriate treatment and hygiene measures. While standard antibiotic susceptibility methods such as agar diffusion and microdilution, remain the methods of choice, molecular biological detection of resistance genes is a useful complementary method, especially in borderline cases where the classical methods are less reliable. Furthermore, the phenotypic expression of methicillin resistance depends on assay conditions such as incubation temperature, pH and the NaCl concentration in the medium [4–6]. Hybridisation experiments with gene probes demonstrate the *mecA* gene in almost all methicillin resistant staphylococci [7–9]. However, the *mecA* gene is not the only chromosomal factor determining expression of methicillin resistance, as the genes *mecR* and *mecI*, and the *femA-femB* operons play an essential regulatory role in methicillin resistance expression [10–12]. The *mecA* genes from MRSA and MRSE have been sequenced and found to be almost identical [13].

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Corresponding author: Dr F-J. Schmitz.

DNA hybridisation has several disadvantages as a diagnostic method; a relatively large number of bacterial cells is required, the procedure is complicated, and radioactive labelled probes are often necessary. The PCR avoids some of these problems in that DNA preparation is simpler and requires only a few hours [1]. Numerous studies have used the PCR technique to detect methicillin resistance in staphylococci [14–19]. In most, extracted DNA was incubated with restriction endonucleases before the start of the assay. However, Hedin and Löfdahl picked *S. epidermidis* colonies directly from agar plates in their PCR method [20], which has advantages for routine use in diagnostic laboratories.

The detection of methicillin resistance is particularly important in *S. aureus*. In most laboratories the presence of coagulase, coded for by the *coa* gene, is used to identify *S. aureus*. There are several other genes that may be used to identify *S. aureus*, e.g., the *nuc* gene coding for thermostable nuclease, and the *gyrA* or *femA* genes [11, 21–23]. Vannuffel *et al.* [24] described a multiplex PCR in which determinants of the genes *mecA*, *femA*, and a staphylococcal specific sequence were co-amplified. Geha *et al.* [25] co-amplified determinants of the *mecA* and 16S rRNA genes in parallel. Both studies used DNA extracted from pre-incubated colonies.

This report describes a novel method in which single colonies are picked from agar plates and transferred directly to a multiplex PCR assay. The study investigated the routine applicability of the multiplex PCR to testing bacterial colonies picked directly from the agar before Gram staining and independent of colonial morphology.

The multiplex PCR used four sets of primers specific for all eubacteria (universal 16S rRNA primer set); for staphylococci (staphylococcal specific 16S rRNA primer set); for the *coa* gene and for the *mecA* gene.

Materials and methods

Staphylococci

A total of 686 staphylococcal isolates from clinical specimens (blood cultures, respiratory secretions, effusion aspirates and wound swabs) was tested (Table 1). Most isolates (586) originated from patient specimens sent to the Institute for Medical Microbiology and Virology in Düsseldorf [26, 27]. In general, each isolate originated from a different patient to ensure the greatest possible variation in strains. A further 100 MRSA isolates originating from seven different countries were kindly provided by Hoffmann LaRoche (Basel; Table 1). Nine ATCC reference strains of staphylococci (ATCC nos. 12600, 13565, 19095, 25923, 27626, 29213, 33591, 33592, 33593) were used as controls.

Staphylococci were identified by the following tests: catalase, tube coagulase, nuclease, (DNAase) (Unipath, Wesel, Germany), anaerobic mannitol digestion and API Staph® (bioMérieux, Germany). Identification as *S. aureus* required a positive reaction in the first four tests and an unequivocal biochemical identification as *S. aureus*.

Other organisms

As a specificity control for the two 16S rRNA primers, 100 non-staphylococcal eubacterial isolates and 20 isolates of *Candida albicans* were tested with all four sets of primers and with the multiplex PCR assay. Eubacterial isolates included: enterococci (10), pneumococci (10), *Micrococcus* spp. (4), *Streptococcus pyogenes* (4), *Str. dysgalactiae* (4), *Str. agalactiae* (4), *Str. mitis* (3), *Str. bovis* (3), *Peptostreptococcus* spp. (3), *Peptococcus* spp. (3), *Neisseria meningitidis* (3), *Branhamella catarrhalis* (2), *Citrobacter freundii* (2), *Escherichia coli* (5), *Klebsiella* spp. (5), *Haemophilus* spp. (6), *Enterobacter* spp. (10), *Proteus* spp. (5), *Shigella* spp. (5), *Yersinia* spp. and *Salmonella* spp. (5).

Susceptibility testing

Staphylococci were tested for resistance to methicillin by a standardised agar diffusion method [28] with Mueller-Hinton agar supplemented with NaCl 2%, a 5-μg oxacillin disk and incubation at 30°C for 48 h. Of the 293 MRSA isolates, three showed low-level resistance is (MIC < 8 μg/ml); of the 100 methicillin-resistant coagulase-negative staphylococci (CNS) two showed low-level resistance.

Oligonucleotides

Based on published DNA sequences for the *mecA* gene [13, 29], the *coa* gene [21] and the 16S rRNA gene [30, 31], the appropriate primers were selected and synthesised by Pharmacia Biotech. The primer sequences were as follows.

mecA gene: 5′-primer: 37-[5′]-GTTGTAGTTGTCGGGTTTGG-[3′]-66 (20-mer); 3′-primer: 178-[5′]-CGGACGTTCAGTCATTTCTAC-[3′]-198 (21-mer) (amplification product length, 161 nucleotides).

coa gene: 5′-primer: 1520-[5′]-GCTTCTCAATATGGTCCGAG-[3′]-1539 (20-mer); 3′-primer: 1631-[5′]-CTTGTTGAATCTTGGTCTCGC-[3′]-1651 (21-mer) (amplification product length, 131 nucleotides).

16S rRNA gene for eubacteria: 5′-primer: 1170-[5′]-AACTGGAGGAAGGTGGGGAT-[3′]-1189 (20-mer) [RW01]; 3′-primer: 1521-[5′]-AGGAGGTGATCCAACCGCA-[3′]-1539 (19-mer) [DG74] (amplification product length, 371 nucleotides).

16S rRNA gene for staphylococci: 5′-primer: 294-[5′]-

GCCGGTGGAGTAACCTTTTAGGAGC-[3′]-318 (25-mer) [RDR327]; 3′-primer: 1522-[5′]-AGGAGGTGA-TCCAACCGCA-[3′]-1540 (19-mer) [DG74] (amplification product length, 106 nucleotides).

The *S. epidermidis* strain ATCC 27626 served as a positive control for the *mecA* gene amplification, *S. aureus* strain ATCC 25923 as the negative control. The ATCC *S. aureus* strains 12600, 13565, 19095, 25923, 29213, 33591, 33592 and 33593 served as positive control for the *coa* gene, the ATCC *S. epidermidis* strain 27626 as the negative control.

PCR amplification

Approximately one-tenth of a single bacterial colony was picked with a plastic pipette tip and mixed gently in the PCR amplification reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 100 μM dNTPs and 0.4 μM of the respective primers at 94°C for 10 min (initial denaturation). In the middle of this step, 5 Units of Ampli*Taq*-DNA polymerase® were added (hot start). Denaturation was followed by 25 amplification cycles as follows: 94°C for 20 s (denaturation), 55°C for 20 s (annealing) and 72°C for 50 s (extension). After the 25th cycle the reaction was achieved with a final extension at 72°C for 5 min.

The end volume of the PCR was 50 μl. DNA was not concentrated in the PCR reaction tube and thus no inhibition occurred.

A GeneAmp PCR System 2400® (Perkin-Elmer, Weiterstadt, Germany) was used for the amplification cycles and all reagents (GeneAmp dNTPs®, Ampli*Taq* DNA polymerase® and GeneAmp 10 × PCR buffer®) were from Perkin-Elmer.

After amplification PCR products were separated in a Metaphor®/Agarose 4% w/v gel. PCR product (15 μl) was mixed with 3 μl of sample buffer (glycerine 30% and BPB 0.1%) and loaded in the gel pockets. Separated PCR products were then visualised with ethidium bromide.

Study design

In initial experiments, the specificity of the *mecA* gene primer set was assessed with methicillin-susceptible and -resistant staphylococcal isolates from clinical sources. The value of the *coa* gene as a marker of *S. aureus* and the two 16S rRNA primer sets as controls for eubacteria and staphylococcal species was evaluated with all the strains listed in Table 1.

After this initial validation all isolates were submitted to the multiplex PCR with the four sets of primers being co-amplified. 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 cross-checked on two occasions.

Table 1. Distribution of staphylococcal isolates

Organism	Number of isolates that were methicillin-susceptible	Number of isolates that were methicillin-resistant	Origin
S. aureus	195	193	Düsseldorf
S. aureus	···	26	Japan
S. aureus	···	23	Brasil
S. aureus	···	11	Switzerland
S. aureus	···	5	Sri Lanka
S. aureus	···	13	Spain
S. aureus	···	14	England
S. aureus	···	8	Hungary
S. epidermidis	50	54	Düsseldorf
S. simulans	11	10	Düsseldorf
S. haemolyticus	12	10	Düsseldorf
S. warneri	2		Düsseldorf
S. auricularis	6	7	Düsseldorf
S. sciuri	4	4	Düsseldorf
S. hominis	2	3	Düsseldorf
S. capitis	3	3	Düsseldorf
S. saprophyticus	2	3	Düsseldorf
S. cohnii	1	1	Düsseldorf
S. lugdunensis	4	4	Düsseldorf
S. schleiferi	1	1	Düsseldorf

Reproducibility

To show the reproducibility of the PCR assays, six *S. aureus* strains (three methicillin-resistant and three methicillin-susceptible) and six CNS strains (three methicillin-resistant and three methicillin-susceptible) were tested 10 times in one PCR assay (within-run precision) and on 10 separate days (day-to-day precision).

Results

Primary validation of all four single sets of primers

The 161-bp *mecA* fragment was obtained from 388 of 393 methicillin-resistant staphylococcal isolates after DNA amplification, and from five phenotypic methicillin-susceptible strains. Amplification of the 131-bp *coa* fragment was obtained with all *S. aureus* strains, whereas it did not occur with any of the CNS or other eubacteria. The 106-bp fragment from 16S rRNA of staphylococci could be detected after amplification in all staphylococcal isolates, regardless of their β-lactam susceptibility or coagulase production. The 371-bp fragment from 16S rRNA of eubacteria was present after amplification of DNA from all eubacterial isolates.

Multiplex PCR assay

On the basis of specific amplifications of the *mecA*, *coa* and 16S rRNA genes, the multiplex PCR allowed the identification of eubacteria, specific identification of

the staphylococcal species (*S. aureus* or CNS) and determination of their methicillin susceptibility. Fig. 1 shows the specific amplified DNA products for different organisms and the different bands that were obtained in the multiplex PCR.

The results of the multiplex PCR amplification are summarised in Table 2. The sensitivity and specificity of the universal 16S rRNA primer set, the staphylococcal specific 16S rRNA primer set as well as that of the *coa* gene primer set were each 100%. Sensitivity and specificity of the *mecA* gene primer set was 98%.

A specific *mecA* fragment was detected in 388 of 393 methicillin-resistant staphylococci; of the five methicillin-resistant isolates which were *mecA* negative, three were *S. aureus*. In contrast 288 of 293 methicillin-sensitive staphylococci were *mecA* negative; of the five methicillin-sensitive staphylococci that were *mecA* positive, three were *S. aureus*. Detection of *coa* and the 16S rRNA determinants identified all the *S. aureus* strains; the *coa* gene was not detected in any CNS isolate.

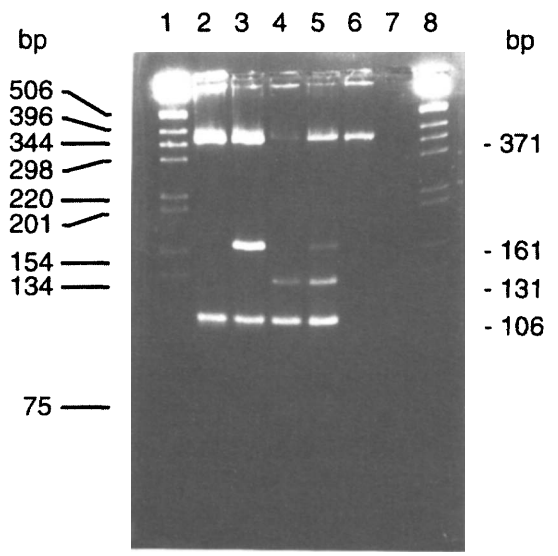

Fig. 1. Results of the multiplex PCR for different bacteria. Lanes **1** and **8**, 1-kb ladder size standard; **2**, methicillin-susceptible, CNS; **3**, methicillin-resistant, CNS; **4**, methicillin-sensitive, coagulase-positive *S. aureus*; **5**, methicillin-resistant, coagulase-positive *S. aureus*; **6**, *E. coli* as positive control for the eubacterial 16S rRNA primers; **7**, H_2O control. 371 bp, eubacterial specific 16S rRNA PCR product; 161 bp, *mecA* gene specific product; 131 bp, *coa* gene specific product; 106 bp staphylococcal specific 16S rRNA PCR product.

Reproducibility of the PCR assay results (within-run precision and day-to-day precision) was excellent. The specific PCR products of the 12 staphylococci tested were always detected in both the individual PCR reactions and the combined multiplex PCR in the intra- and inter-assay analyses.

Discussion

MRSA play an increasingly important role in nosocomial infection [2, 3] and the rapid detection of MRSA and reliable distinction from CNS are important in infection control, particularly in intensive care units.

At a molecular level, intrinsic methicillin resistance is due to the production of an additional penicillin-binding protein (PBP 2a), which has less affinity for the β-lactam antibiotic molecule. The PBP 2a protein is coded for by the *mecA* gene, and, therefore, this gene plays an essential role in methicillin resistance [1, 32, 33]. Other genes have been identified which, when inactivated, lead to a reduction in methicillin resistance and decrease in the peptidoglycan synthesis [4, 12, 32, 34]. The *mecR*, *mecI* and *femA* genes have a regulatory role in *mecA* expression and are also necessary for the expression of methicillin resistance [10, 11]. DeLencastre and Tomasz [34] found 10–12 other genes that may play a part in methicillin resistance.

The phenotypic expression of methicillin resistance is dependent on laboratory test conditions such as incubation temperature, pH and concentration of salts in the medium [5, 6]. As a result of this, the National Committee for Clinical Laboratory Standards (NCCLS) has given recommendations concerning the detection of methicillin resistance [35, 36]. Although the breakpoint set by NCCLS for methicillin resistance is a MIC $> 2\ \mu g/ml$, many have described 'borderline' or 'low-level' resistant (MIC

Table 2. Results of the multiplex PCR

Organisms	Methicillin sensitivity	Number of isolates	16S rRNA gene [E]*		16S rRNA gene [S]†		*coa* gene		*mecA* gene	
			positive	negative	positive	negative	positive	negative	positive	negative
S. aureus	Sensitive	195	195	0	195	0	195	0	3	192
	Resistant	293	293	0	293	0	293	0	290	3
CNS	Sensitive	98	98	0	98	0	0	98	2	96
	Resistant	100	100	0	100	0	0	100	98	2
Other eubacteria	N/A	100	100	0	0	100	0	100	0	100
Candida albicans	N/A	20	0	20	0	20	0	20	0	20

CNS, coagulase-negative staphylococci.
*16S rRNA gene [E]; eubacterial specific 16S rRNA product.
†16S rRNA gene [S]; staphylococcal specific 16S rRNA product.

1–8 μg/ml) *S. aureus* isolates which do not synthesise PBP 2a [37–40]. Such a 'low-level resistance' in *mecA* negative staphylococci may result from increased synthesis of β-lactamase [41], the production of normal PBPs with a reduced affinity for the β-lactam molecule [40, 42], the formation of a newly described methicillinase [39] or from other unidentified factors [32, 34, 42]. Despite this, hybridisation experiments show that the *mecA* gene is present in nearly all methicillin-resistant staphylococci. Detection of *mecA* gene to indicate methicillin resistance is thus of practical value.

A number of studies have compared PCR detection of the *mecA* gene with either hybridisation methods or with antibiotic susceptibility testing (agar diffusion or microdilution) [14–19]. The various results show a correspondence of >95% for MRSA strains and a slightly lower correspondence for MRCNS [1]. The present study found a correspondence of >98% between the presence of the *mecA* gene and the results of susceptibility testing. In only five phenotypic methicillin-resistant strains was the *mecA* gene not detected. In all five strains the MIC of methicillin was >8 μg/ml and excess production of β-lactamase was excluded. One of the other mechanisms of methicillin resistance described above may have been responsible, or a modified *mecA* gene sequence in these strains may have resulted in failure of primer attachment. In a further five strains a PCR product for the *mecA* gene was found in the presence of phenotypic methicillin susceptibility. In these strains a mutation of the *mecA* gene may have resulted in dysfunction or other genetic factors necessary for the expression of methicillin resistance may have been absent. In clinical practice, such *mecA* positive strains should probably be considered methicillin resistant, as the potential for resistance is present.

In most reports on *mecA* PCR, DNA has been extracted by incubation of bacteria with lysostaphin, lysozyme, achromopeptidase and proteinase K, before amplification. Only Hedin and Löfdahl [20] have reported the use of *S. epidermidis* colonies directly from agar plates for the PCR reaction and their results were comparable to results with pre-extracted DNA. The present findings confirm the applicability of mixing colonies directly into the PCR reaction and furthermore show that this procedure can be combined with a multiplex PCR. In diagnostic laboratories, besides the detection of methicillin resistance the accurate identification of *S. aureus* is also important. The coagulase enzyme has eight variants, all coded by alleles of the 2-kb coagulase gene, *coa*. Sequence analysis of the *coa* gene shows that three regions can be differentiated from each other [21, 29]. The sequence for the prothrombin-binding domain is localised at the N-terminal end, the function of the highly conserved middle region is unknown, and at the C-terminal end are four-to-eight repeat sequences. The sequence amplified in the multiplex PCR described in this paper for the detection of the *coa* gene was in the middle region and all *S. aureus* strains were identified correctly.

To reduce the number of false positive and negative results it is essential to include in each amplification a staphylococcal specific primer set as a positive control for the amount of DNA available as a template. This serves as a control for cell lysis as CNS in particular can pose lysis problems [43]. The staphylococcal specific primers used had a sensitivity and specificity of 100% and were ideal positive controls.

Amplification of the 16S rRNA region conserved in all eubacteria was used as an additional internal control. Greisen *et al.* [30] amplified this region in 176 phylogenetically different species and showed the conserved nature of this sequence. In the present study both the specificity and sensitivity of this sequence in the multiplex PCR were 100%. The failure of an amplification to produce a product corresponding to the target sequence indicates an error in the experimental conditions and the necessity of repeating the procedure. Possible errors include incorrect concentrations of the separate additives, insufficient or excess DNA template, a PCR cycle error or a pipetting error.

The main advantage of the multiplex PCR described here is that bacterial colonies picked directly from agar plates can be analysed without preceding DNA preparation providing information on taxonomy, pathogenicity and methicillin resistance in a single process within 4 h. This has the potential to improve both patient management and hospital infection control procedures. Furthermore, the procedure is quick and simple enough to be used in a routine diagnostic laboratory.

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