

Use of a DNA Microarray for Simultaneous Detection of Antibiotic Resistance Genes among Staphylococcal Clinical Isolates[∇]

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We developed a multiplex asymmetric PCR (MAPCR)-based DNA microarray assay for characterization of the clinically relevant antibiotic resistance genes leading to penicillin, methicillin, aminoglycoside, macrolide, lincosamide, and streptogramin B (MLS_B) resistance in staphylococci. The DNA-based assay involves detection of specific conserved regions of the *mecA*, *blaZ* (methicillin and penicillin resistance), *aac(6′)-Ie-aph(2′)* (aminoglycoside resistance), *ermA* and *ermC* genes (MLS_B resistance), and the *msrA* gene (macrolide and streptogramin B resistance). The microarray uses a variable sequence region of the 16S rRNA gene to broadly differentiate between *Staphylococcus aureus* and other coagulase-negative staphylococci (CoNS). The performance of the microarray was validated with a total of 178 clinically important *S. aureus* and 237 CoNS isolates, with correlations of 100% for *S. aureus* to CoNS discrimination and more than 90% for antibiotic resistance between the genotypic analysis determined by the microarray and the phenotype determined by standard methods of species identification and susceptibility testing. The major discrepant results were 17 *mecA*-positive CoNS and 60 *aac(6′)-Ie-aph(2′)*-positive CoNS isolates measured by microarray that were susceptible to the corresponding antibiotics based on disk diffusion assay. Overall, this microarray-based assay offers a simultaneous, fast (≤5 h), and accurate identification of antibiotic resistance genes from a single colony, as well as species classification. Our extensive validation of the microarray suggests that it may be a useful tool to complement phenotypic susceptibility testing in clinical laboratories and to survey the spread of antibiotic resistance determinants in epidemiological studies.

Three groups of important antibiotics commonly used in treatment of staphylococcal infections include beta-lactams, particularly lactamase-resistant oxacillin, aminoglycoside, and macrolide, lincosamide, and streptogramin B (MLS_B). However, resistance to these antibiotics is increasingly prevalent among staphylococci (20, 31, 32). For instance, PBP 2a protein, encoded by the *mecA* gene, is responsible for oxacillin (methicillin) resistance in staphylococci (16, 34, 41). β-Lactamase encoded by the *blaZ* gene accounts for the resistance to penicillins. Schmitz et al. (31) have reported that the *aac(6′)-Ie-aph(2′)* gene, encoding a bifunctional enzyme AAC(6′)/APH(2′), is the most frequently encountered (70 to 90%) aminoglycoside resistance mechanism among staphylococcal isolates. Ribosome modification confers MLS_B resistance (9, 39), principally by a single base change in the 23S rRNA by methylases encoded by erythromycin ribosomal methylase (*erm*) genes *ermA* or *ermC*. Resistance to macrolides and streptogramin B (MS resistance) can also occur in staphylococci with active efflux by a membrane-bound transporter protein (*msrA* gene) (30). These different antibiotic resistance genes are either chromosomally encoded (*mecA*) (34), or carried by

transferable genetic elements such as transposons [*blaZ*, *aac(6′)-Ie-aph(2′)*, and *ermA*] (2, 23) and plasmids (*ermC* and *msrA* gene) (18, 30).

Accurate and rapid antibiotic susceptibility information is crucial for clinicians to make appropriate therapy decisions (3, 4). Microarray technology, which allows for the simultaneous analysis of a large amount of genetic information in a single assay (29), has recently been developed to analyze specific bacterial species or test for a few microbial antibiotic resistance determinants (5, 14, 27, 40). Here we have developed a new multiplex PCR-based DNA microarray for detection of six antibiotic resistance genes in staphylococci, including *mecA*, *blaZ*, *aac(6′)-Ie-aph(2′)*, *ermA*, *ermC*, and *msrA*, using one sequence-specific probe for each gene. The microarray also had probes specific to a variable region of 16S rRNA gene, simultaneously differentiating between *S. aureus* and other coagulase-negative staphylococci (CoNS) isolates. Validation of the microarray with 415 nonduplicate staphylococcal isolates has demonstrated that this platform will be a suitable complement for phenotypic susceptibility testing and will provide a rapid guide for appropriate antimicrobial therapy as well as infection control.

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MATERIALS AND METHODS

Bacterial strains. Table 1 lists the descriptions of the reference strains used in the present study. A total of 178 clinically important *S. aureus* and 237 CoNS isolates previously characterized (42) from blood (*n* = 145), pus (*n* = 110),

TABLE 1. Reference strains used in this study

Strain	Species	Origin	Relevant resistance gene(s) ^a	Inhibition zone diam (mm) with ^b :					
				PEN	OX	FOX	GM	CM	ER
TR146	<i>S. aureus</i>	Pus	None	41	19	27	25	25	25
J143	<i>S. epidermidis</i>	Sputum	<i>mecA</i>	25	15	24	29	30	34
R3k728	<i>S. epidermidis</i>	Urine	<i>msrA</i>	36	21	30	22	26	9
TR1780	<i>S. epidermidis</i>	Sputum	<i>ermC</i>	29	24	29	29	MLS _B ⁱ	6
B150	<i>S. aureus</i>	Pus	<i>blaZ</i>	18	22	29	25	27	28
TR1708	<i>S. aureus</i>	Pus	<i>aac6</i>	32	18	26	14	21	24
7605	<i>S. simulans</i>	Sputum	<i>ermA</i> , <i>ermC</i>	40	26	27	23	MLS _B ⁱ	6
6314	<i>S. aureus</i>	Sputum	<i>blaZ</i> , <i>aac6</i> , <i>msrA</i>	16	22	22	14	29	9
TR558	<i>S. aureus</i>	Sputum	<i>blaZ</i> , <i>mecA</i> , <i>aac6</i> , <i>ermA</i> , <i>ermC</i>	6	6	11	10	6	6
7622	<i>S. auricularis</i>	Sputum	<i>blaZ</i> , <i>mecA</i> , <i>aac6</i> , <i>ermC</i> , <i>msrA</i>	6	6	6	6	6	6

^a *aac6*: *aac(6')*-Ie-*aph(2')*.

^b OX, oxacillin; FOX, cefoxitin; PEN, penicillin; GM, gentamicin; CM, clindamycin; ER, erythromycin.

respiratory tract ($n = 92$), urine ($n = 44$), and other tissue sources ($n = 24$) were used to validate the microarray. The isolates were collected from inpatients of the hospital departments and intensive care units of the Beijing Hospital, the Beijing Tiantan Hospital, the Beijing Tongren Hospital, and the Peking Union Medical College Hospital from January to May 2003. Duplicate samples from the same patient were excluded. The isolates were cultured at 35°C on blood agar (Jinzhang Co., Ltd., Tianjin, China) before testing. All isolates were confirmed as *S. aureus* or CoNS by colony morphology, Gram stain, catalase test, and coagulase test and by the Vitek 2 system (bioMérieux, France). For CoNS, the collection comprised 144 *S. epidermidis*, 41 *S. haemolyticus*, 18 *S. auricularis*, 13 *S. simulans*, 7 *S. hominis*, 4 *S. capitis*, 4 *S. sciuri*, and 6 other CoNS strains.

Antimicrobial susceptibility tests. Antimicrobial susceptibility was tested by the agar disk diffusion method on Muller-Hinton agar (Tiantan Biotechnology Co., Ltd., Beijing, China) according to the National Committee for Clinical Laboratory Standards guidelines (25). Disks (Tiantan Biotechnology) were preloaded with the following antibiotics at the specific absolute concentrations indicated in parentheses: penicillin G (10 U), oxacillin (1 µg), cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg), and clindamycin (2 µg). Plates with disks were incubated at 35°C for 24 h to measure the inhibition zone diameters. *S. aureus* ATCC 25923 was included for quality control. The diameters of the zones of inhibition (in millimeters) used for interpretation were as indicated for penicillin (resistant [R] ≤ 28, susceptible [S] ≥ 29) and oxacillin (R ≤ 10, I = 11 to 12, S ≥ 13 for *S. aureus*; R ≤ 17, S ≥ 18 for CoNS), gentamicin (R ≤ 12, I = 13 to 14, S ≥ 15), erythromycin (R ≤ 13, I = 14 to 22, S ≥ 23), clindamycin (R ≤ 14, I = 15 to 20, S ≥ 21), and cefoxitin (R ≤ 19, S ≥ 20 for *S. aureus* and *S. lugdunensis*; R ≤ 24, S ≥ 25 for CoNS) (26).

D-zone test. The D-zone test was performed as described by Fiebelkorn et al. (11) for the detection of MLS_Bⁱ (inducible macrolide, lincosamide, and streptogramin B resistance) strains. Quality control was performed with *S. aureus* ATCC 25923.

β-Lactamase assays. The microtiter nitrocefin (Calbiochem, San Diego, CA) method was used (1). Quality controls included *Staphylococcus aureus* ATCC 29213 as a positive control and *Staphylococcus aureus* ATCC 25923 as a negative control.

Oligonucleotide primers and probes. The complete list of oligonucleotide primers and probes is shown in Table 2. One primer of each primer pair was designed to be tagged with an unrelated universal sequence at its 5' end (named the UT primer) for efficient multiplex asymmetric PCR amplification, and another was sequence specific. The fluorescent dye TAMRA, labeled at the 5' end of the UT primer, was simultaneously incorporated into the PCR products for subsequent hybridization detection. Oligonucleotide probe sequences were designed by multiple-sequence alignment analysis of the sequences available in GenBank by using the DNAMAN (version 4.0) program. The probes were chosen according to the consensus sequences for the resistance genes and to several species-specific sequence regions of the 16S rRNA gene for differentiation of *S. aureus* from CoNS. The lengths of these probes were about 20 to 30 nucleotides, with melting temperatures (T_m) between 60 and 65°C. The 5' end of each probe was modified by adding a spacer with 12 consecutive thymines and an amino-linker group (BioAsia Co., Ltd., Shanghai, China) for covalent immobilization on the aldehyde-coated glass surface.

Eight-plex asymmetric PCR amplification. Analysis of the antibiotic resistance genes and species identification was performed by eight-plex asymmetric PCR amplification. In brief, bacteria were lysed by vortexing a single fresh colony

suspended in 100 µl of 1× TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) with 50 mg of glass beads (Sigma). The resulting lysate was boiled for 5 min and then centrifuged to collect the supernatant as a crude template. Each PCR contained 250 µM concentrations of each deoxynucleoside triphosphate, 1 U of *Taq* DNA polymerase (Tianwei Times Technology Co., Ltd., Beijing, China), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 1 µl of lysate supernatant as a template source. The final primer mixture was optimized, where the concentration of each primer was 0.05 µM, except the concentrations of the *blaZ*-ur and *aac6*-ur primers were both 0.2 µM and the concentrations of the 16S-uf and 16S-r primers were 0.5 and 0.25 µM, respectively. Finally, a 1 µM final concentration of a universal primer (its sequence identical to the unrelated universal sequence at the 5' end of the UT primer) was added to the PCR mixture to further balance the amplification efficiency for each gene. The reaction was performed by using a two-round amplification on the thermal cycler PTC-200 (MJ Research, Inc.). After an initial denaturation step for 3 min at 94°C, 20 cycles of the first-round amplification were performed as follows: denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 90 s. Then, the second-round amplification of 20 cycles was performed as follows: denaturation at 94°C for 30 s, followed by annealing and extension at 72°C for 120 s. The PCR products were visualized after electrophoresis through a 1.2% agarose gel and ethidium bromide staining.

Fabrication of DNA microarray. Microarrays were produced by using a Smart-Array-48 microarrayer (CapitalBio Co., Ltd., Beijing, China). The oligonucleotide probes were spotted onto the surface of the aldehyde-activated slides (CapitalBio) at a concentration of 10 µM in DNA spotting solution (CapitalBio) and covalently immobilized on the slides by the mediation of an amino group at their 5' ends. In each array, four control probes were printed including a fluorescent dye HEX-labeled oligonucleotide as a spotting and position control, an oligonucleotide complementary to a synthetic template included in the hybridization mixture as a hybridization positive control to monitor the hybridization process, an oligonucleotide with the consensus sequence of 23S rRNA gene as a process control, and an oligonucleotide designed to not hybridize to any sequences present in the hybridization mixture as the negative control for background signal corrections.

DNA hybridization and detection. The fluorescently labeled PCR products (8 µl) were resuspended in 10 µl of hybridization buffer (5× Denhardt solution, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.2% sodium dodecyl sulfate, and 10% dextran sulfate) containing 0.01 µM TAMRA-labeled oligonucleotide as the target of the hybridization positive control probe. The resulting hybridization mixture was heat denatured, cooled on ice immediately, and then applied to the microarray. Hybridization was performed for 1.5 h at 56°C. After hybridization, the slides were washed once with 2× SSC plus 0.1% sodium dodecyl sulfate at room temperature for 5 min and then washed twice with distilled water at room temperature for 1 min. Slides were dried by brief centrifugation and subsequently scanned with a LuxScan-10K scanner (CapitalBio). The setting used for the scanner was laser power 90 and PMT 70. The fluorescence intensities of the spots were quantified by the SpotData Pro 2.1 (CapitalBio). The mean of the fluorescence signals for the quadruplicate spots was calculated after subtraction of the fluorescence intensity of the negative control. A positive spot was defined as having a signal intensity of more than 1,000.

TABLE 2. Primers and probes used in this study

Primer or probe	Sequence (5'-3') ^a	Target (length in bp)
Primer		
blaZ-f	CAACGTCTAAAAGAAGACTAGGAGA	<i>blaZ</i> (259)
blaZ-ur	TAMRA-Uni-TAGTCTTTTGGAACACCGTCT	
mecA-f	GATGGCTATCGTGTCACAATC	<i>mecA</i> (352)
mecA-ur	TAMRA-Uni-TGAGTTGAACCTGGTGAAGT	
aac6-f	AGCCTTGGGAAGATGAAGTT	<i>aac(6')</i> - <i>Ie-aph(2'')</i> (513)
aac6-ur	TAMRA-Uni-GCCACACTCATAACCACTAC	
ermA-f	CCTGTCCGGAATTGGTTTTTTAG	<i>ermA</i> (452)
ermA-ur	TAMRA-Uni-CGGTAAACCCCTCTGAGAATA	
ermC-f	AGTAATGCCAATGAGCGTTTT	<i>ermC</i> (303)
ermC-ur	TAMRA-Uni-GGTGTAATTCGTAACTGCCA	
msrA-f	TACTTGAAGCTATTTACCACCA	<i>msrA</i> (256)
msrA-ur	TAMRA-Uni-TAATTTCTGTTCTTTCCCCACC	
23S-uf	TAMRA-Uni-AACGGTCTAAGGTAGCGAA	23S rRNA gene (231)
23S-r	GGCTCCTACCTATCCTGTACA	
16S-uf	TAMRA-Uni-AGAGTTTGTCTGGCTCAG (10)*	16S rRNA gene (~1,500)
16S-r	AAGGAGGTGATCCAGCC (19)*	
Uni	GGTTTCGGATGTTACAGCGT	
Probes		
16S-U	NH ₂ -T ₁₂ -GCTGCCTCCCGTAGGAGT (7)*	Bacteria 16S universal
16S-G-	NH ₂ -T ₁₂ -AGGGCCATGATGACTTGACG (13)*	Gram negative 16S specific
16S-G+	NH ₂ -T ₁₂ -AAGGGGCATGATGATTTGACGTC	Gram positive 16S specific
16S-Str	NH ₂ -T ₁₂ -GTTAGCCGTCCTTTCTGG (12)*	<i>Streptococcus</i> 16S genus specific
16S-Ent	NH ₂ -T ₁₂ -GTTTCCAAGTGTTATCCC	<i>Enterococcus</i> 16S genus specific
16S-Sta	NH ₂ -T ₁₂ -TCCTCCATATCTCTGCGCAT	<i>Staphylococcus</i> 16S genus specific
16S-SA	NH ₂ -T ₁₂ -AGAAGCAAGCTTCTCGTCCG	<i>S. aureus</i> 16S species specific
16S-CoNS	NH ₂ -T ₁₂ -GGAGCAAGTCTTCTCTGTTTC	CoNS 16S specific
blaZ	NH ₂ -T ₁₂ -CTGCTTTTCGGTAAGACTTTAAATAAACTT	<i>blaZ</i>
mecA	NH ₂ -T ₁₂ -TATCCACCCTCAAACAGGTGAATT	<i>mecA</i>
aac6	NH ₂ -T ₁₂ -ATTGGAGTAAAGGAATTGGTACAAGAT	<i>aac(6')</i> - <i>Ie-aph(2'')</i>
ermA	NH ₂ -T ₁₂ -ATAGTAAACCCAAAGCTCGTTGC	<i>ermA</i>
ermC	NH ₂ -T ₁₂ -TTGGAAATTATCGTGATCAACAAGTT	<i>ermC</i>
msrA	NH ₂ -T ₁₂ -GCAAATGGCATACTATCGTCAACT	<i>msrA</i>
H	NH ₂ -TCACTTGCTTCCGTTGAGG-HEX	Position control
IC	NH ₂ -T ₁₂ -AYGGGGTCTTTCCGCTCCTGT	Internal control (23S rRNA gene)
NC	NH ₂ -T ₁₂ -CAAGCAGCCACGCCAGTAC	Negative control
EC	NH ₂ -T ₁₂ -CCTCAACGGAAGCAAGTGAT	External control
ECT	TAMRA-ATCACTTGCTTCCGTTGAGG	Target of external control

^a *, The source reference is indicated in parentheses. All other primers and probes were designed in the present study. Y = T or C; R = A or G. TAMRA, 6-carboxy-tetramethyl-rhodamine; HEX, hexachloro-6-carboxy-fluorescein; T₁₂, 12 consecutive thymines.

RESULTS

Eight-plex asymmetric PCR amplification. Before undertaking the multiplex reaction, we confirmed that the single PCR amplifications yielded the expected amplicons. An optimized multiplex asymmetric PCR (MAPCR) was then used to simultaneously amplify the six antibiotic resistance genes [*mecA*, *blaZ*, *aac(6')*-*Ie-aph(2'')*, *ermA*, *ermC*, and *msrA*], the 16S rRNA genes, and the 23S rRNA genes. This MAPCR reliably amplifies multiple targets and efficiently generates single-stranded products in a linear manner after the exponential phase by use of the UT primers and an elevated annealing temperature (72°C). Agarose gel electrophoresis results showed that a fragment about 1,500 bp corresponding to the double-stranded products of the 16S rRNA gene and a second fragment of <1,000 bp corresponding to the molecular size of its single-stranded products were seen in all of the isolates (data not shown), indicating that the MAPCR produced single-strand products efficiently. The amplification products of the antibiotic resistance genes and the 23S rRNA gene were be-

tween about 230 to 500 bp. Although the products of these genes would not be clearly observed and differentiated due to their short lengths, they hybridized on the microarray with high sensitivity and specificity.

Specificity and sensitivity of DNA microarray. Resistance genes assayed by the microarray test were selected based on clinical considerations. Our DNA microarray contained six probes specific to the consensus region of some six prevalent antibiotic resistance genes associated with resistance of staphylococci to clinically relevant antibiotics. In addition, eight sequence-specific probes based on known 16S rRNA gene sequences were included for differentiation between *S. aureus* and CoNS of other staphylococcal species. Included in this set were universal genus probes for streptococcus and enterococcus targets which were used as the negative controls for identification of staphylococcal species. The printed panel is shown in Fig. 1A.

To evaluate the specificity of the antibiotic resistance gene- and 16S rRNA gene-specific probes, we performed microarray

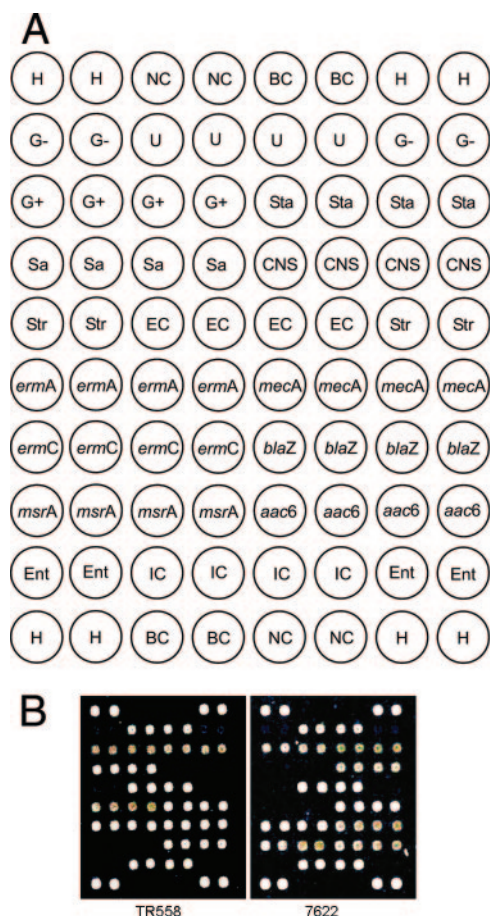


FIG. 1. DNA microarray hybridization patterns of staphylococcal isolates. (A) Layout of the oligonucleotide probes on the array surface. H, position control; NC, negative control; BC, blank control (50% dimethyl sulfoxide); U, bacterial 16S rRNA gene universal; G⁻, gram-negative 16S rRNA gene specific; G⁺, gram-positive 16S rRNA gene specific; Str, *Streptococcus* 16S rRNA gene genus specific; Ent, *Enterococcus* 16S rRNA gene genus specific; Sta, *Staphylococcus* 16S rRNA gene genus specific; Sa, *S. aureus* 16S rRNA gene species specific; CNS, CoNS 16S rRNA gene specific; IC, internal control (23S rRNA gene); EC, external control. *ermA*, *mecA*, *ermC*, *blaZ*, *msrA*, and *aac6* represented six antibiotic resistance genes prevalent in staphylococci. (B) Examples of DNA microarray hybridization patterns for *S. aureus* TR558 [with *mecA*, *blaZ*, *ermA*, *ermC*, and *aac(6')*-*Ie-aph(2')* genes] and CoNS 7622 [with *blaZ*, *mecA*, *aac(6')*-*Ie-aph(2')*, *ermC*, and *msrA* genes].

hybridization with the MAPCR amplicons from five reference strains (Table 1), each of which harbored only a single antibiotic resistance gene and belonged either to *S. aureus* or to CoNS. Because no strain containing only the *ermA* gene was available, strain 7605 with the two genes *ermA* and *ermC* was used to assess the specificity of the *ermA* probe. Each of the probes hybridized specifically to its corresponding target resistance gene, and no obvious cross-hybridization with other targets was observed (data not shown). The 16S rRNA gene-specific probes also clearly differentiated *S. aureus* from CoNS. *S. aureus* TR146 did not contain any of the six tested resistance genes; thus, no signal could be detected on the probes of the tested resistance genes. Furthermore, the blank control (dou-

ble-distilled H₂O) gave no hybridization signals at the antibiotic resistance gene probes and 16S or 23S rRNA gene-specific probes, except for the expected signal at the external hybridization positive control. The hybridization signal intensities varied slightly due to the different thermal stabilities of the different probe sequences when bound to their targets and the variation in the input of PCR amplicons. Nonetheless, detection of antibiotic resistance profiles was not affected. To assess the detection limit of the multiplex PCR-based microarray assay, we performed a serial dilution test with *S. aureus* 6314, *S. aureus* TR558, and CoNS 7622. Reliable detection of each of the six specific resistance gene targets could be made with a minimum of 10³ *S. aureus* or CoNS cells. This result indicates that the microarray assay could reliably and directly detect the antibiotic resistance genes from a single colony which contains about 10⁷ to 10⁸ cells (28).

In addition, tests using the reference strains showed that the signal intensities of the specific probes were only decreased marginally (20 to 30%) if the hybridization mixture was not denatured before hybridization (data not shown). The time of hybridization could also be shortened to 30 min with a similar small reduction of the signal intensities (a decrease of 10 to 30%) compared to the 1.5-h hybridization (data not shown).

Microarray testing of clinical isolates. We validated the microarray assay using some 415 confirmed clinical staphylococcal isolates recovered from different patients by comparison with the phenotypic results by classical disk diffusion assay. The hybridization results showed that the microarray differentiated *S. aureus* from other CoNS isolates.

The accuracy of this differentiation between *S. aureus* and CoNS isolates was independently confirmed in all cases by analysis with the Vitek 2 system, which provides species identification (36). An overview of the resistance genotypes of all 415 clinical isolates determined with the DNA microarray is shown in Table 3, and two examples of the microarray hybridization patterns are shown in Fig. 1B. A total of 29 different resistance genotypes were detected in the clinical isolates by the DNA microarray. Interestingly, the resistance genotypes harbored in *S. aureus* and CoNS isolates were to some extent different. Many clinical isolates contained multiple antibiotic resistance genes. For example, 68 *S. aureus* isolates harbored *blaZ*, *mecA*, *aac(6')*-*Ie-aph(2')*, *ermA*, and *ermC*, and 35 isolates harbored *blaZ*, *mecA*, *aac(6')*-*Ie-aph(2')*, and *ermA*, whereas for CoNS, 41 isolates harbored *blaZ*, *mecA*, *aac(6')*-*Ie-aph(2')*, and *ermC*, and 34 isolates harbored *blaZ*, *mecA*, *aac(6')*-*Ie-aph(2')*, *ermC*, and *msrA*.

Relationship between the antibiotic resistance genes and the phenotypic resistance. Table 4 shows the relationships between the microarray results and the phenotypic resistance determined by the disk diffusion methods and the microtiter nitrocefin method. The MAPCR-based microarray results correlated well with phenotypic antimicrobial susceptibility testing, and accurately differentiated the phenotypically resistant isolates from the phenotypically susceptible isolates, with a sensitivity of >90% for all of the tested antimicrobials. There were no significant differences between the genotypes and phenotypes for resistance to oxacillin, penicillin (detected by the nitrocefin method), and gentamicin in *S. aureus* and to penicillin (detected by the nitrocefin method), clindamycin, and erythromycin in CoNS (all *P* values were >0.05 as determined

TABLE 3. Resistance genotypes of the 415 different staphylococcal isolates tested

Resistance genotype ^a	No. of strains	
	CoNS	<i>S. aureus</i>
Wild type	12	8
<i>aac6</i>		1
<i>blaZ</i>	4	22
<i>ermC</i>	8	4
<i>mecA</i>	4	
<i>msrA</i>	9	
<i>blaZ aac6</i>	1	6
<i>blaZ ermC</i>		16
<i>blaZ mecA</i>	9	
<i>blaZ msrA</i>	1	
<i>ermA ermC</i>	1	
<i>mecA aac6</i>	6	
<i>mecA ermC</i>	6	
<i>mecA msrA</i>	1	
<i>blaZ aac6 msrA</i>		3
<i>blaZ mecA aac6</i>	10	4
<i>blaZ mecA ermA</i>	1	3
<i>blaZ mecA ermC</i>	18	
<i>blaZ mecA msrA</i>	17	
<i>mecA aac6 ermA</i>	1	
<i>mecA aac6 ermC</i>	22	
<i>mecA aac6 msrA</i>	2	
<i>blaZ mecA aac6 ermA</i>		35
<i>blaZ mecA aac6 ermC</i>	41	7
<i>blaZ mecA aac6 msrA</i>	22	
<i>blaZ mecA ermC msrA</i>	5	
<i>blaZ mecA aac6 ermA ermC</i>	1	68
<i>blaZ mecA aac6 ermA msrA</i>	1	1
<i>blaZ mecA aac6 ermC msrA</i>	34	
Total	237	178

^a *aac6*, *aac(6')*-Ie-*aph(2')*.

by the McNemar matched chi-square test; Table 4). Furthermore, the microarray results for *mecA* gene were found to be 100% consistent with the results of the "gold standard" *mecA*-PCR (26; data not shown), suggesting that the microarray assay would be acceptable for the detection of methicillin resistance in the patient care setting, such as a positive blood culture.

The microarray assay could detect >95% of the phenotypically resistant *S. aureus* and CoNS isolates for methicillin, penicillin (detected by the nitrocefin method), and gentamicin; however, for penicillin resistance in CoNS the sensitivity was only 91.9% using the penicillin disks. For clindamycin and erythromycin, the microarray assay detected 98.6 and 97.9%, respectively, of the phenotypically resistant CoNS isolates, while its accuracy was only 91.1 and 92.5%, respectively, in *S. aureus* isolates. Overall, the specific probes on the microarray can detect $\geq 90\%$ of *S. aureus* and CoNS isolates that are phenotypically resistant to the antibiotics most frequently used for staphylococci infections.

The specificity of the microarray results with the phenotypic susceptibility was good for *S. aureus* isolates, with >90% specificity for all of the tested antimicrobials except for gentamicin. This result further indicates that the absence of the corresponding antibiotic resistance gene(s) is highly correlated with the phenotypic susceptibility in *S. aureus* isolates. However, for CoNS, the microarray results were only highly related to the phenotypic susceptibility results for MLS_B, macrolide, and penicillin (when detected by the nitrocefin method) with a specificity of >90%. For penicillin (detected by penicillin disk) and oxacillin (detected by cefoxitin disk), the specificity relationship was much lower at 75 and 65.3%, respectively. Furthermore, the specificity of gentamicin susceptibility was low both in *S. aureus* (89.5%) and in CoNS (62.0%) isolates. Many

TABLE 4. Relationship between resistance gene status determined by microarray and phenotypic resistance

Parameter ^a	Relationship ^b					
	<i>mecA</i> to OX	<i>blaZ</i> to nitrocefin	<i>blaZ/mecA</i> to PEN	<i>aac(6')</i> -Ie- <i>aph(2')</i> to GM	<i>ermA/ermC</i> to CM	<i>ermA/ermC/msr</i> to ER
<i>S. aureus</i> (<i>n</i> = 178)						
No. of gene-positive isolates	118	165	165	125	134	137
% Sensitivity (%)	98.3 (116/118)	99.4 (164/165)	95.4 (165/173)	98.3 (119/121)	91.1 (133/146 ^c)	92.5 (136/147)
% Specificity (%)	96.7 (58/60)	92.3 (12/13)	100.0 (5/5)	89.5 (51/57)	96.9 (31/32)	96.8 (30/31)
<i>P</i>	1.000	1.000	0.008	0.289	0.002	0.006
CoNS (<i>n</i> = 237)						
No. of gene-positive isolates	201	165	207	139	139	191
% Sensitivity	97.9 (184/188)	98.1 (158/161)	91.9 (203/221)	100 (79/79)	98.6 (139/141 ^d)	97.9 (191/195)
% Specificity	65.3 (32/49)	90.8 (69/76)	75.0 (12/16)	62.0 (98/158)	100.0 (96/96)	100.0 (42/42)
<i>P</i>	0.007	0.344	0.004	<0.001	0.500	0.125
Total (<i>n</i> = 415)						
% Sensitivity	98.0 (300/306)	98.8 (322/326)	93.4 (368/394)	99.0 (198/200)	94.8 (272/287)	95.6 (327/342)
% Specificity	82.6 (90/109)	91.0 (81/89)	81.0 (17/21)	69.3 (149/215)	99.2 (127/128)	98.6 (72/73)
<i>P</i>	0.015	0.388	<0.001	<0.001	0.001	0.001

^a Sensitivity was calculated as the number of resistance gene-positive strains with phenotypic resistance/the number of strains with phenotypic resistance (indicated in parentheses). Specificity was calculated as the number of resistance gene-negative strains with phenotypic susceptibility/the number of strains with phenotypic susceptibility (indicated in parentheses). The *P* value was calculated using the McNemar matched chi-square test (SPSS 13.0; SPSS, Inc., Chicago, IL).

^b OX, oxacillin (phenotypic oxacillin results were detected by using a cefoxitin disk); PEN, penicillin; GM, gentamicin; CM, clindamycin; ER, erythromycin. The status of the related gene(s) was determined by microarray hybridization.

^c Includes 22 *S. aureus* isolates with MLS_B resistance.

^d Includes 45 CoNS isolates with MLS_Bi resistance.

aac(6')-Ie-*aph(2'')*-positive isolates were phenotypically susceptible to gentamicin, especially among CoNS isolates ($n = 60$). For all strains with a discrepancy between phenotype and genotype, the MICs of the corresponding antibiotics were determined by the agar dilution method on Muller-Hinton agar with an inoculum of 10^4 CFU per spot according to the NCCLS (24), using the breakpoints of the MICs as follows (in mg/liter): oxacillin ($R \geq 4$, $S \leq 2$ for *S. aureus*; $R \geq 0.5$, $S \leq 0.25$ for CoNS), gentamicin ($R \geq 8$, $S \leq 4$), clindamycin ($R \geq 4$, $S \leq 0.5$), and erythromycin ($R \geq 8$, $S \leq 0.5$). The MIC results showed that most of the phenotypically susceptible CoNS strains containing related resistance genes [*mecA* or *aac(6')*-Ie-*aph(2'')*] were borderline resistant or susceptible [15 of 17 *mecA*-positive CoNS isolates and 43 of 60 *aac(6')*-Ie-*aph(2'')*-positive CoNS isolates]. The high percentage of oxacillin borderline-resistant CoNS isolates may be the cause of the lower specificity for CoNS and oxacillin resistance as evaluated using the cefoxitin disk compared to that reported by Swenson et al. (38). In addition, five (2.8%) *S. aureus* (MLS_{B/C} phenotypic resistance) contained the *ermB* gene (PCR and DNA sequence analysis [data not shown]), conferring the resistant MICs to clindamycin and erythromycin.

DISCUSSION

We describe here a MAPCR-based microarray assay that can be used to survey clinically relevant antibiotic resistance genes frequently encountered in staphylococci. One of the major advantages of our method over other multiplex PCR-based or microarray-based systems described previously (27, 37) is that it reliably detects the most prevalent different groups of antibiotic resistance genes in staphylococci, and simultaneously differentiates *S. aureus* from other CoNS. These properties are necessary for its potential use for clinical diagnosis of the antibiotic resistance of staphylococcal infections. Probes for some infrequently encountered antibiotic resistance genes, such as *ermB*, were not included in the microarray assay, with the consequence that about 0.6 to 3% of the MLS_B resistance cannot be detected (32, 40). In addition, the genes *aphA3* coding for APH(3')III enzyme and *aadC* coding for ANT(4',4'') enzyme, which combined contribute only 10 to 30% of the resistance to aminoglycosides and are not considered clinically relevant because they mediate resistance to aminoglycosides not usually prescribed to treat staphylococcal infections (33), were also not present in the microarray assay. The low number of abundant resistance genes detected by the current MAPCR-based microarray assay was intentional, since it allows for sufficient numbers of samples of each genotype to be validated statistically. In the future, we may need to analyze more samples to ensure the accuracy of the microarray platform over a wider diversity of different resistance genotypes. We intend also to develop a second generation of MAPCR-based microarray by increasing the range of resistance genes to include less frequently encountered resistance mechanisms such as those encoded by the *ermB*, *aphA3*, and *aadC* genes and others.

Hamels et al. (15) also described a microarray for simultaneous identification of *Staphylococcus* species and methicillin resistance. However, other prevalent antibiotic resistance genes could not be detected in that assay. The MLS_{B/C} pheno-

type is not easily detected by standard susceptibility test methods, while failure to identify MLS_{B/C} resistance may lead to clinical failure when clindamycin therapy is used (8, 35). It is increasingly important to distinguish the MLS_{B/C} strains from MS-resistant strains that contain the *msrA* gene. Thus, the second benefit of our microarray is that it clearly identifies strains that remain susceptible to clindamycin but have MS resistance and the MLS_{B/C} phenotype. In the clinical setting, the simultaneous identification of the bacteria and determination of its susceptibility to antibiotics generally require 48 h (3), whereas in our method the detection time can be shortened to 5 h from a cultured isolate. In addition, unlike the traditional multiplex PCR which requires extensive optimizations (17), the MAPCR reaction used in the present study was easily optimized and reproducibly achieved efficient multiplex amplification by simple adjustment of the individual primer concentrations, without additional optimization of either the reaction components or annealing temperatures. Initially, we used the equimolar concentrations of each primer pair in the multiplex asymmetric amplification. However, the hybridization signals of the 16S rRNA, *blaZ*, and *aac(6')*-Ie-*aph(2'')* genes were weak or undetected (data not shown). When we increased the concentration ratios of these three primer pairs to 2:1 for the 16S rRNA gene and to 4:1 for the *blaZ* and *aac(6')*-Ie-*aph(2'')* genes, strong hybridization signals were seen for all genes without further optimization. Since the microarray was evaluated using isolated bacterial colonies, which required culture prior to analysis, further work will focus on increasing the sensitivity of the methods so that this assay could be adapted to use in direct detection from positive blood cultures and from normally stable clinical samples such as sputum or urine. In addition, the probes for detection of additional clinically relevant resistance genes in staphylococci such as *vanA* and *vanB* for glycopeptide resistance and the mutations of DNA topoisomerases for fluoroquinolone resistance, and the resistance genes of other clinically significant isolates, such as *Streptococcus* and *Enterococcus*, can be designed and incorporated into a system for extensive detection of possible infection and antibiotic resistance profiles of gram-positive bacteria.

We have compared this microarray assay for the detection of antibiotic resistance genes with traditional phenotypic methods for the determination of antibiotic susceptibility. Overall, we found correlations were more than 90% for detection of the phenotypic resistance and 100% for species differentiation. However, some of the discrepancies between the microarray results and the disk diffusion results are significant ($P < 0.05$ as determined by the McNemar matched chi-square test; Table 4) and were due mainly to the phenotypically susceptible but resistance gene-positive isolates. For example, we encountered oxacillin-susceptible but *mecA*-positive CoNS ($n = 17$), presumably because of the known heterogeneous expression of *mecA* gene in *Staphylococcus* in vitro (6). For more frequently encountered gentamicin-susceptible but *aac(6')*-Ie-*aph(2'')*-positive *S. aureus* ($n = 6$) and CoNS ($n = 60$) isolates, the discrepancy in genotype and phenotype might be attributed to the so-called silent antibiotic resistance gene that might become activated to express the resistance (21), and their presence may also facilitate the spread to other bacteria. From a clinical perspective, a susceptible strain harboring but not expressing an antibiotic resistance gene should be regarded as

potentially resistant to that antibiotic (22). Thus, detection of the discrepancies between the presence of the resistance gene and the phenotypic susceptibility (false-positive) is important for physicians to guide prescription of appropriate functional antibiotic therapy so as to control the spread of the resistance due to antibiotic selection. Furthermore, there are also discrepancies between the absence of the antibiotic gene test on the microarray and the phenotypic resistance (false negative). For example, approximately 7 to 10% of MLS_B resistance in *S. aureus* could not be detected by the microarray assay because of the presence of the *ermB* gene or other mechanisms, which results in a discrepancy of detection of clindamycin resistance in *S. aureus* between phenotype and genotype that is significant ($P = 0.002$ as determined by the McNemar matched chi-square test). We intend to develop the microarray to increase the genotypic testing of such related resistance genes so that it can also be readily adopted for detection of clindamycin resistance in samples such as skin or soft tissue isolates of *S. aureus*.

In conclusion, the MAPCR-based microarray assay provides a rapid, simple, and reliable tool for parallel detection of the prevalent antibiotic resistance genes in staphylococci and a definite discrimination between *S. aureus* and CoNS in a 5-h procedure after pure culture isolation. This approach appears to be highly robust and highly informative, can be adapted to analyze the clinically important staphylococcal isolates for diagnosis-based studies, and could supplement or provide an early indication of likely antibiotic resistances. This early detection would allow clinicians initially to avoid potentially inappropriate treatment options and allow prompt intervention in infection control issues. In the future, we will work on increasing the sensitivity of the microarray for nonculture diagnoses of bacterial infections and expanding the repertoire of the antibiotic resistance genes for detection of more phenotypic resistance.

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