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RESEARCH ARTICLE

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Multiplex PCR for simultaneous detection of enterococcal genes *vanA* and *vanB* and staphylococcal genes *mecA*, *ileS-2* and *femB*

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Abstract The experimental transfer of the *vanA* gene cluster from *Enterococcus faecalis* to *Staphylococcus aureus* has raised fears about the occurrence of such genetic transfer in clinical isolates of methicillin-resistant staphylococci. Recently, infections by a *S. aureus* strain carrying the enterococcal vancomycin resistance *vanA* gene cluster were reported. The possible emergence and dissemination of these strains is a serious health threat and makes optimization of prevention strategies and fast detection methods absolutely necessary. In the present study, we developed a PCR protocol for simultaneous detection of enterococcal *vanA* and *vanB* genes, the staphylococcal methicillin and mupirocin resistance markers *mecA* and *ileS-2,* and identification of *S. aureus*. As no vancomycin-resistant *S. aureus* isolates were available for our study, we used mixtures of enterococcal and staphylococcal colonies that harbored the different resistance markers to show that these genes could be detected simultaneously. This protocol could be used to facilitate the detection and identification of predictable *S. aureus* or methicillin-resistant strains carrying *vanA* or *vanB*.

Keywords *Staphylococcus aureus* · Antibiotic resistance · Glycopeptides · Vancomycin-resistant *S. aureus* · Methicillin-resistant *S. aureus*

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Introduction

Staphylococcus aureus is one of the most common causes of nosocomial and community-acquired infections worldwide [2, 6]. Since the emergence of methicillin-resistant strains of *S. aureus* (MRSA), the glycopeptide vancomycin has been the antibiotic of choice for serious MRSA infections [5]. In 1996, a *S. aureus* strain with intermediate resistance to vancomycin (VISA) (vancomycin MIC = 8μg /ml) was first isolated from a patient in Japan [10]. Shortly afterward, VISA strains were isolated in USA, Europe and other Asian countries [9, 24], arousing considerable concern regarding the emergence of *S. aureus* strains for which there will be no effective therapy. Characterization of these VISA strains indicates that the mechanisms of resistance are complex and involve changes in cell wall content and composition [3, 22]. In addition, the appearance of a powerful glycopeptide resistance mechanism among clinical isolates of enterococci, associated with the *vanA* and *vanB* gene clusters [4, 13, 15, 21], and the demonstration of experimental transfer of the *vanA* gene cluster from *Enterococcus faecalis* to *S. aureus* [16] have raised fears about the occurrence of such genetic transfer in clinical isolates of methicillin-resistant staphylococci. This type of transfer has already been reported in clinical isolates of *Streptococcus bovis* (*vanB*) [20] and *Bacillus circulans* (*vanA*) [12].

In June 2002, the world's first reported clinical infection due to *S. aureus* with high resistance to vancomycin (VRSA) (vancomycin MIC > 128 μg/ml) was diagnosed in a patient in the USA [23]. A second isolate was reported in October of the same year [14]. These isolates contain the *vanA* genes from enterococci and the methicillin-resistance gene *mecA*. The possible emergence and dissemination of VRSA strains is a serious health threat and makes it absolutely necessary to optimize prevention strategies and fast detection methods.

In the present study, we developed a multiplex PCR protocol that allows the simultaneous detection of the

two most widespread, transferable vancomycin resistance genes, *vanA* and *vanB*, and the methicillin resistance gene *mecA*, as well as the identification of *S. aureus* (*femB*). In addition, since mupirocin has been suggested as a reasonable adjuvant agent to prevent staphylococcal infections, and high resistance is already widespread [5, 18, 19], detection of the *ileS-2* gene was also included.

Material and methods

Bacterial strains and DNA extraction

As there were no vancomycin-resitant *S. aureus* isolates available for our study, mixtures of enterococcal and staphylococcal colonies that harbored the different resistance markers were used to show that these genes could be detected simultaneously. Bacterial strains and susceptibility testing methods were previously described [17, 18]. The following strains were used: *E. faecalis* V583 (vancomycin-resistant, VanB), *E. faecalis* ATCC 29212 (vancomycin-susceptible), *E. faecium* BM4147 (vancomycin-resistant, VanA), *S. aureus* ATCC 29213 (methicillin- and mupirocin-susceptible), *S. aureus* SEIMC (methicillin-resistant and mupirocin-susceptible), *S. aureus* isolate 242 (methicillin- and mupirocin-resistant) and *Staphylococcus epidermidis* 8859-65 (methicillin- and mupirocin-resistant). For DNA extraction, bacteria were grown overnight on BHI agar plates. One colony from a *Staphylococcus* strain and one from a *Enterococcus* strain were mixed and resuspended in 25 µl of sterile distilled water, and heated at 100 °C for 15 min. A 5-µl aliquot of this suspension was directly used as template for PCR amplification.

Simultaneous detection of *vanA*, *vanB*, *femB*, *mecA* and *ileS-2* by PCR

The primers used in this study are listed in Table 1. Multiplex PCR assays were carried out with the DNA suspension obtained from the *Staphylococcus*/*Enterococcus* mixtures. We ensured that our PCR protocol was adequate for the individual amplification of each DNA fragment: *vanB* (1,145 bp), *vanA* (732 bp), *fem*B (651 bp), *ileS-2* (456 bp) and *mec*A (310 bp) (not shown). Multiplex PCR was then done using the following mixtures: *E. faecalis* V583 (*vanB*)/*S. aureus* 242 (*femB*, *mecA*, *ileS-2*), *E. faecium* BM4147 (*vanA*)/*S. aureus* 242 (*femB*, *mecA*, *ileS-2*), *E. faecalis* ATCC 29212/*S. aureus* ATCC 29213 (*femB*), *E. faecalis* V583 (*vanB*)/*S. aureus* ATCC 29213 (*femB*), *E. faecium* BM4147 (*vanA*)/*S. aureus* ATCC 29213 (*femB*), *E. faecalis* V583 (*vanB*)/*S. aureus* SEIMC (*femB*, *mecA*), *E. faecium* BM4147 (*vanA*)/*S. aureus* SEIMC (*femB*, *mecA*), *E. faecalis* V583 (*vanB*)/*S. epidermidis* (*mecA*, *ileS-2*), *E. faecium* BM4147 (*vanA*)/*S. epidermidis* (*mecA*, *ileS-2*). The PCR conditions used were modifications of protocols previously described by us [17, 18]. A 5-µl aliquot of the DNA suspension was added to 28.5 µl of PCR mixture consisting of 1× reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8)], 0.2 mM concentration of each of the four dNTPs (Promega Corp., Madison, Wis.), 3 mM $MgCl_2$, 1.5 µM of each *femB* primer, 0.5 µM of each *vanA* and *vanB* primer, 0.4 µM of each *ileS-2* primer and 0.4 µM of each *mecA* primer, and 1.25 U of Taq DNA polymerase (Bioline, London, UK). The *fem*B primers were used to identify *S. aureus* strains, and the *mecA, ileS-2*, *vanA* and *vanB* primers to detect the respective resistance markers. In order to reduce the formation of nonspecific extension products, the protocol included a "hot-start". DNA amplification was carried out in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, Calif., USA) with the following thermal cycling profile: an initial denaturation step at 94 °C for 5 min was followed by 10 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 45 s), and another 25 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 2 min), ending with a final extension step at 72 °C for 10 min. After PCR amplification, 5 µl were removed and subjected to agarose gel electrophoresis (2% agarose, 1× TBE, 100 V) to estimate the sizes of the amplification products by comparison with a 100-bp DNA ladder (Roche Diagnostics, Mannheim, Germany). The gel was stained with ethidium bromide and the amplicons were visualized under a UV light.

Results and discussion

The reaction conditions for the multiplex PCR assay were optimized to ensure that each target sequence was satisfactorily amplified. The primers used in this study differ in annealing temperatures, which increased the possibility of occurrence of unwanted bands that originated from nonspecific amplification. Therefore, two rounds of amplification with different annealing temperatures were carried out. Multiplex PCR with targets that differ widely in size often favors amplification of the shorter target over the longer ones [18]. Thus, to ensure amplification of each target, different primer concentrations, template DNA preparations, and $MgCl_2$ concentrations were assayed, and those described above were chosen.

Figure 1 shows the results obtained with the optimized multiplex PCR assay as described in Material and methods; amplification of *vanA, vanB*, *femB*, *ileS-2* and *mecA* targets produced distinct bands corresponding to their respective molecular size that were easily recognizable. *vanA* or *vanB* could be detected in mixtures containing *femB* (lanes 4 and 5), *femB* and *mecA* (lanes 6 and 7), and *femB, mecA*, and *ileS-2* (lanes 1 and 2).

Table 1 PCR primers used in this study

Primer pair	Target gene	5′–3′ Sequence	Product length (bp)	Reference
VanBfor VanBrev	*vanB*	GTGCTGCGAGATACCACAGA CGAACACCATGCAACATTTC	1,145	This study
VanA1 VanA2	*vanA*	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	[7]
FemB1 FemB2	*femB*	TTACAGAGTTAACTGTTACC ATACAAATCCAGCACGCTCT	651	[11]
MupA MupB	*ileS-2*	TATATTATGCGATGGAAGGTTGG AATAAAATCAGCTGGAAAGTGTTG	456	[1]
MecA1 MecA2	*mecA*	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310	[8]

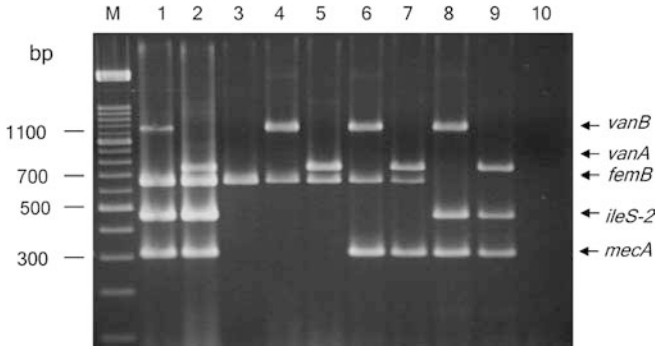

Fig. 1 Agarose gel electrophoresis of multiplex PCR amplification products from different enterococcal/staphylococcal mixes. *Lanes*: *M* 100-bp DNA ladder (Roche Diagnostics, Mannheim, Germany), *1 Enterococcus faecalis* V583 (*vanB1*)/*Staphylococcus aureus* 242 (*femB*, *mecA*, *ileS-2*), *2 Enterococcus faecium* BM4147 (*vanA*)/*S. aureus* 242 (*femB*, *mecA*, *ileS-2*), 3 *E. faecalis* ATCC 29212/*S. aureus* ATCC 29213 (*femB*), *4 E. faecalis* V583 (*vanB1*)/*S. aureus* ATCC 29213 (*femB*), *5 E. faecium* BM4147 (*vanA*)/*S. aureus* ATCC 29213 (*femB*), *6 E. faecalis* V583 (*vanB1*)/*S. aureus* SEIMC (*femB*, *mecA*), *7 E. faecium* BM4147 (*vanA*)/*S. aureus* SEIMC (*femB*, *mecA*), *8 E. faecalis* V583 (*vanB1*)/*S. epidermidis* (*mecA*, *ileS-2*), *9 E. faecium* BM4147 (*vanA*)/*S. epidermidis* (*mecA*, *ileS-2*), *10* control without a DNA template

vanA, vanB, *ileS-2* and *mecA* were also simultaneously detected in the presence of the *S. epidermidis* (femB$^-$) DNA. No amplification of the antibiotic resistance markers was observed in the control without DNA (lane 10) or in the mixture containing two susceptible strains (*E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213) (lane 3). The entire protocol for the multiplex PCR assay, including the DNA extraction and electrophoresis, can be completed in less than 5 h.

Since vancomycin is frequently the drug of choice for the treatment of infections caused by now common MRSA, the recent appearance of a fully vancomycin-resistant strain of *S. aureus* has confronted us with the possible existence of non-tractable infections. The protocol described here could be used to facilitate the detection and identification of *S. aureus* or MRSA strains carrying *vanA* or *vanB*.

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