J Korean Med Sci 2003; 18: 631-6
ISSN 1011-8934

Multiplex PCR for the Detection of Genes Encoding Aminoglycoside Modifying Enzymes and Methicillin Resistance among Staphylococcus Species

We developed multiplex polymerase chain reaction (PCR) to detect *aac(6′)/aph(2″)*, *aph(3′)-IIIa*, and *ant(4′)-Ia*, the genes encoding the most clinically relevant aminoglycoside modifying enzymes (AME), and simultaneously, the methicillin resistant gene, *mecA*, in Staphylococcus species. Clinical isolates of 45 *S. aureus* and 47 coagulase negative staphylococci (CNS) from tertiary university hospitals were tested by conventional susceptibility testing, using the agar dilution method and by multiplex PCR. Of a total of 92 isolates, 61 isolates were found to be methicillin-resistant. Of these, 54 isolates (89%) were found to be harboring *mecA*. Seventy-five percent of the 92 isolates demonstrated resistance to at least one of the aminoglycosides tested. Moreover, resistance to aminoglycosides was closely associated with methicillin-resistance ($p<0.05$). The most prevalent AME gene was *aac(6′)/aph(2″)* which was found in 65% of the isolates, and *ant(4′)-Ia* and *aph(3′)-IIIa* were present in 41% and 9% of the isolates, respectively. The concordance between methicillin-resistance and the presence of *mecA* gene was 98% in *S. aureus* and 81% in CNS. The concordance between gentamicin resistance and the presence of *aac(6′)/aph(2″)* gene was 100% in *S. aureus* and 85% in CNS. The multiplex PCR method that we developed appears to be both a more rapid and reliable than conventional method.

Key Words : *Antibiotics, Aminoglycoside; Aminoglycoside Modifying Enzyme; Polymerase Chain Reaction; Staphylococcus aureus; Methicillin Resistance*

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Received : 22 May 2003
Accepted : 23 July 2003

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*This paper was presented at the 42nd ICAAC in the San Diego, California, September 27-30, 2002. (Poster no. C2-712)

INTRODUCTION

Methicillin-resistant staphylococci have become a major pathogen of nosocomial infections (1-3). According to the 1996 national nosocomial infection surveillance in Korea, *Staphylococcus aureus* (17.2%) was the most commonly isolated organism, and 78.8% of *S. aureus* were methicillin-resistant (4). Since the phenotypic expression of methicillin-resistance is influenced by many factors-such as inoculum size, incubation time and temperature, pH of the medium, salt concentration of the medium, and exposure to β-lactam antibiotics, the performance of susceptibility tests has been erratic (5, 6). Moreover, methicillin-resistant staphylococci are also resistant to other antibiotics, including β-lactams, aminoglycosides, and macrolides (7). Considering the importance of antibiotic resistance in treatment, more rapid and reliable diagnostic methods that can determine their resistance profiles are required.

Methicillin-resistance in *S. aureus* and coagulase negative staphylococci (CNS) is primarily mediated by the overproduction of penicillin-binding protein (PBP) 2a, an altered PBP with extremely low affinities for β-lactam antibiotics (8). The *mecA* gene encoding PBP 2a, has very high levels of homology in methicillin-resistant *S. aureus* (MRSA) and MRCNS, and unlike PBP 2a, can be detected independently of growth conditions (9). The *mecA* gene is therefore considered a useful molecular marker of methicillin-resistance in all staphylococci.

The main mechanism of aminoglycoside resistance is drug inactivation by plasmid- or transposon-mediated aminoglycoside modifying enzymes (AME). The bifunctional enzyme AAC(6′)/APH(2″) encoded by the *aac(6′)/aph(2″)* gene is the most frequently encountered AME in staphylococcal isolates. Additional enzymes, such as APH(3′)-III encoded by *aph(3′)-IIIa* gene and the ANT(4′)-I by *ant(4′)-Ia* gene, are also found in Staphylococcus species (10, 11).

Investigators have tried different approaches to detect these resistance genes. In particular, polymerase chain reaction (PCR) appears to be the more rapid, sensitive, and specific assay for such detection than southern blot hybridization, macrorestriction, fingerprinting, and determining the minimal inhibitory concentration (MIC) of methicillin or oxacillin (12-14). In particular, multiplex PCR that detects several genes simultaneously in the same PCR tube, has the advantage of identifying genotypic resistance for several antibiotics more rapidly and reliably (5, 15). The aims of the present study were: (i)

to develop a rapid multiplex PCR assay for the simultaneous detection of genes encoding AME and PBP 2a; (ii) to compare this multiplex PCR assay with standard microbiological methods of susceptibility testing; and (iii) to evaluate the prevalence of these resistance genes in Staphylococcus species.

MATERIALS AND METHODS

Bacterial Strains

Between June 2000 and August 2000, a total of 45 *S. aureus* and 47 CNS from various sources (blood, sputum, urine, and pus) were collected from 3 tertiary teaching hospitals located in geographically different regions of Korea. Staphylococcal strains were identified by using the MicroScan Pos Combo Panel Type 6 (Baxter Diagnostics, West Sacramento, CA, U.S.A.). The CNS were speciated by biochemical tests, using API-Staph (bioMérieux, Marcy, L'Etoile, France). They consisted of *S. epidermidis* (29), *S. haemolyticus* (6), *S. capitis* (4), *S. chromogenes* (3), *S. lugdunensis* (2), *S. hominis* (1), and *S. warneri* (1), *S. simulans* (1). Stock cultures were stored frozen (-70℃) in brain-heart infusion (BHI), containing 10% glycerol.

Susceptibility Testing and Determination of Minimal Inhibitory Concentration (MIC)

Susceptibility tests for oxacillin, gentamicin, tobramycin, kanamycin, and amikacin (Sigma, St. Louis, MO, U.S.A.) were done by using the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (16). Two μL of bacterial suspension, diluted to a final concentration of 10^7 CFU/mL (inoculum size of 2×10^4 CFU/spot), were inoculated on Mueller-Hinton agar containing each antibiotics from 0 to 512 μg/mL. The inoculated plates were then incubated at 37℃ for 24 hr. The MIC was defined as the lowest concentration of antibiotics that prevented visible growth.

According to the current oxacillin breakpoint for methicillin susceptibility, an oxacillin MIC breakpoint of ≤ 2 μg/mL was considered as methicillin susceptible in *S. aureus* and ≤ 0.25 μg/mL in CNS. Gentamicin and tobramycin MICs of ≤ 4 μg/mL, and kanamycin and amikacin MICs of ≤ 16 μg/mL were considered to be susceptible to each aminoglycoside. *S. aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as standard strains. This testing was performed two times to ensure accuracy.

DNA Purification from Culture Samples

Using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany), DNA was purified from staphylococcal isolates. A single colony of the isolates was inoculated into brain heart infusion broth (Becton Dickinson, Sparks, U.S.A.) and was incubated for 24 hr at 37℃ The bacterial pellet was then obtained by centrifugation, resuspended in 200 μL of lysis buffer (containing 400 μg of lysozyme) and incubated at 37℃ for 1 hr. The sample was then lysed by incubating at 56℃ for 30 min in the presence of 20 μL of proteinase K (10 mg/mL) and 200 μL of AL buffer. To extract the DNA, 200 μL of 100% ethanol was added and the lysate was transferred to a QIAamp spin column, centrifuged at 8,000 rpm for 1 min, washed in 500 μL of AW1 and AW2 buffer, and centrifuged to remove all remaining AW1 and AW2 solution. The DNA was then extracted according to the manufacturer's instructions.

Primers for PCR and Multiplex PCR

Using a PCR primer selection program (GeneFisher, Bioinformatics Bielefeld, Germany), four sets of primers specific for *aac(6′)/aph(2″), aph(3′)-IIIa, ant(4′)-Ia*, and *mecA* gene were designed from a site within the nucleotide sequence of the published region of each resistance gene (Table 1).

DNA solution (2 μL) as processed above was added to a PCR mixture containing 0.2 μM of the respective primers, 10 μL of a 10-fold concentrate of PCR buffer, 200 μM of deoxynucleoside triphosphates, and 0.5 U of *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). A Genecycler (Bio-Rad laboratories, Hercules, CA, U.S.A.) was used for DNA amplification. After an initial denaturation (for 5 min at 95℃), 30 cycles of amplification were performed as follows: denaturation at 95℃ for 2 min, annealing at 58℃ for 30 sec, and DNA extension at 72℃ for 30 sec. The reaction was achieved with a final extension at 72℃ for 7 min. Ten μL of the PCR products were then loaded onto a 2.5% agarose gel and electrophoresis was performed in Tris-borate-EDTA buffer 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.

S. aureus USS1504 (possessing *mecA*), *S. aureus* BM3002/piP52 [possessing *ant(4′)-Ia*], and *Enterococcus faecalis* BM6217 [possessing *aph(3′)-IIIa, aac (6′)/aph(2″)*] served as the positive controls for each resistance gene.

Statistic Analysis

Calculations were performed by using SPSS software ver-

Table 1. Oligonucleotides used in this study

Target genes	Primer sequence	Size
aac(6′)/aph(2″)	5′-GAAGTACGCAGAAGAGA-3′ 5′-ACATGGCAAGCTCTAGGA-3′	491 bp
aph(3′)-IIIa	5′-AAATACCGCTGCGTA-3′ 5′-CATACTCTTCCGAGCAA-3′	242 bp
ant(4′)-Ia	5′-AATCGGTAGAAGCCCAA-3′ 5′-GCACCTGCCATTGCTA-3′	135 bp
mecA	5′-CCTAGTAAAGCTCCGGAA-3′ 5′-CTAGTCCATTCGGTCCA-3′	314 bp

sion 10.0. The correlation between methicillin-resistance and aminoglycoside resistance was evaluated by using the χ^2 test and Fisher's exact test. $p<0.05$ was considered statistically significant. Comparisons of PCR assay results and the results of conventional susceptibility testing were made by using a matched 2-by-2 table.

RESULTS

Prevalence of Antimicrobial Resistance by Conventional Methods

Of total 92 staphylococcal isolates, 61 (66%) isolates were methicillin-resistant (*S. aureus*, 53%; CNS, 79%). Of these, 54 isolates (89%) were harboring *mecA* based on PCR analysis. Overall, 66% of the isolates were resistant to gentamicin, 71% to kanamycin, 71% to tobramycin, and 23% to amikacin (Table 2). Sixty-nine (75%) staphylococcal isolates demonstrated resistance to at least one of the aminoglycosides tested. Of the *S. aureus*, the proportion of MRSA that showed resistance to the aminoglycosides was 4 times higher than that of the methicillin susceptible *S. aureus* (MSSA; odd ratio, 95% CI, 2.03-7.93; $p<0.05$). Of the CNS, the percentages of MRCNS showing resistance to the aminoglycosides were 11.76 times higher than methicillin susceptible CNS (MSCNS; odd ratio, 95% CI, 4.36-33.46; $p<0.05$). Resistance to aminoglycosides was closely associated with methicillin-resistance ($p<0.05$).

Distribution of Resistance Gene as Detected by Multiplex PCR

Amplified DNA fragments of four different sizes (135, 242, 314, and 491 bp) were detected on agarose gel electrophoresis (Fig. 1). Each amplified DNA fragment was consistently observed in the positive control strains. No specific background amplification products were encountered in this assay. Therefore, the specificity of the primers selected for this study was confirmed.

Of 92 staphylococcal isolates, 57 (62%) were *mecA* positive (*S. aureus*, 55.6%; CNS, 68.1%, Table 3). *aac(6′)/aph(2″)* was encountered most frequently (65%) and 50% of the isolates carried this gene in combination with either *ant(4′)-Ia* and/or *aph(3′)-IIIa.* The *ant(4′)-Ia* and *aph(3′)-IIIa* were present in 41% and 9%, respectively (Table 3). The most frequent combination of genes was *aac(6′)/aph(2″)* with *ant(4′)-Ia* (28%). One isolate possessing the triple combination *aac(6′)/aph(2″), ant(4′)-Ia,* and *aph(3′)-IIIa* was highly resistant to gentamicin (MIC, 256 μg/mL), kanamycin (>256 μg/mL), tobramycin (>256 μg/mL), and amikacin (128 μg/mL). Seventeen isolates that were not detected to have any AME genes were determined to be susceptible to all aminoglycosides tested, except for 4 of these isolates that were intermediate to tobramycin (MIC, 8 μg/mL).

Accuracy of Multiplex PCR

The correlation between methicillin-resistance (OX^r) and the presence of the *mecA* gene is summarized in Table 4. For *S. aureus*, there were 24 strains of OX^r/*mecA*$^+$ and 20 strains of OX^s/*mecA*$^-$ isolates. For CNS, there were 30 strains of OX^r/*mecA*$^+$ and 8 strains of OX^r/*mecA*$^-$ isolates. As a result, the concordance between phenotype and genotype of methicillin-resis-

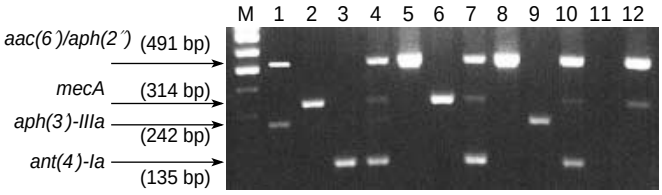

Fig. 1. Gel electrophoresis of DNA fragments amplified by multiplex PCR from reference strains and clinical isolates of Staphylococcus species. Lanes: M, 1,000 bp DNA ladder (Bio-Rad Laboratories, Hercules, CA, USA); 1, *Enterococcus faecalis* BM6217 [possessing *aph(3′)-IIIa* and *aac(6′)/aph(2″)*]; 2, *S. aureus* USS1504 (possessing *mecA*); 3, *S. aureus* BM 3002/piP524 (possessing *ant(4′)-Ia*); 4-8, *S. aureus* (OX 128, GM 256); (OX 0.25, GM 32); (OX >256, GM 0.25); (OX 16, GM 64); (OX 0.5, GM 128); 9-12, CNS (OX 0.125, GM <0.125); (OX >256, GM 256); (OX <0.125, GM <0.125); (OX 8, GM 256) [The parenthesis indicates MIC (μg/mL) of gentamicin (GM) and oxacillin (OX)].

Table 2. The distribution of aminoglycoside resistance in *S. aureus* and coagulase negative staphylococci in relation to methicillin resistance

Species	No. of isolates (%)	Gentamicin	Tobramycin	Kanamycin	Amikacin
MSSA	21 (23)	11 (52)	11 (52)	13 (62)	1 (5)
MRSA	24 (26)	20 (83)	22 (92)	20 (83)	10 (42)
MSCNS	10 (11)	2 (20)	2 (20)	2 (20)	0 (0)
MRCNS	37 (40)	28 (76)	30 (81)	30 (81)	10 (27)
Total	92 (100)	61 (66)	65 (71)	65 (71)	21 (23)

Abbreviations: MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; MSCNS, methicillin-susceptible CNS; MRCNS, methicillin-resistant CNS.

Table 3. The distribution of genes encoding aminoglycoside modifying enzymes as determined by multiplex PCR assay in Staphylococcus species in relation to methicillin resistance

Resistance genes	MSSA n=21 (%)	MRSA n=24 (%)	MSCNS n=10 (%)	MRCNS n=37 (%)	Total n=92 (%)
aac(6′)/aph(2″)	11 (52)	20 (83)	4 (40)	25 (68)	60 (65)
aph(3′)-IIIa	3 (14)	5 (21)	0 (0)	0 (0)	8 (9)
ant(4′)-Ia	1 (5)	10 (42)	10 (100)	17 (46)	38 (41)
mecA	1 (5)	24 (100)	2 (20)	30 (81)	57 (62)

Abbreviations: MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; MSCNS, methicillin-susceptible CNS; MRCNS, methicillin-resistant CNS.

Table 4. Correlation between oxacillin resistance and the presence of the *mecA* gene

Species	No. of strains tested by agar dilution with OX	PCR results for *mecA*	
		No. of +	No. of –
S. aureus	24 (resistant)	24	0
	21 (susceptible)	1	20
CNS	37 (resistant)	30	7
	10 (susceptible)	2	8

Abbreviations: CNS, coagulase negative staphylococci; OX, oxacillin resistance; +, positive; –, negative.

Table 5. Correlation between gentamicin resistance and the presence of the *aac(6′)/aph(2″)* gene

Species	No. of strains tested by agar dilution with GM	PCR results for *aac(6′)/aph(2″)*	
		No. of +	No. of –
S. aureus	31 (resistant)	31	0
	14 (susceptible)	0	14
CNS	30 (resistant)	26	4
	17 (susceptible)	3	14

Abbreviations: CNS, coagulase negative staphylococci; GM, gentamicin resistance; +, positive; –, negative.

tance was 98% in *S. aureus* and 81% in CNS. This resulted in a positive predictive value (PPV) of 95% and a negative predictive value (NPV) of 80% for the *mecA* assay.

The correlation between gentamicin resistance and the presence of *aac(6′)/aph(2″)* gene is summarized in Table 5. In *S. aureus,* 100% concordance was observed. However, in CNS, the concordance was 81%. The discrepancy was due to 7 strains of CNS [3 GM^S/*aac(6′)/aph(2″)*$^+$ and 4 GM^r/*aac(6′)/aph(2″)*$^-$ isolates]. The *aac(6′)/aph(2″)* gene detection by multiplex PCR had a PPV of 95% and a NPV of 88% for gentamicin resistance.

The concordances between kanamycin, tobramycin, amikacin resistance and the presence of *aac(6′)/aph(2″)* gene were 91%, 96%, and 78%, respectively, in *S. aureus* (vs. 81%, 77%, and 51% in CNS).

DISCUSSION

Although culture-based methods are generally reliable for detecting methicillin-resistant staphylococci, the detection of *mecA* gene by PCR assay is now considered as the gold standard. In particular, multiplex PCR assay that simultaneously detects several genes in a single reaction has the advantage of identifying genotypic resistance for several antibiotics more rapidly and reliably. Several studies have used multiplex PCR for the detection of genes encoding methicillin-resistance and/or genes for species identification in cases of staphylococcal infection (11, 14, 17). We designed 4 sets of primers, which

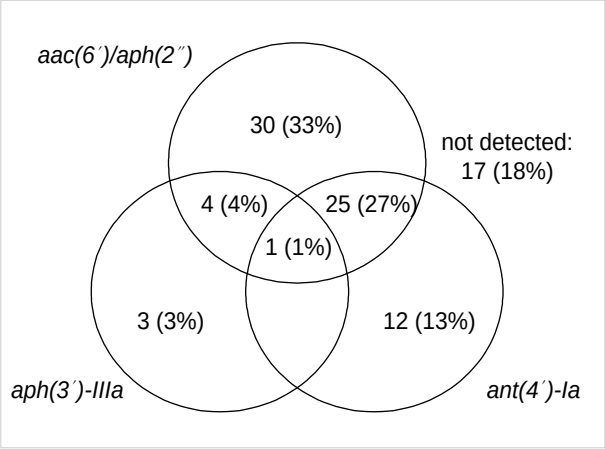

Fig. 2. The incidence of genes encoding aminoglycoside modifying enzymes, as determined by multiplex PCR in 92 isolates of Staphylococcus species.

were specific for *aac(6′)/aph(2″), aph(3′)-IIIa,* and *ant(4′)-Ia,* the genes encoding the most clinically relevant AME, and *mecA* gene. We successfully developed a rapid multiplex PCR method, which was performed in less than 6 hr. Moreover, the accuracy of this method was reliable, compared with conventional testing. Recently, the breakpoint for oxacillin resistance in CNS was lowered from 2 μg/mL to 0.25 μg/mL (18). The use of the recently determined oxacillin MIC interpretative breakpoints resulted in decreased percentages of OX^S/*mecA*$^+$ CNS (19). As a result, we observed that the concordance between genotype and phenotype increased from 68% to 81% in this study. However, 11% (10/92) of the strains were discordant, and most of these were CNS (7 strains of OX^r/*mecA*$^-$ and 2 strains of OX^S/*mecA*$^+$). In the case of OX^r/*mecA*$^-$ strains, non-PBP 2a-dependent mechanisms such as hyperproduction of β-lactamase and alteration of PBP types may participate in the expression of resistance (20, 21). We performed nitrocefin disk test (Becton Dickinson, Sparks, U.S.A.) for the 5 strain out of 7 discordant OX^r/$mecA^-$ strain and 3 strain showed positive results. And additional study to confirm the mechanism of oxacillin resistance will be needed. Two OX^S/*mecA*$^+$ strains were identified as *S. hominis* and *S. capitis* and the MIC for these strains were 0.125 μg/mL. The *mecA* gene is not consistently expressed and certain auxiliary genes, such as *femA, mecR,* and the gene encoding the β-lactamase plasmid may participate in the control of its expression.

Aminoglycosides play an important role in serious staphylococcal infections despite reports of increased resistance to these drugs. Gentamicin and tobramycin are the most active against staphylococci, and are often used in combination with either a β-lactam or a glycopeptide, especially in the treatment of staphylococcal endocarditis. Drug inactivation by AME is the main mechanism of aminoglycoside resistance. The bifunctional enzyme, AAC(6′)/APH(2″), encoded by the *aac(6′)/aph(2″)* gene, is the most frequently encountered AME in

staphylococcal isolates and mediates resistance to gentamicin, tobramycin, dibekacin, netilmicin, amikacin, and isepamicin (11). In the present study, the concordances between gentamicin, kanamycin, tobramycin, and amikacin resistance and the presence of *aac(6′)/aph(2″)* gene were 100%, 91%, 96%, and 78%, respectively, in *S. aureus* (vs. 81%, 81%, 77%, and 51% in CNS). In 4 GM^r/*aac(6′)/aph(2″)*$^-$ isolates of 7 discordant CNS, other mechanisms, such as a loss of permeability and ribosomal alteration, may mediate resistance. Three isolates of GM^s/*aac(6′)/aph(2″)*$^+$ CNS showed MICs of 0.25 μg/mL, 2 μg/mL, and 4 μg/mL, respectively, and those were relatively higher than the usual MIC of 0.015-0.125 μg/mL in gentamicin susceptible *S. epidermidis*. This may be due to the partial expression of *aac(6′)/aph(2″)* gene (15).

An ANT(4″)-I enzyme encoded by *ant(4′)-Ia* is known to mediate resistance to neomycin, kanamycin, tobramycin and amikacin in staphylococci and resistance to neomycin, and kanamycin is conferred by an APH(3′)-III enzyme encoded by *aph(3′)-IIIa* (11). However, the concordance between kanamycin resistance and the presence of *aph(3′)-IIIa,* tobramycin resistance and the presence of *ant(4′)-Ia,* and amikacin resistance and the presence of *ant(4′)-Ia,* was less than 50% (data was not shown). Ida et al. reported that *ant(4′)-Ia* is the most prevalent gene in MRSA, and the isolates possessing this gene presented high level of resistance to tobramycin (22). In our study, the concordance between tobramycin resistance and the presence of *ant(4′)-Ia* gene was only 45%. In addition, we found no significant difference in the MICs of *ant(4′)-Ia* positive and negative isolates.

Several reports have stated that aminoglycoside resistance is closely related to methicillin-resistance (10, 11, 23, 24). Our study also shows a significant correlation between aminoglycoside and methicillin-resistance. This is presumed to be due to the adjacent locations of *mecA* gene and of the AME encoding genes, and further study is needed on this topic (22). In the clinical setting, such as in MRSA endocarditis, which requires combination therapy with aminoglycosides, a more prudent choice of aminoglycosides is required.

The mechanisms of aminoglycoside resistance have become more complex with the increased usage of aminoglycosides over time. Moreover, there is no rapid and reliable method for detecting aminoglycoside resistance. To the best of our knowledge, this is the first report about AME and its corresponding genes, using multiplex PCR in Korea and the result of this study reiterates the usefulness of DNA-based assays for the detection of antibiotic resistance genes associated with staphylococcal infections. However, for the application of this method in the clinical practice, additional data for a large number of clinical isolates will be needed. Also, the clinical result of the antimicrobial therapy for the isolates according to the resistant pattern should be evaluated. Considering the high prevalence of aminoglycoside resistance, observed in this study, periodic surveillance of aminoglycoside resistance and of the corresponding genes is needed. We believe that such PCR assays allow for the faster establishment of effective antibiotic therapies, and will lead to improved therapeutic success and reduced empirical treatments with broad-spectrum antibiotics, which are costly and have high toxicities, and eventually slow potential development of antibiotic resistant organisms. In terms of infection control programs, such rapid detection of resistance could be used to prevent nosocomial spread of MRSA in advance.

ACKNOWLEDGMENT

We are grateful to Dr. P. Courvalin (L'Unité des Agents Antibactériens of the L'Institut Pasteur, France) for donating the reference strains.

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