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Article

Evaluation of the *mec*A *fem*B Duplex Polymerase Chain Reaction for Detection of Methicillin-Resistant *Staphylococcus aureus*

D. Jonas, H. Grundmann, D. Hartung, F.D. Daschner, K.J. Towner

Abstract This study systematically evaluated a recently described duplex polymerase chain reaction test for methicillin-resistant *Staphylococcus aureus* with 25 different German epidemic strains of methicillin-resistant *Staphylococcus aureus* and 66 staphylococci other than methicillin-resistant *Staphylococcus aureus*, including 17 different coagulase-negative staphylococcal species and subspecies, that were either oxacillin susceptible or oxacillin resistant. The results were compared with those of conventional cultural identification and susceptibility testing. Of the 91 isolates tested, all 25 confirmed strains of methicillin-resistant *Staphylococcus aureus* were identified correctly. None of the remaining strains of methicillin-susceptible *Staphylococcus aureus* was misidentified as methicillin-resistant *Staphylococcus aureus*. It was concluded that the duplex polymerase chain reaction appears to offer a time-saving and accurate method of detection of methicillin-resistant *Staphylococcus aureus*.

Introduction

The prevalence of colonisation or infection by methicillin-resistant *Staphylococcus aureus* (MRSA) continues to increase [1, 2], with considerable variation between countries [3] or hospitals in the same area [4]. Conventional microbiological culture and sensitivity techniques require several days to confirm the presence of MRSA, and sensitive procedures faster than cultural identification are therefore increasingly desirable for appropriate treatment and timely directed containment measures in hospital epidemiology. Several different polymerase chain reaction (PCR) protocols have been proposed, including multiplex PCRs that simultaneously amplify DNA sequences specific for both the species and methicillin resistance. All of these methods detect the *mec*A gene as the resistance marker but use different target sequences, such as *nuc* [5], *coa*A [6], *fem*A [7] or *fem*B [8], to indicate the species *Staphylococcus aureus*.

Recently a *mec*A *fem*B duplex PCR method was described and has been used successfully to examine patient screening samples for MRSA in the UK [9]. As this method has so far been evaluated only with staphylococci from one area of the UK, the aim of the current study was threefold. First, the procedure was evaluated with 25 different epidemic German MRSA isolates. Second, as the *fem*B gene codes for an enzyme important in crosslinking peptidoglycan in various different *Staphylococcus* spp. and the corresponding DNA sequence data of coagulase-negative staphylococci (CNS) are limited, DNA from 17 different species or subspecies of CNS was examined to investigate whether amplification occurred with the particular *fem*B primers used in the duplex PCR to recognise *Staphylococcus aureus*. Third, the technical limitations of a duplex PCR amplifying two different targets simultaneously were examined.

D. Jonas (✉), D. Hartung, F.D. Daschner
Institute of Environmental Medicine and Hospital Epidemiology, University Hospital Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany

H. Grundmann, K. Towner
Department of Microbiology & Public Health Laboratory Service Laboratory, University Hospital, Queens Medical Centre, Nottingham NG7 2UH, UK

Materials and Methods

Bacterial Strains. The clinical isolates studied comprised 25 German isolates of different, typed MRSA strains provided by

the Nationales Referenzzentrum für Staphylokokken (Wernigerode, Germany), 32 different isolates of methicillin-susceptible *Staphylococcus aureus* (MSSA) and 16 different isolates of *Staphylococcus epidermidis* from the intensive care unit of the University Hospital Freiburg, which were isolated from specimens taken from different patients over a period of 12 months. In addition, a collection of 18 different *Staphylococcus* type strains was obtained from the Centre National de Référence des Staphylocoques (Lyon, France), including one further MSSA strain and one additional methicillin-susceptible *Staphylococcus epidermidis* strain (Table 1).

Species Identification and Susceptibility Testing. *Staphylococcus epidermidis* isolates were identified by the BBL Crystal Gram-positive ID system (Becton Dickinson, Germany). Oxacillin susceptibility was tested by inoculation onto Mueller-Hinton agar (Merck, Germany) supplemented with 4% NaCl and 6 μg/ml oxacillin (Heipha Diagnostika, Germany), followed by incubation at 30 °C for 24 h. MICs were determined by the microdilution broth method in Mueller-Hinton broth (Merck) supplemented with 2% NaCl [10]. MSSA isolates were identified routinely by Staphyslide and API Staph (bioMérieux, Germany). Oxacillin susceptibility was tested initially with the ATB system (bioMérieux) or by the method of Bauer-Kirby [11] on Mueller-Hinton agar.

Preparation of DNA. Colonies (3–5) from a fresh overnight culture were resuspended in 100 μl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 50 U/ml lysostaphin (Sigma, Germany) in a microfuge tube and incubated for 30 min at 35 °C, followed by 10 min at 95 °C. After addition of 900 μl distilled H_2O, bacterial debris was pelleted by centrifugation at 15 000 × *g* for 20 s. The supernatant was transferred into a fresh microfuge tube and stored at –20 °C.

Polymerase Chain Reaction. The multiplex PCR for detection of MRSA was performed essentially as described previously [9]. Primers (Pharmacia Biotech, Germany) used for detection of the *mec*A gene were MecA1 (5′ GTA GAA ATG ACT GAA CGT CCG ATA A 3′) and MecA2 (5′ CCA ATT CCA CAT TGT TTC GGT CTA A 3′) [12], yielding a 310 bp product, while the *fem*B gene was detected with primers FemB1 (5′ -TTA CAG AGT TAA CTG TTA CC 3′) and FemB2 (5′-ATA CAA ATC CAG CAC GCT CT 3′) [8], yielding a 651 bp target. Both upstream primers (MecA1 and FemB1) were fluorescently labelled with Cy-5 during manufacture. The PCR was performed in a total volume of 25 μl, with 4 μl of the bacterial lysate being added to a PCR mix comprising 0.2 mM dNTPs (Pharmacia Biotech), 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1 U *Taq* DNA polymerase (Boehringer Mannheim, Germany) and, unless otherwise indicated, 2.5 pmol of each MecA primer and 7 pmol of each FemB primer. The PCR comprised an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 50 °C, 60 s at 72 °C, and a final extension step at 72 °C for 2 min. PCR products were detected either by agarose gel electrophoresis (1% agarose, 1 × TBE, 1 μg/ml ethidium bromide, 90 V for 90 min) of a 10 μl portion or by analysis of a 1 μl portion on an Automated Laser Fluorescence (ALF) Express DNA Sequencer (Pharmacia) as described previously [13]. In the latter case, the amount of the different PCR products was determined from the peak area, expressed in arbitrary units, by means of Fragment Manager software V1.2 (Pharmacia). An inter-16S-23S rRNA PCR [14] was used as an amplification control to exclude inhibition of the PCR.

16S rRNA Sequencing. Sequencing of the small subunit rRNA gene for definitive species identification was done with a MicroSeq 16S rRNA Gene Kit (PE Applied Biosystems, Germany). Sequencing products were separated on an ABI Prism 377 DNA Sequencer (PE Applied Biosystems). Each sequence was compared to known sequences contained in the EMBL and GenBank databases by using the gapped basic local alignment search tool algorithm [15].

Table 1 Type strains of different *Staphylococcus* species and subspecies

Staphylococcus species and subspecies	Original strain designation
S. aureus	CCM 885
S. auricularis	ATCC 33753
S. capitis subsp. *capitis*	CCM 2734
S. capitis subsp. *ureolyticus*	ATCC 49326
S. caprae	CCM 3573
S. cohnii subsp. *cohnii*	CCM 2736
S. cohnii subsp. *urealyticum*	ATCC 49330
S. epidermidis	CCM 2124
S. haemolyticus	CCM 2737
S. hominis	DSM 20328
S. lugdunensis	ATCC 43809
S. pasteuri	ATCC 51129
S. saprophyticus	CCM 883
S. schleiferi subsp. *coagulans*	GA 211
S. schleiferi subsp. *schleiferi*	ATCC 43808
S. simulans	ATCC 27848
S. warneri	CCM 2730
S. xylosus	ATCC 29971

Results

Determination of the Optimal Primer Concentrations. The molar ratio of both primer pairs can be expected to influence the yield of their respective amplification products. In order to optimise the duplex reaction, different amounts of both primer pairs were added to a PCR volume of 25 μl. Following the PCR, 1 μl of each reaction was analysed on an ALF Express sequencing gel and the amounts of the *mec*A and *fem*B PCR products were estimated in terms of their peak area (expressed in arbitrary units). As shown in Table 2, 2.5 pmol of the MecA primers and 7 pmol of the FemB primers produced an optimum even amplification of both target gene sequences.

Screening. DNA from 91 isolates of staphylococci was screened with the optimised PCR protocol. Figure 1 shows an agarose gel stained with ethidium bromide to illustrate the typical results obtained with the duplex PCR. DNA from three different strains of MRSA, MSSA and methicillin-resistant *Staphylococcus epidermidis* (lanes 1–3, respectively) gave rise to clearly detectable and distinguishable amplification products. Lane 4 shows the PCR products from a methicillin-resistant *Staphylococcus epidermidis* isolate that showed visible growth on the oxacillin agar screen only after incubation for 48 h. The methicillin-susceptible *Staphylococcus epidermidis* isolate (lane 5) did not yield any PCR products. For all isolates that failed to yield PCR products with either the MecA or FemB primers, the presence of DNA and absence of inhibitors was confirmed by means of the inter-16S-23S rRNA PCR (data not shown).

Table 2 Influence of the molar ratio of the primers used in the duplex PCR on the yield of their respective amplification product

Amount of primer		Amount of gene-specific PCR product[a]		Ratio of both PCR products
MecA (pmol)	FemB (pmol)	*mec*A (peak area[b])	*fem*B (peak area[b])	peak area$_{mecA}$ peak area$_{femB}$
1.7	10	579	5231	0.11
2.5	7	3319	4051	0.82
2.5	2.5	3796	96	39.5

[a] Data shown are average values calculated from two independent experiments
[b] Arbitrary units

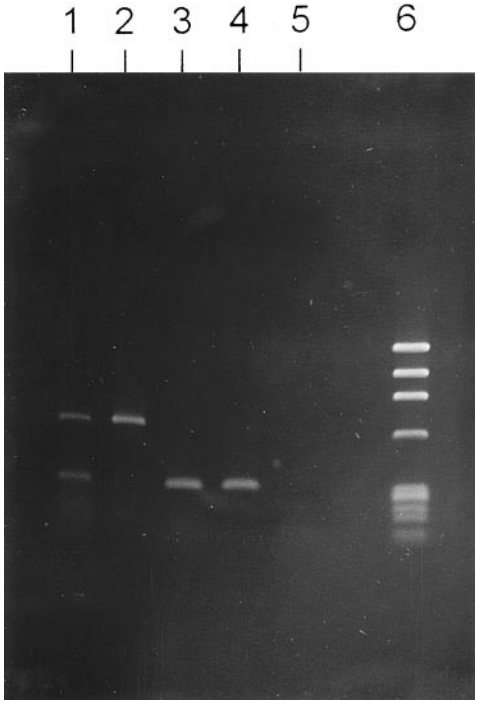

Figure 1 Detection of different oxacillin-susceptible and -resistant staphylococci by *mec*A *fem*B duplex PCR. *Lane 1* MRSA; *lane 2* MSSA; *lane 3* methicillin-resistant *Staphylococcus epidermidis*; *lane 4* methicillin-resistant *Staphylococcus epidermidis*; *lane 5* methicillin-susceptible *Staphylococcus epidermidis*; *lane 6* marker (PhiX-174 *Hae* III digested DNA)

The overall results for the 91 isolates tested are summarised in Table 3. All 25 confirmed isolates of MRSA were identified correctly, but one MSSA isolate failed to yield the expected *fem*B PCR product. The original biochemical identification of this isolate as *Staphylococcus aureus* was confirmed by sequence analysis of the 16S rRNA, which revealed identity of >99% with the corresponding sequence of *Staphylococcus aureus*. With the exception of the type strain ATCC 33753 of *Staphylococcus auricularis*, none of the CNS staphylococcal isolates or type strains yielded a *fem*B amplification product.

One isolate originally identified as MRSA did not yield a *mec*A PCR product. Subsequent examination of this isolate revealed that it was oxacillin susceptible, and the loss of the *mec*A gene was confirmed by the National Reference Center for Staphylococci (W. Witte, personal communication). In contrast, one isolate initially identified as MSSA was found to yield a *mec*A PCR product. Repeated susceptibility tests showed that this isolate was indeed oxacillin resistant, thereby confirming the PCR result. In addition, five isolates of *Staphylococcus epidermidis*, initially reported as oxacillin susceptible, yielded a *mec*A-positive PCR result. These isolates showed visible growth only after incubation for 2 days on the oxacillin agar screen. When the MIC was determined by the microdilution broth method in Mueller-Hinton broth supplemented with 2% NaCl (National Committee for Clinical Laboratory Standards), in all five cases the MIC was determined to be ≥ 32 μg/ml after incubation at 30 °C for 24 h. The MIC did not increase by more than two dilution steps after incubation for 48 h, which could be an indication for strong repression of the *mec*A gene [16].

Discussion

Rapid and accurate detection of MRSA is of major importance in clinical microbiology and hospital

Table 3 Results of duplex PCR amplification with type strains

Staphylococci	Oxacillin susceptibility	No. of isolates tested	PCR product *mec*A *fem*B	No. of isolates with PCR result			
				+ +	Ø +	+ Ø	Ø Ø
S. aureus	R	25		25			
	S	33			32		1
CNS	R	14				14	
	S	19			1		18

S, susceptible; R, resistant; +, positive; Ø, negative; CNS, coagulase-negative staphylococci

epidemiology. Spread of multiresistant bacteria has to be limited by careful directed use of costly containment measures, while adequate treatment of infections caused by *Staphylococcus aureus* requires accurate sensitivity testing, as delayed appropriate antimicrobial therapy of MRSA will cause treatment failures. However, unnecessary use of glycopetide antibiotics will rapidly lead to the emergence of resistance, as has been seen with enterococci [17] and staphylococci showing intermediate resistance [18, 19].

Numerous PCR methods have been proposed for the detection of methicillin resistance in *Staphylococcus aureus*, all of which detect the sequence of the *mec*A gene as an antimicrobial resistance marker. Definitive identification of the species *Staphylococcus aureus* could be achieved by sequencing the ribosomal genes, but different PCR methods focusing on these target sequences yield multiple amplification products [20, 21], which are difficult to analyse accurately on a daily routine basis. Several alternative PCR targets have been proposed in an attempt to identify the species *Staphylococcus aureus* by a single amplification product, including the *nuc* gene [22], the *coa* gene [6] or the genes encoding the factors essential for methicillin resistance [23], *fem*A [24, 25] or *fem*B [8]. However, the absence of any of these genes does not completely rule out the presence of *Staphylococcus aureus*, as any of these target sequences can be mutated. Thus, in the present study, the absence of amplifiable *fem*B DNA from an MSSA isolate with a *Staphylococcus aureus*-specific 16S rRNA gene could be shown in one instance. However, in contrast to mutations in genes coding for coagulase or DNase, a mutation in the *fem* operon will render a *mec*A-positive *Staphylococcus aureus* methicillin susceptible [26]. Thus, even the rare event of a *fem*B mutation in an isolate of *Staphylococcus aureus* would probably not, by definition, lead to a missed isolate of MRSA.

Another objection to the *mec*A *fem*B duplex PCR method is that other CNS of clinical importance could also be *fem*B-positive with the specific primers used and hence would be misidentified as *Staphylococcus aureus*. However, of the different type strains and isolates of CNS examined, only the type strain of *Staphylococcus auricularis*, and none of the clinical CNS isolates, yielded a *fem*B amplification product. The habitat of *Staphylococcus auricularis* is the external auditory meatus of the ear [27], although one study of 162 CNS isolated from various skin diseases has been published in which two isolates of *Staphylococcus auricularis* were isolated from skin lesions [28]. As *Staphylococcus auricularis* is not of clinical importance and appears not to colonise body sites normally screened for MRSA, this single specific false-positive *fem*B amplification is probably not of practical significance.

Routine diagnostic laboratories still have great difficulty in identifying MRSA correctly, and one *Staphylococcus aureus* isolate reported to be oxacillin susceptible by the ATB Staph system was identified as an isolate of MRSA by both the PCR and dilution broth techniques. A recent study revealed a marked number of false-susceptible reports with *Staphylococcus aureus* isolates when testing oxacillin resistance by the ATB Staph system [29]. Similarly, difficulties in unambiguous determination of oxacillin resistance in culture of CNS are well known [30], and prolonged incubation of broth dilutions is recommended [10], so that the molecular biology approach seems to offer considerable advantages over the conventional cultural approach in determining this special type of antimicrobial resistance. A duplex PCR allows this approach to be combined with species identification in one reaction, but simultaneous amplification of different targets may lead to preferential amplification of the smaller target sequence [31]. As demonstrated in the present study, this requires careful optimisation of the relative concentrations of both pairs of primers.

Overall, this study provided evidence that the *mec*A *fem*B duplex PCR method is generally applicable, and not just to special clones in one European region. As originally reported with isolates from the UK [9], the objection that other CNS of clinical importance could be *fem*B-positive with the specific primers used, and would thus be misidentified as *Staphylococcus aureus*, does not seem to be of significant importance. The technical problem of a multiplex reaction needing to amplify several different targets in a single PCR can be simply solved if there are only two targets to be considered. In conclusion, it seems that the multiplex PCR method is superior to conventional culture methods and offers a fast and unambiguous test of oxacillin resistance in *Staphylococcus aureus*.

Careful evaluation using patient specimens in a clinical situation should prove whether this PCR method is superior to conventional methods of MRSA identification. The potential benefits to infection control teams and hospital budgets resulting from accurate and speedy detection of MRSA should outweigh the additional costs of this molecular test, especially in hospitals with a problem of endemic MRSA infections.

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