J. Microbiol. Biotechnol. (2007), **17**(4), 673–676

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Detection of Methicillin Resistance in *Staphylococcus aureus* Isolates Using Two-Step Triplex PCR and Conventional Methods

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Received: July 11, 2006
Accepted: October 3, 2006

Abstract A two-step triplex PCR assay targeting the *mecA*, *femA*, and *nuc* genes was developed for the detection of methicillin resistance genes harbored by some *Staphylococcus aureus* isolates and for the simultaneous identification of such isolates at the species level. The triplex PCR revealed the presence of the *femA* and *nuc* genes in all the *S. aureus* isolates examined (n=105). Forty-four clinical isolates were *mecA* positive and no foodborne isolates were *mecA* positive. The PCR results had a 98 or 99% correlation with the results of PBP2a latex agglutination tests or oxacillin susceptibility tests, respectively.

Keywords: Two-step triplex PCR, methicillin-resistant *S. aureus* (MRSA), *mecA* gene, *femA* gene, *nuc* gene

Staphylococcus aureus represents one of the most significant human pathogens, causing both nosocomial and community-acquired infections. β-Lactams are preferred antibiotics to treat serious *S. aureus* infections. However, since 1961 when methicillin was introduced for clinical use, the occurrence of methicillin-resistant *S. aureus* (MRSA) strains has been steadily increased and MRSA infections have become a serious problem [2, 6, 8]. Thus, rapid and reliable detection methods of MRSA strains are prerequisites to secure the optimal treatments of patients suffering from staphylococcal infections and the proper procedures to control the infections [9, 10, 12].

Conventional detection methods, such as disk diffusion methods or agar dilution methods, are laborious and time-consuming. In addition, methicillin resistance is influenced by culture conditions, such as inoculum sizes, incubation times and temperatures, pH and salt concentrations of the medium, and exposure to β-lactam antibiotics. These factors make the detection of methicillin resistance complicated, especially when the resistances are detected at low levels [10, 18, 19]. The shortcomings of such conventional typing methods based on phenotypes have led to the development of genotype-based typing methods based on DNA sequences. As for MRSA detection, various uniplex and multiplex PCR techniques have been described in recently published reports [10, 15, 18, 20, 22, 23, 29].

Although other mechanisms have been implicated, methicillin resistance in *S. aureus* is primarily mediated by the overproduction of penicillin-binding protein 2a (PBP2a), an additional altered penicillin-binding protein with extremely low affinities for β-lactam antibiotics. The *mecA* gene, the structural determinant encoding PBP2a, has very high levels of homology in MRSA, is absent from susceptible staphylococcal isolates, and unlike PBP2a, can be detected independently of growth conditions. The *mecA* gene is therefore considered a useful molecular marker of methicillin resistance in MRSA strains [20, 24]. 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* [26]. Interestingly, *femA* appears to be present in all *S. aureus* strains, but not in other *Staphylococcus* species strains. 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. On the other hand, the *nuc* gene encodes staphylococcal thermostable nuclease, part of which was used in this paper as an additional *S. aureus*-specific molecular marker and as an internal control for potential false-negative results [28]. Consequently, we have designed a two-step triplex PCR strategy that optimizes amplification reactions using three sets of primers specific for the *mecA*, *femA*, and *nuc* genes. This strategy was evaluated for specific differential detection

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of MRSA isolates among *S. aureus* from food samples and clinical patients. The results were compared with those of slide latex agglutination tests and oxacillin susceptibility tests.

A total of 105 previously identified *S. aureus* isolates consisting of 55 clinical isolates and 50 foodborne isolates were used in this study [13, 14]. Two reference strains of *S. aureus* (ATCC 43300 and ATCC 19095) were also used as control strains for MRSA and methicillin-susceptible *S. aureus*, respectively.

The *S. aureus* isolates were phenotypically characterized by determination of thermonuclease (TNase) activity, a PBP2a latex agglutination test (LAT), and an oxacillin susceptibility test. The detection of TNase by the *S. aureus* isolates was examined using a toluidine blue-DNA-agar plate method [17]. The PBP2a LAT was performed by using a slide latex agglutination test kit (Oxoid, Basingstoke, U.K.) according to the manufacturer's instructions. The oxacillin susceptibility test was done by 1-μg oxacillin disk diffusion testing as described by the National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

For two-step triplex PCR amplifications, the template DNA was isolated as described previously [13]. Three sets of primers were selected according to the known sequences of *S. aureus mecA*, *femA*, and *nuc* genes. Table 1 summarizes the DNA sequences for, and the amplicon sizes from, the PCR primers used in this study. Two-step triplex PCR amplifications were routinely carried out in a 50 μl reaction volume, which consisted of 2 μl DNA templates; primers femA1 and femA2 at 0.6 μM each; 2 mM $MgCl_2$; dATP, dCTP, dGTP, and dTTP (Bioneer, Seoul, Korea) at 200 μM each; 1 U of *Taq* DNA polymerase (Bioneer, Seoul, Korea); and 1× PCR buffer (pH 8.0) supplied by the manufacturer. The reaction mixture was overlaid with 30 μl of light mineral oil and heated to 94°C for 2 min. Amplification was carried out in an automated DNA thermal cycler (PTC-100, MJ Research Inc., MA, U.S.A.) for a 10-reaction cycle. Parameters for amplification consisted of 0.5 min at 94°C (DNA denaturation), 0.5 min at 55°C (primer annealing), and 1 min at 72°C (primer extension). Subsequently, primers RSM2647 and RSM2648 (0.4 μM each) and primers NUC1 and NUC2 (0.2 μM each) were added to the reaction mixture and PCR was continued for 20 cycles. Two-step triplex PCR amplifications were completed with a final primer extension at 72°C for 5 min. The amplified DNA products (10 μl) were electrophoretically separated in a 1.5% agarose gel. The results of the two-step triplex 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.

Before the triplex PCR reaction was optimized, we ensured that the single PCR amplifications yielded amplicons of the expected sizes. Amplicon sizes ranged from 270 to 686 bp, differing by at least 150 bp to facilitate electrophoretic separation (Table 1). The reaction conditions for the triplex PCR were optimized to ensure that all of the three target genes were satisfactorily amplified. The primers used in this study differ in annealing temperatures. There is evidence that multiplex PCR with targets that differ widely in size often favors amplification of the shorter target over the longer one, resulting in different amounts of amplified products [3, 21]. In addition to the optimized concentrations of PCR ingredients, therefore, the two-step triplex PCR amplification was needed to obtain evenly intensified distinct bands from target genes.

As methicillin (oxacillin) resistance mediated by PBP2a, the low-affinity penicillin-binding protein encoded by the *mecA* gene, is often heterogeneously expressed in staphylococci [5, 7, 27], PCR detection of the *mecA* gene is the "gold standard" for the detection of methicillin resistance [2, 5, 11, 15, 25]. Thus, a primer pair targeting a *mecA* region was included in the two-step triplex PCR assay. The *femA* gene has been characterized as being essential for the expression of methicillin resistance in *S. aureus* [4]. Thus, a primer pair targeting an *S. aureus*-specific *femA* region was included as well. To verify the efficiency of the amplification, an internal control primer pair targeting an *S. aureus*-specific *nuc* region was included in the two-step triplex PCR assay [1]. The two-step triplex PCR included RSM2647 and RSM2648, a primer pair for the specific detection of methicillin resistance gene; NUC1 and NUC2, a primer pair for the species-specific detection; and femA1 and femA2, a primer pair for both. Therefore, including these three primer pairs facilitates the definite identification of *S. aureus* isolates as well as the determination of their methicillin resistance genotypes.

Table 1. Characteristics of the primer pairs used in the two-step triplex PCR assay.

Target gene	Primer pair[a]	Sequence (5'→3')	Amplicon size (bp)	Source
femA	femA1	CTT ACT TAC TGG CTG TAC CTG	686	[29]
	femA2	ATG TCG CTT GTT ATG TGC		
mecA	RSM2647	AAA ATC GAT GGT AAA GGT TGG	533	[19]
	RSM2648	AGT TCT GCA GTA CCG GAT TTG C		
nuc	NUC1	GCG ATT GAT GGT GAT ACG GTT	270	[6]
	NUC2	AGC CAA GCC TTG ACG AAC TAA AGC		

[a]For each target, the first primer is positive sense and the second primer is negative sense.

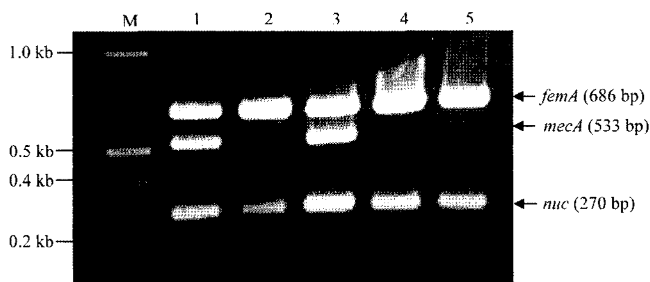

Fig. 1. Gel electrophoresis of DNA fragments generated by the two-step triplex PCR amplification, showing the typical patterns of MRSA and methicillin-susceptible *S. aureus*.
The positions of the genes corresponding to the amplified fragments and of the molecular size markers on the right and left, respectively. Lane 1, *S. aureus* ATCC 43300 (MRSA); lane 2, *S. aureus* ATCC 19095 (methicillin-susceptible *S. aureus*); lane 3, a typical clinical isolate (MRSA); lane 4, a typical clinical isolate (methicillin-susceptible *S. aureus*); lane 5, a typical foodborne isolate (methicillin-susceptible *S. aureus*); and lane M, molecular size markers (in kb).

Fig. 1 illustrates the typical results on an agarose gel obtained with the optimized two-step triplex PCR assay. Amplification of *femA*, *mecA*, and *nuc* target genes produced evenly intensified distinct corresponding bands. The 533-bp *mecA* fragment was obtained from all of the resistant *S. aureus* isolates after DNA amplification, whereas no amplification product was detected for any oxacillin-susceptible isolate except one (Table 2). Production of the 686-bp *femA* fragment occurred in all *S. aureus* isolates (Table 2). The 270-bp control fragment from the *nuc* gene was present after DNA amplification from all *S. aureus* isolates, regardless of patterns of methicillin susceptibility or PBP2a production (Table 2). On the other hand, neither the *femA* or *nuc* fragment was obtained from Gram-negative bacteria (n=5) or coagulase-negative staphylococci (n=4) (data not shown). Therefore, the two-step triplex PCR procedure allowed the specific identification of MRSA strains on the basis of specific amplifications of the *mecA*, *femA*, and *nuc* genes (Fig. 1).

We compared methicillin (oxacillin) susceptibility results determined by the disk diffusion method and the PBP2a LAT test for 105 *S. aureus* isolates with the results obtained by the two-step triplex PCR assays for the detection of methicillin resistance genes. The PCR results for *nuc* detection were in agreement with those of conventional methods for 100% of the isolates (n=105). Discrepancies between phenotypic oxacillin susceptibility and the presence of an amplified *mecA* gene fragment were observed for one isolate, and those between phenotypic PBP2a production and the presence of the amplified fragment were observed for two isolates (Table 2). Therefore, the PCR results had a 99% or 98% correlation with the results of oxacillin susceptibility tests or PBP2a LAT tests, respectively. In regard to methicillin resistance, these results may point out the advantages of the PCR strategy over the conventional laboratory methods, which can be hampered by the great variability of growth conditions for bacteria.

In our study, we used a comprehensive two-step triplex PCR assay, which combines the advantages of the previous strategies by simultaneously identifying three genetic markers that characterize the methicillin resistance determinant, the methicillin resistance mechanism, and a species-specific consensus sequence used as the control. Amplification of the *mecA*, *femA*, and *nuc* genes in the same PCR tube allows a straightforward detection of *S. aureus* together with detection of the methicillin-resistant phenotype.

Acknowledgment

This work was supported by Korea Research Foundation Grant (KRF-2003-003- F00046).

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Table 2. Comparison of two-step triplex PCR and conventional methods for the detection of methicillin resistance in *Staphylococcus aureus* isolates (n=105).

Isolates (n=105)	Conventional method			Two-step triplex PCR		
	PBP2a LAT detection	Oxacillin resistance	TNase activity	*mecA* PCR	*femA* PCR	*nuc* PCR
Clinical (n=55)						
Positive	42	43	55	44	55	55
Negative	13	12	0	11	0	0
Foodborne (n=50)						
Positive	0	0	50	0	50	50
Negative	50	50	0	50	0	0

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