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RESEARCH ARTICLE

Simple and Robust Array-Based Methods for the Parallel Detection of Resistance Genes of *Staphylococcus aureus*

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ABSTRACT: The diagnostic use of DNA arrays in microbiology allows the simultaneous detection of a multitude of different target genes. To develop an array-based procedure that can be performed in a routine diagnostic laboratory, *Staphylococcus aureus* was selected as a target organism. A DNA array comprising probes for species-specific controls, different genes for antibiotic resistance properties including *mecA*, and for the gene of the toxic shock syndrome toxin was constructed. This array was applied for hybridizations with labeled genomic DNA from clinical isolates of *Staphylococcus aureus* and from other *Staphylococcus* species. Labeling and detection of hybridizations were performed by four different protocols. These results were compared with each other and with data from conventional susceptibility testing showing a high degree of accordance. A protocol using a sequenase-driven random amplification followed by incorporation of biotin-labeled nucleotides and a detection by a streptavidin-gold-induced silver precipitation appeared to be the most sensitive and most economical method for the detection of hybridizations on the array. Thus, DNA arrays may become a useful tool for the study of the genetic epidemiology of antimicrobial resistance, as well as for the rapid identification of staphylococcal toxins.

Keywords: DNA Array, *Staphylococcus aureus*, Antimicrobial resistance.

1. INTRODUCTION

The diagnostic use of DNA arrays in medicine and microbiology offers the possibility to analyze multiple target genes at once, saving time and resources. Numerous research groups are currently working on different array-based approaches for these applications [1]. One technique uses standard format glass slides with modified surfaces on which oligonucleotides, polymerase chain reaction (PCR) products, peptides, or proteins are spotted [2]. Commercial spotting machines are available for the deposition of the probes on the chip which use needles or piezoelectric cartridges (http://www.gene-chips.com; http://ihome.cuhk.edu.hk/~b400559/array.html). Hybridization against the probe arrays usually is performed with fluorescence-labeled target nucleic acids. For the subsequent readout of the slide, confocal laser scanning microscopes or CCD-camera-based detection systems are required ([3] and http://129.143.8.2/biotech/rubric/products3/p00-03-a3.pdf).

The resulting scan images are analyzed and processed by specific software (http://ihome.cuhk.edu.hk/~b400559/arraysoft.html; http://www.cs.tcd.ie/Nadia.Bolshakova/softwaretotal.html).

Other approaches are based on *in situ* synthesis of arrays [4–6], electrically controlled immobilization [7], and micro wet printing technology [8]. The resulting arrays differ in formats, hardware requirements, and pricing. Some of these systems are available as commercial ready-to-use systems (e.g., http://www.affymetrix.com; http://www.nanogen.com; http://www.clondiag.com). However, up to now no proce-

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dures or commercial test kits have been available for routine clinical tasks.

For the application of DNA arrays in routine medical analyses, high standards of reproducibility and quality have to be fulfilled. A good approach for the standardization of array experiments was elaborated by the MGED group (see [9] and http://www.mged.org/Workgroups/MIAME/miame_checklist.html). Furthermore, an array-based system must be easy to handle, compatible with preexisting laboratory equipment, and affordable in terms of purchasing price and maintenance costs.

To develop and optimize a diagnostic procedure using an array-based assay, *Staphylococcus aureus* was selected as a model organism, as it has several well-characterized genes for antibiotic resistance. Furthermore, this organism can be cultured *in vitro* easily, providing a simple possibility for verification of DNA-based results by standard susceptibility testing.

The following genes were included in this array:

CoA is the gene for coagulase. This is a species-specific virulence factor of *S. aureus*, stimulating the conversion of fibrinogen to fibrin [10]. Several assays and commercial kits use coagulase for quick identification of that germ since other staphylococci lack this enzyme. Thus, these species are known as coagulase-negative staphylococci (CNS). *NorA* encodes a membrane-associated protein that could be used as a second species-specific marker for *S. aureus*. Initially it was identified as a fluoroquinolone resistance gene [11]. Subsequently it was shown that it also confers resistance on other compounds [12]. Detection of that gene cannot predict resistance to fluoroquinolones. The overexpression rather than the presence of *norA* can cause decreased susceptibility as well as mutations in gyrase or topoisomerase [13]. *CatA* is a gene for catalase, and *femA* encodes a factor involved in peptidoglycan synthesis [14]. Although coagulase-negative staphylococci also have catalase and genes homologous to *femA*, probes were designed to be species specific for *S. aureus*.

The *blaZ* gene encodes a beta-lactamase, conferring resistance to penicillin G but not to penicillinase-resistant penicillins, such as methicillin or flucloxacillin [10]. The presence of the *mecA* gene defines a methicillin-resistant *S. aureus* (MRSA) [10]. It encodes a modified penicillin-binding protein (PBP2′) that causes resistance not only to methicillin but to all beta-lactam antibiotics. Both genes are known to occur in *S. aureus* as well as in coagulase-negative staphylococci.

ErmA and *ermC* are, according to major European studies [15, 16], the most prevalent genes causing resistance to erythromycin and related compounds in *S. aureus*. *Erm* genes can also cause an inducible or a constitutive resistance to clindamycin [17]. *LinA* encodes an enzyme that modifies lincosamides, thus also causing resistance to clindamycin [18].

AacA-aphD and *aphA3* encode the aminoglycoside modifying enzymes AAC(6′)-APH(2″) and APH(3′), respectively. These enzymes cause resistance to aminoglycosides. They can be identified by susceptibility patterns, with the use of several of these drugs as substrates. *AacA-aphD* confers resistance to gentamicin, whereas the presence of *aphA3* can be assumed in strains that are susceptible to that drug but resistant to kanamycin or neomycin [19, 20].

DfrA encodes a dihydrofolate reductase responsible for trimethoprim resistance. *TetK* and *tetM* are genes causing resistance to tetracyclines. They appear to be the most prevalent *tet* genes in *S. aureus* [21]. *TetK* encodes an active efflux pump, and the product of *tetM* protects bacterial ribosomes. *Sat* genes confer resistance to streptothricin antibiotics [22], which are used in livestock feeding only.

Tst-1 is the gene for the toxic shock syndrome toxin (TSST) [23, 24]. It is a superantigen and it causes a nonspecific activation of T-cells, resulting in severe shock and subsequent desquamation. *Tst-1* was included in the DNA array because there is no routine assay for this toxin, although PCR is performed in some reference laboratories. The clinical effects of TSST are so severe that a rapid diagnostic test might be helpful for the management of patients.

2. MATERIALS AND METHODS

2.1. Strains and Culture

Staphylococcus strains were isolated from clinical samples (Table I). Samples were spread on Columbia blood agar (OXOID, Wesel, Germany), incubated overnight at 37 °C, and cloned by the picking of single colonies. Screening for the clumping factor and coagulase was performed with a commercial kit (Staphytect Plus; OXOID) and rabbit plasma. Identification and susceptibility testing was performed with the VITEK I system (BioMerieux, Nürtingen, Germany), following standard procedures supplied by the manufacturer. Susceptibility test results indicating methicillin resistance were confirmed with the MRSA-screen agglutination assay (Innogenetic, Ghent, Belgium), which detects PBP2′.

In addition, susceptibility testing for neomycin was performed with a standard agar dilution technique with Mueller Hinton plates containing drug concentrations from 0.5 to 128 μg/ml.

Trimethoprim was tested with the use of disk diffusion (1.25 μg per disk, OXOID).

The identification of epidemic strains of MRSA, confirmation of susceptibility testing results for these isolates, and PCR assays for detection of *tst-1* were kindly performed by the German National Reference Centre for Staphylococci at Wernigerode.

2.2. DNA Preparation

For DNA preparation, cultures on Columbia blood agar were incubated overnight at 37 °C. For harvesting, staphylococci were suspended in 5 ml of sterile isotonic saline (0.9% NaCl) and centrifuged at 3000 rpm for 10 min. The sediment

Table I. *Staphylococcus spec.* isolates used for the present study.

Sample ID	Species	MRSA Epidemic strain	Source
B 23	*S. epidermidis*	—	Blood culture, surgical ward
B 43	*S. aureus*	—	Hickman catheter, pediatric ward
B 50	*S. haemolyticus*	—	Blood culture, hematological ward
B 101	*S. hominis*	—	Blood culture, pediatric-hematological ward
V 568	*S. aureus*	—	Ventriculo-peritoneal shunt, neurosurgical ward
V 8510	*S. aureus*	MRSA Hannover	Swab from diabetic food ulcer, medical OPD
V 14652	*S. aureus*	Possibly MRSA Southern Germany	Bronchial aspirate, surgical ICU
V 14700	*S. aureus*	Possibly MRSA Berlin	Nasal swab, urological ward
V 21179	*S. aureus*	Possibly MRSA Berlin	Swab from diabetic food ulcer, medical OPD
V 23881	*S. aureus*	—	Swab, gynecological ward
V 29618	*S. aureus*	MRSA Berlin	Swab from diabetic food ulcer, medical OPD
V 30827	*S. aureus*	MRSA Southern Germany	Swab from diabetic food ulcer, medical ward

OPD = Outpatient Department, ICU = Intensive Care Unit.

was resuspended in 1000 μl of a lysis buffer containing 200 mg lysostaphin (Sigma, Steinheim, Germany), 2 g lysozyme (Sigma), 2 g RNase (Sigma), 2 ml Tris/HCl (20 mM, pH 8.0), 2 ml EDTA (2 mM), and 1.2 ml Triton X-100 per 100 ml of distilled water.

The suspension was incubated on a rotary shaker (300 rpm) for 30 min at 37 °C. Then, 125 μl Proteinase K (from Qiagen kit DNeasy, catalog no. 69506; Qiagen, Hilden, Germany) and 1000 μl of a second lysis buffer (Buffer AL from Qiagen kit DNeasy) were added, and the sample was incubated for 30 min at 56 °C. After the addition of 1000 μl ethanol, the sample was divided into five aliquots, which were transferred to Qiagen columns (Qiagen kit DNeasy) for DNA purification according to the manufacturer's specifications.

The quality of each DNA preparation was controlled by agarose gel electrophoresis and subsequent ethidium bromide staining.

2.3. DNA Array Preparation

Gene sequences and sources that were used for the present study are shown in Table II. Probe sequences were derived from published sequences with the Array Design Software Package by CLONDIAG chip technologies (Jena, Germany). As far as possible, more than one source for sequence information was used to cover possible sequence diversity. To prevent a high divergence of signal strengths, probes were designed to have similar lengths, GC contents, and melting temperatures. In addition, probe sequences were blasted against the PUBMED database (http://www.ncbi.nlm.nih.gov/BLAST/). For the genes included in the given array we found neither evidence for relevant sequence variation nor possible cross-reactions with genes other than the intended target genes.

For the spotting procedure of the probes on the array chips, 5′ amino-modified oligonucleotides with these sequences were purchased from MWG Biotech GmbH (Ebersberg, Germany). They were applied in a final concentration of 10 μM dissolved in Spotting Buffer 1 (Quantifoil Micro Tools GmbH, Jena, Germany). Then, arrays were spotted with a Microgrid II spotting machine (BioRobotics Inc./Apogent Discoveries Europe, Cambridge, England), which was used following the procedure supplied by the manufacturer. Arrays that were to be used in experiments using labeling based on biotin were inserted in ArrayTubes by CLONDIAG chip technologies. A typical probe layout on the array is shown in Figure 1 (*catA, femA*, and *dfrA* are not shown in this figure because they were included in a later version of the array).

2.4. Labeling

For fluorescence labeling with terminal transferase, 6 μg genomic staphylococcal DNA, 6 μl 10 × NEBuffer4, 6.0 μl 2.5 mM $CoCl_2$, 2.0 μl terminal transferase (20 U/μl; New England Biolabs M0252L), 3.0 μl *HhaI* (20 U/μl; New England Biolabs R0139S), 3.0 μl *NlaIII* (10 Uμl; New England BioLabs R0125S), and 0.6 μl Renaissance Cy3-dCTP (25 nM; Perkin Elmer NEL576) were mixed, and the solution was filled with distilled water to a volume of 36 μl. Then the mix was incubated for 1 h at 37 °C and 550 rpm. After denaturation (5 min at 95 °C) the sample was cooled down (3 min at 4 °C), precipitated with 1/10 vol. 5 M NaCl and 2.5 vol. 96% ethanol (−20 °C), and washed two times with ice-cold 70% ethanol. The precipitate was transferred into 3 μl distilled water and used for hybridization.

For the labeling with biotin-N4-dCTP and terminal transferase [42], a biotin labeling mix was prepared from 10 μg staphylococcal DNA, 3.6 μl 10 × NEBuffer 4, 3.6 μl 2.5 mM $CoCl_2$, 1.2 μl terminal transferase (20 U/μl), 1.8 μl *HhaI* (20 U/μl), 1.8 μl *NlaIII* (10 U/μl), and 1.0 μl Renaissance biotin-N4-dCTP (25 nM; Perkin Elmer NEL509). Distilled water was added to the solution, resulting in a volume of 60 μl. The sample was incubated for 1 h at 37 °C at 550 rpm. After denaturation (5 min at 95 °C), it was cooled down (3 min at 4 °C), precipitated with 1/10 vol. 5 M NaCl and 2.5 vol. 96% ethanol (−20 °C), and washed

Table II. Probe sequences and sources for sequence data on which the probes are based.

Gene	Probe ID	Probe sequence	Source	Genebank
catA	1	GAGGATTTGCGTTAAAGTTCTACACTGA	[25]	AJ000472
	2	CACGATGAGTATCCTCTAATTGAAGTTG		
coA	1	GAAATCACAAGTTAATGCTGGGAGTAA	[26]	X17679
	2	AGCAGAAGAAGATAAAGCAACTAAGGAAG		
	3	GTAAAGCTGAAGAAACAACACAACCAGT		
	4	ATATAACGTAACAACACATGGAAACGG		
femA	1	ATGAGTTAAAGCTTGCTGAAGGTTATGA	[27]	X17688
	2	TGAATTACCTATCTCTGCTGGTTTCTTC		
	3	GGTAGTGGTTATGATAGTGTGGCATATTT		
norA	1	GATTTGGGATTAACTGGTAGTGATTTAGG	[11], [28],	D90119, AB019536,
	2	GATTTATGGCAGAAGTTTCACATCGTA	[29]	M80252
	3	GAAGCACCAATTTATATGGCTATAGGTG		
	4	GCTATAGGTGTTTCATTAGCAGGTGTT		
mecA	1	AGCGATAATGGTGAAGTAGAAATGACTG	[30]	X52593
	2	GGTATGTGGAAGTTAGATTGGGATCATA		
	3	CAGGTGAATTATTAGCACTTGTAAGCAC		
	4	CTATTAACTGATGGTATGCAACAAGTCG		
blaZ	1	CATGTAATTCAAACAGTTCACATGCC	[31], [32],	U58139, X16471,
	2	GAGATTTGCCTATGCTTCAACTTCA	[33], [34]	X04121, M15526
	3	TTTGTTTATCCTAAGGGCCAATCTG		
	4	ATAAGTGAAACCGCCAAGAGTGTAA		
ermC	1	TAATATCTTTGAAATCGGCTCAGGA	[35]	M17990
	2	TCGTAACTGCCATTGAAATAGACCA		
	3	TATTTAATCGTGGAATACGGGTTTG		
	4	ACATGCAGGAATTGACGATTTAAAC		
ermA	1	CACACGAATATCAGTAAACAAGACAACG	[36]	D86934
	2	TCAAGTGACTAAAGAAGCGGTAAACC		
	3	GCAACGAGCTTTGGGTTTACTATTAA		
	4	GGTAAACCGTGAATATCGTGTTCTTT		
linA	1	TGGAAGTAACTCATTGGTTAGATGGAG	[17]	J03947
	2	GATGCCTTCACGTATGGAACTTAAA		
	3	TGATGGTTCCATTACTCAAGCAAAC		
	4	ATACCATGCATTTCCAAAGAAGCTC		
aphA3	1	GGAAGGAATGTCTCCTGCTAAGGTATATAA	[22], [37]	U51474, M36771
	2	GAAGAGTATGAAGATGAACAAAGCCCT		
	3	AGATGGCAAAGTAAGTGGCTTTATTG		
	4	GGGAAGAACAGTATGTCGAGCTATTT		
aacA-aphD	1	GAAAGTGTATCAAACTGCTAAATCGGTA	[38]	M18086
	2	AATAATCCAAGAGCAATAAGGGCATAC		
	3	GGTAGTGGTTATGATAGTGTGGCATATTT		
	4	GACAAATGCACGGTTTAGATTATACAGA		
dfrA	1	TAAAGCATATTAAACAACTGACCACTGG	[39]	AF051916
	2	TCGTACTCACTAACCAAGCTTCATTTC		
tetM	1	TAACAGTGGAGCGATTACAGAATTAGG	[40]	M21136
	2	CAGAATTGTTCTCTGTTCCCTCTTTATC		
	3	GCTCGGTTTCTCTTGGATACTTAAATC		
	4	TACAAATGGGCTTAGTGTTTGTTTAGC		
tetK	1	GGAATTACAAACTGGGTAAACACTGC	[41]	S67449
	2	AATTGTAGCTTTAGGTGAAGGGTTAGGT		
	3	TCAAGTAGTCTTTCTGAAGAAGAAGTTGC		
	4	AGCAATTGTAGGAGGTTTATTGTCACTAC		
sat	1	AGGGCACCTGAAAGATATCGATAAA	[22]	U51474
	2	TGAGGTGATAGGTAAGATTATACCGAGGT		
	3	CGAGAATTGGACCTTTACAGAATTACTC		
	4	AGGCAGATTGCCTTGAATATATTGAC		
tst-1	1	CCCTGTTCCCTTATCATCTAATCAAA	[24]	J02615
	2	GCGAAGGAACTTATATCCATTTCCA		
	3	ACTTTGAAATTCGTCATCAGCTAACTC		
	4	CGGATCCACATATCAAAGTGATTTATC		

91: POSITION MARKER	92: aphA3, 2	93: aphA3, 3	94: aphA3, 3	95: aphA3, 4	96: linA, 1	97: linA, 2	98: linA, 3	99: linA, 4	100: POSITION MARKER
81: POSITION MARKER	82: sat, 1	83: sat, 2	84: sat, 2	85: sat, 3	86: sat, 3	87: sat, 4	88: sat, 4	89: aphA3, 1	90: aphA3, 1
71: norA, 4	72: norA, 4	73: tst-1, 1	74: tst-1, 1	75: tst-1, 2	76: tst-1, 2	77: tst-1, 3	78: tst-1, 3	79: tst-1, 4	80: tst-1, 4
61: tetK, 3	62: tetK, 3	63: tetK, 4	64: tetK, 4	65: norA, 1	66: norA, 1	67: norA, 2	68: norA, 2	69: norA, 3	70: norA, 3
51: tetM, 2	52: tetM, 2	53: tetM, 3	54: tetM, 3	55: tetM, 4	56: tetM, 4	57: tetK, 1	58: tetK, 1	59: tetK, 2	60: tetK, 2
41: aacA-aphD, 1	42: aacA-aphD, 1	43: aacA-aphD, 2	44: aacA-aphD, 2	45: aacA-aphD, 3	46: aacA-aphD, 3	47: aacA-aphD, 4	48: aacA-aphD, 4	49: tetM, 1	50: tetM, 1
31: ermC, 4	32: ermC, 4	33: ermA, 1	34: ermA, 1	35: ermA, 2	36: ermA, 2	37: ermA, 3	38: ermA, 3	39: ermA, 4	40: ermA, 4
21: blaZ, 3	22: blaZ, 3	23: blaZ, 4	24: blaZ, 4	25: ermC, 1	26: ermC, 1	27: ermC, 2	28: ermC, 2	29: ermC, 3	30: ermC, 3
11: coA, 1	12: coA, 2	13: coA, 3	14: coA, 3	15: coA, 4	16: coA, 4	17: blaZ, 1	18: blaZ, 1	19: blaZ, 2	20: blaZ, 2
1: POSITION MARKER	2: mecA, 1	3: mecA, 2	4: mecA, 2	5: mecA, 3	6: mecA, 3	7: mecA, 4	8: mecA, 4	9: coA, 1	10: POSITION MARKER

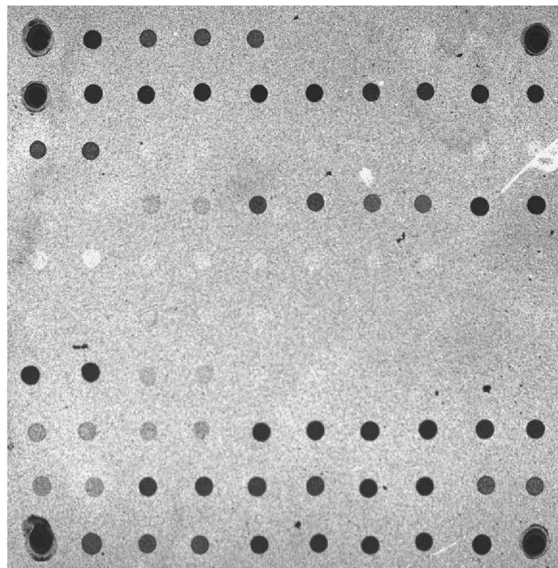

Figure 1. Layout and microphotograph of an array hybridized with terminal transferase/biotin-labeled DNA from MRSA V 21179.

two times with ice-cold 70% ethanol. The precipitate was transferred to 3 μl distilled water and then used for hybridization.

The labeling using a random primed sequenase reaction was performed according to the protocol published under http://www.microarrays.org/pdfs/Round_A_B_C.pdf, which is based on a method by Bohlander [43]. In short, the sample DNA was amplified by sequenase, with the use of a primer that contains a random sequence at its 3′ end and a defined sequence at its 5′ end. This resulted in a multitude of fragments that are reamplified in a PCR reaction, with this defined 5′ sequence as a primer. In a third round of amplification, these PCR products were used as templates in a repetition of that PCR incorporating either Cy3-dCTP or biotin-16-dUTP.

Negative controls containing distilled water instead of DNA preparations were subjected to the same amplification rounds, agarose gel electrophoresis, and subsequent ethidium bromide staining to rule out possible contamination.

2.5. Hybridization Reactions and Measurements

After washing and blocking following manufacturer's procedures, the arrays or, respectively, the array tubes were hybridized with labeled sample DNA (as described above). The samples were dissolved with 80 μl (Cy3-labeled DNA), or approximately 100 μl (biotin-labeled DNA) hybridization buffer (Quantifoil Micro Tools GmbH, Jena, Germany), denatured (3 min at 95 °C), cooled immediately for 2 min on ice, and then hybridized for 1 h at 50 °C.

When the Cy3-labeled DNA was used, the arrays were washed after hybridization in 800 μl 2× standard saline citrate (SSC)/0.2% sodium dodecyl sulfate (SDS), 800 μl 2× SSC, and 800 μl 0.2× SSC (see [44]; each step 5 min at room temperature and 550 rpm) and vacuum-dried after the procedure. Then they were analyzed with a fluorescence microscope (Axioskop II Zeiss, 100W HBO; Sensicam, Zeiss; filter set number 26).

When the biotin-labeled DNA was used, the ArrayTubes were washed after hybridization in 800 μl 2× SSC + 0.2% SDS, 800 μl 2× SSC, and 800 μl 0.2× SSC (10 min at 550 rpm) and blocked in blocking solution according to the manufacturer's specifications (CLONDIAG chip technologies) (15 min at 37 °C). Then the ArrayTubes were incubated with 0.946 μl streptavidin-gold conjugate (British Biocell) in 100 μl 6× SSPE + 0.1% SDS (15 min at 30 °C and 350 rpm). Following this, the arrays were washed with 500 μl 2× SSC + 0.2% SDS, 500 μl 2× SSC, and 500 μl 0.2× SSC (each step 5 min at room temperature and 550 rpm). Silver-based developing solution was prepared by mixing equal volumes of the enhancer (British Biocell) and initiator solution (British Biocell) and adding them to the ArrayTube (100 μl per array). Then, the silver staining procedure and online detection were performed in an ArrayTube reader (atr01; CLONDIAG chip technologies) for 40 min at 25 °C, with the use of manufacturer's specifications and software.

For analysis of data from experiments, signal intensity and local background measurements were obtained for each spot on the array with the use of Iconoclust software (CLONDIAG chip technologies GmbH). Extinctions of local backgrounds were subtracted from extinctions of spots. Resulting values below 0.1 were considered negative (−) and those above 0.3 as positive (+). Values between 0.1 and 0.3 were regarded as ambiguous (+/−). Threshold values were derived from a series of measurements applying

the biotin-N4-dCTP/terminal transferase protocol on *coA* and *mecA* probes. These genes were selected for the determination of thresholds because their presence can be predicted reliably by phenotypic markers. *CoA* is positive in *S. aureus* and negative in CNS and *mecA* is present in PBP2′-positive MRSA strains and absent in PBP2′-negative methicillin-susceptible strains.

To determine the presence of a gene in a sample, average values for all measurements with all probes for that gene within a given protocol were calculated. Again, mean values below 0.1 were considered negative and those above 0.3 as positive.

3. RESULTS AND DISCUSSION

3.1. Conventional Tests

Conventional susceptibility testing and identification methods yielded the results summarized in Table III. Isolates were regarded as susceptible, intermediate, or resistant, according to VITEK I data. For neomycin, MIC values are stated, as there were no defined breakpoints available. All isolates that were considered resistant to trimethoprim showed no inhibition, whereas susceptible isolates had growth inhibition zones with diameters of more than 22 mm.

One *S. aureus* isolate (V 568) was positive in a PCR for *tst*-1, although it was isolated from a neurosurgical patient without symptoms of the Toxic Shock syndrome.

3.2. Hybridizations

A microphotograph of a hybridized array is shown in Figure 1.

In the present study, the results of conventional testing were to be compared with the results from array-based hybridizations using different methods for detection. All protocols yielded comparable results, although generally the silver precipitation methods appeared to be more sensitive.

All *S. aureus* isolates tested positive for *coA*. Three isolates of coagulase-negative staphylococci (*S. epidermidis, S. haemolyticus*, and *S. hominis*) were included in the study, and all of them were negative for *coA*. The *norA*, *femA*, and *catA* were detected in all *S. aureus* isolates with the use of the described set of probes that were designed to be specific for that species. Coagulase-negative staphylococci were negative, but in some cases weak (borderline) signals were detected.

MecA was detected in all isolates showing oxacillin resistance, including the three isolates of oxacillin-resistant coagulase-negative staphylococci. None of the susceptible isolates showed hybridization signals.

The *blaZ* gene, which encodes a penicillinase, was detected in all tested isolates, in *S. aureus* as well as in coagulase-negative isolates. All of them were penicillin G resistant in conventional tests.

Erm genes were found in six isolates (*S. aureus* B 43, V 8510, V 14652, V 14700, V 21179, and V 30827). Phenotypically, all of them were resistant. Two isolates were resistant to erythromycin, although no signals (*S. epidermidis* V 23) or very weak signals in some assays (*S. haemolyticus* B 50) for *erm* genes were detected. No isolate was tested positive for *linA*.

The *S. aureus* isolates V 8510, V 14652, V 14700, V 21179, V 29618, and V 30827 as well as the *S. haemolyticus* isolate (B50) were positive for the *aphA3* gene. Phenotypically, they had elevated MIC values for neomycin (8-64 μg/ml), except for one *S. aureus* isolate (V 30827), which had a MIC of only 2 μg/ml. The remaining isolates, which were *aphA3*-negative, had MIC values of 0.5, with the exception of one isolate (*S. hominis* B 101: MIC 4 μg/ml). Four *S. aureus* isolates (V 8510, V 14652, V 14700, and V 30827), the *S. epidermidis* isolate (B 23), and the *S. haemolyticus* isolate (B 50), which were resistant to gentamicin, gave positive hybridization signals for the *aacA-aphD* gene. All susceptible isolates (with the exception of the *S. hominis* isolate B 101) were *aacA-aphD*-negative.

One *S. aureus* isolate (V 14700) and the *S. epidermidis* isolate B 23 were positive for *dfrA*. They were resistant to trimethoprim. Two *dfrA*-negative isolates (*S. aureus* V 8510 and *S. hominis* B 101) showed resistance to that drug. The remaining *dfrA*-negative isolates were susceptible.

One isolate resistant to tetracycline (*S. aureus* V 8510) was positive for the *tetM* gene, another one (*S. hominis* B 101) for *tetK*. One isolate (*S. haemolyticus* B 50) showed intermediate susceptibility, but it was clearly negative in all hybridization assays. The gene *sat* for streptothricin resistance was included, although no results of susceptibility tests were available. Seven isolates, including the *S. haemolyticus* isolate (B 50), showed positive reactions with the *sat* probes.

The gene for toxic shock toxin (*tst*-1) was found in one isolate (V 568), which was isolated from a neurosurgical patient without symptoms of TSS. The hybridization results were confirmed by PCR.

3.3. Discussion

Screening of bacterial strains for resistance genes with the use of DNA arrays could contribute to our understanding of how a given sequence translates in a resistance phenotype, as this technology makes it possible to screen high numbers of sample strains for a multitude of target genes. This knowledge makes it possible not only to infer the underlying molecular mechanism from resistance patterns, but also to predict cross-resistances and to ensure the quality of conventional susceptibility test results [19, 20]. For organisms that are not easily grown *in vitro*, DNA arrays might even become a routine tool for the detection of resistance genes. Differences between conventional and DNA-based methods, however, can occur. Some strains could be susceptible to a specific antibiotic compound, although they contain a gene for resis-

Table III. Results of conventional and array-based tests.

		B 23					B 43					B 50					B 101				
Gene	Property	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio
catA	Species identification	*Staphylo-*	−	−	−	−	*Staphylo-*	nt	+	+	+	*Staphylo-*	nt	−	−	−	*Staphylo-*	nt	−	−	−
coA		*coccus*	−	−	−	−	*coccus*	−	+/−	−	+	*coccus*	−	−	+/−	−	*coccus*	−	−	−	−
femA		*epider-*	+	−	−	+/−	*aureus*	nt	+	−	+	*haemo-*	nt	−	−	−	*hominis*	nt	−	−	−
-norA		*midis*	+/−	−	−	−		−	+	+/−	+	*lyticus*	−	−	+/−	−		−	−	−	−
mecA	Resistance to oxacillin	Resistant	+	+	+/−	+	Susceptible	−	−	−	−	Resistant	+/−	+	+	+	Resistant	+/−	+	+/−	+
blaZ	Beta-lactamase	Positive	+/−	+/−	−	+	Positive	+	+	−	+	Positive	+/−	+/−	+/−	+/−	Positive	+	+	+/−	+
ermC	Resistance to erythromycin and clindamycin	Erythr. resistant / Clinda. susceptible	−	−	−	−	Erythr. resistant / Clinda. inducable resistant	+	+	+/−	+	Resistant	+/−	+/−	−	−	Susceptible	−	−	−	−
ermA			−	−	−	−		−	−	−	−		−	−	−	−		−	−	−	−
linA			−	−	−	−		−	−	−	−		−	−	−	−		−	−	−	−
aphA3	MIC for neomycin	0.5	−	−	−	−	0.5	+/−	−	−	−	8	−	+	+	+	4	−	−	−	−
aacA-aphD	Resistance to gentamicin	Resistant	+	+	−	+	Susceptible	−	−	−	−	Resistant	+	+	+	+	Susceptible	+/−	+	+/−	+
dfrA	Resistance to trimethoprim	Resistant	+	+	+	+	Susceptible	nt	−	−	−	Susceptible	nt	−	−	−	Resistant	nt	−	−	−
tetM	Resistance to tetracycline	Susceptible	−	−	−	−	Susceptible	−	−	−	−	Interm	−	−	−	−	Resistant	−	−	−	−
tetK			−	−	−	−		−	−	−	−		−	−	−	−		+	+	+	+
sat	Resistance to streptothricin	nt	−	−	−	−	nt	+	−	−	−	nt	−	+	+	+	nt	−	−	−	−
tst1	Toxic shock syndrome	nt	−	−	−	−	nt	−	−	−	−	nt	−	−	−	−	nt	−	−	−	−

TT = terminal transferase; RP = Random Primed; cy3 = labeled with Cy3; bio = labeled with biotin-streptavidin-gold; nt = not tested. +, +/−, and −: average values as described in "Hybridization reactions and measurements."

Table III. Continued

Gene	Property	V 568					V 8510					V 14652					V 14700				
		Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conventional tests	TT-cy3	TT-bio	RP-cy3	RP-bio
catA	Species	*Staphylo-*	nt	nt	+/−	+	*Staphylo-*	nt	+	+/−	+	*Staphylo-*	nt	+	+/−	+	*Staphylo-*	nt	+	+/−	+
coA	identification	*coccus*	+/−	+/−	+	+	*coccus*	+	+	+	+	*coccus*	+	+/−	+/−	+	*coccus*	nt	+	+/−	+
femA		*aureus*	nt	nt	−	+	*aureus*	nt	+	−	+	*aureus*	nt	+	−	+/−	*aureus*	nt	+	+	+
norA			+	+	+/−	+		+/−	+	+/−	+		+	+	+/−	+		nt	+	+/−	+
mecA	Resistance to oxacillin	Susceptible	−	−	+/−	−	Resistant	+	+	+/−	+	Resistant	+	+	+/−	+	Resistant	nt	+	+/−	+
blaZ	Beta-lactamase	Positive	+	+	−	+	Positive	+/−	+	+/−	+	Positive	+	+	−	−	Positive	nt	+	+/−	−
ermC	Resistance to	Susceptible	−	+/−	−	−	Resistant	−	−	−	−	Resistant	−	−	−	−	Resistant	nt	+	−	+
ermA	erythromycin		−	−	−	−		+	+	+	+		+	+	+/−	+		nt	+/−	−	−
linA	and clindamycin		−	−	−	−		−	−	−	−		−	−	−	−		nt	−	−	−
aphA3	MIC for neomycin	0.5	−	−	+/−	−	16	+/−	+	+	+	8	+	+	+	+	64	nt	+	+/−	+
aacA-aphD	Resistance to gentamicin	Susceptible	−	−	−	−	Resistant	+	+	−	+	Resistant	+	+	−	+	Resistant	nt	+	+	+
dfrA	Resistance to trimethoprim	Susceptible	nt	nt	−	−	Resistant	nt	−	−	−	Susceptible	nt	−	−	−	Resistant	nt	+	+	+
tetM	Resistance to	Susceptible	−	−	−	−	Resistant	+	+	+	+	Susceptible	−	−	−	−	Susceptible	nt	−	−	−
tetK	tetracycline		−	−	−	−		−	+/−	−	−		+/−	−	+/−	−		nt	+/−	−	−
sat	Resistance to streptothricin	nt	−	−	−	−	nt	+	+	+/−	+	nt	+	+	+/−	+	nt	nt	+	+/−	+
tst1	Toxic shock syndrome	Positive	+	+	+/−	+	nt	−	−	−	−	nt	−	−	−	−	nt	nt	−	+/−	−

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Table III. Continued

Gene	Property	V 21179					V 23881					V 29618					V 30827				
		Conven-tional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conven-tional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conven-tional tests	TT-cy3	TT-bio	RP-cy3	RP-bio	Conven-tional tests	TT-cy3	TT-bio	RP-cy3	RP-bio
catA	Species	*Staphylo-*	nt	+	+	nt	*Staphylo-*	nt	+	+	+	*Staphylo-*	nt	+/−	+	+	*Staphylo-*	nt	+	+/−	+
coA	identification	*coccus*	+	+	+	+	*coccus*	+/−	+/−	+	+	*coccus*	+	+	+	+	*coccus*	+/−	+/−	+/−	+
femA		*aureus*	nt	+	+	+	*aureus*	nt	+	−	+	*aureus*	nt	+	+/−	+	*aureus*	nt	+	−	+
norA			−	+	+	+		+	+	+	+		+	+	+	+		+	+/−	−	+
mecA	Resistance to oxacillin	Resistant	+	+	+	+	Susceptible	−	−	+/−	−	Resistant	+	+	+	+	Resistant	+	+	−	+
blaZ	Beta-lactamase	Positive	+	+/−	−	+	Positive	+	+	+/−	+	Positive	+/−	+/−	+/−	+	Positive	+	+	−	+/−
ermC	Resistance to	Resistant	+	+	+/−	+	Susceptible	−	−	−	−	Susceptible	−	−	−	−	Resistant	−	−	−	−
ermA	erythromycin		−	−	−	−		−	−	−	−		−	−	−	−		+	+/−	−	+
linA	and clindamycin		−	−	−	−		−	−	−	−		−	−	−	−		−	−	−	−
aphA3	MIC for neomycin	32	+	+	+	+	0.5	−	−	−	−	32	+/−	+	+	+	2	+	+	+/−	+
aacA-aphD	Resistance to gentamicin	Susceptible	−	−	−	−	Susceptible	−	−	−	−	Resistant	−	−	−	−	Resistant	+	+/−	−	+/−
dfrA	Resistance to trimethoprim	Susceptible	nt	−	−	−	Susceptible	nt	−	−	−	Susceptible	nt	−	−	−	Susceptible	nt	−	−	−
tetM	Resistance to	Susceptible	−	−	−	−	Susceptible	−	−	−	−	Susceptible	−	−	−	−	Susceptible	−	−	−	−
tetK	tetracycline		−	−	−	−		−	−	−	−		−	−	−	−		−	−	−	−
sat	Resistance to streptothricin	nt	+	+	+/−	+	nt	−	−	−	−	nt	+	+	+	+	nt	+	+	−	+
tst1	Toxic shock syndrome	nt	−	−	−	−	nt	−	−	−	−	nt	−	−	−	−	nt	−	−	−	−

tance toward that drug. The potential for finding these genes could be regarded as an advantage for DNA-based methods, as the expression of these genes can be induced by exposure to the antibiotic compound, leading eventually to therapy failure. A disadvantage for DNA-based methods is that resistances cannot be detected if the responsible genes are not included in the test panel. This might happen if a previously unknown gene were present or if its sequence was unpublished or stored in databases without public access. DNA chip technology, however, makes it easy to add probes for such genes as soon as there are sequences available, without major changes and costs. Because the number of target genes on an array is not an issue, the panel could also be enlarged by adding relevant genes of related organisms. That could be important if a gene transfer is expected, as was the case in the first vancomycin-resistant MRSA that carried *vanA* from enterococci [45]. Another problem for any susceptibility testing method that is based on the detection of specific genes is that resistance properties can be caused by point mutations, such as the fluoroquinolone and rifampicin resistance in *S. aureus*. These resistances could be detected with the use of a set of matching and mismatching probes that are specific for single nucleotide polymorphisms. This potential of the array technology has been shown recently for other target genes [46, 47].

Despite all of these potentials, there is a major drawback for all array-based systems that are currently available. All approaches are expensive, time consuming, and technically demanding, which restricts their use in specialized research rather than clinical routine. The primary aim of the present study was to apply different techniques for the detection of hybridizations of sample DNA fragments at a set of specific probes under the conditions of a clinical laboratory. When we compared these protocols concerning the results, some differences were found.

Methods using biotin-labeled nucleotides were more sensitive than comparable Cy3-based detections. This could be explained by the fact that silver precipitation detection is a catalytic post-hybridization signal amplification reaction. Streptavidin-gold causes the precipitation of elementary silver out of a solution containing silver ions. Once elementary silver is present, it also catalyzes further precipitation, resulting in an exponential increase of precipitated silver.

Interestingly, methods based on random priming usually gained stronger signals than protocols using terminal transferase. A possible explanation is that this enzyme attaches several labeled nucleotides consecutively at the overlapping 3′ end of a DNA fragment. Because of the resulting high density of biotin molecules and the relative size of the streptavidin-gold complexes, a substantial proportion of the biotin molecules might be sterically hindered from binding at streptavidin-gold. This could decrease sensitivity. When labeled nucleotides are incorporated into the DNA fragments, the density of biotin molecules is more equally distributed over the DNA fragment. This should result in a higher efficiency of the formation of biotin-streptavidin-gold complexes and, therefore, in a greater signal strength.

Other differences between the protocols concern handling and affordability, although the prices for reagents are similar. The silver precipitation method can be performed with the use of digital cameras or scanning devices for detection and documentation, which are clearly more affordable than confocal scanning devices or UV-microscopes needed for fluorescence-based methods. The terminal transferase protocols require a comparatively large amount of sample DNA (6–10 μg), as there is no target amplification before hybridization. For that reason, these protocols are virtually resistant to contamination, rendering them rather robust under routine conditions. Contrarily, the random priming methods could be affected by contamination during three amplification rounds before hybridization. These steps, however, make it possible to work with much smaller amounts of sample DNA (usually 0.5 μg, but amounts of 0.05 μg are possible) and could eventually facilitate the testing of original patient samples rather than of overnight cultures. That would also make it possible to detect genes in organisms that are not cultured as easily as *S. aureus*.

In the present study, *S. aureus* was chosen as a target organism because it is easy to verify the results of DNA-based tests by comparison with conventional susceptibility testing. *S. aureus* is easily cultured, and many resistance properties are caused by distinctive genes [48]. We used an experimental array with the most prevalent resistance determinants of *S. aureus*. Some genes were omitted, such as *msrA* for macrolide resistance or *aadD* for aminoglycoside resistance. Cases of resistance phenotypes, for which no responsible gene was detected, possibly could be attributed to such genes. Another reason for an apparently false negative reaction might be the presence of a variant of a gene that cannot be recognized by a given probe. Although there were no indications of sequence variation concerning the targets of the probes included in the present study, several different probes (usually four) were included for every target gene. This should increase the probability of detecting a gene even if there were sequence variations, point mutations, or partial deletions.

Another problem is that even if probes were equimolar and had identical Tm values, the signal strengths might differ. This is caused by sequence-related factors, such as the formation of duplex structures [49]. When a set of probes is hybridized against a complete but fragmented genome, several factors, such as fragment lengths, competition with other DNA fragments, stable tertiary interactions, diffusion kinetics, and even point mutations in target genes, cause different signal strengths. It is theoretically possible that these problems could be diminished by the use of longer oligonucleotides as probes, but that would cause more nonspecific (i.e., false-positive) signals. Therefore, we decided to opt for sets of short oligonucleotides, accepting divergent signal strengths and using average results for analysis.

Generally, we found a high accordance between conventional phenotyping and array-based genotyping results.

Species identification of *S. aureus* with the use of probes for the gene *coA* (coagulase) yielded the same results as conventional testing. Probes for staphylococci *norA, femA*, and *catA* also proved to be species specific. However, there are homologous genes in coagulase-negative staphylococci with partially different sequences. This could facilitate the design of a set of genus-specific and species-specific probes based on these genes, allowing both detection and identification of staphylococci.

The prediction of oxacillin resistance was made possible by detection of the *mecA* gene in *S. aureus* and in coagulase-negative staphylococci. The *blaZ* gene and penicillinase activity were found in all isolates. This result was not surprising, inasmuch as the vast majority of staphylococci are resistant to penicillin G [10]. *Erm* genes were found in six isolates that were resistant to erythromycin. In two resistant isolates, no *erm* gene was detected, but that resistance could have been caused by another determinant (*msrA*) that was not included in the present array. The phenotype of one of these isolates (*S. epidermidis*, resistant to erythromycin but fully susceptible to clindamycin) also might indicate a resistance caused by something other than *erm*. Neither of the isolates used showed positive signals for the *linA* gene, although some isolates were phenotypically resistant to clindamycin. This property can also be attributed to *erm* genes [16], and all of these isolates actually were positive for *ermA* or *ermC* genes.

Concerning aminoglycoside resistance, there is a distinction between the phenotypic effects of the *aphA3* gene (resistance to neomycin) and *aacA-aphD* (resistance to gentamicin; see [19, 20]). In the present study, *aphA3*-positive isolates, with one exception, had elevated MIC values for neomycin (8–64 μg/ml). One isolate with a slightly elevated MIC (*S. hominis* B 101: 4 μg/ml) was negative for *aphA3*. This could be attributed to a gene not included in the array, such as *aadD*.

All isolates that were resistant to gentamicin contained *aacA-aphD*, and all susceptible isolates, again with one exception, lacked that gene.

Two *dfrA*-positive isolates were resistant to trimethoprim, but two *dfrA*-negative isolates also showed resistance to that drug. This may indicate that there were resistance determinants in addition to *dfrA* that were not included in the array. One tested isolate was positive for the *tetM* gene, another one for *tetK*. Both of them were resistant. One isolate with intermediate susceptibility (*S. haemolyticus* B 50) did not contain either of these genes. All other isolates were negative for *tetM* and *tetK*, and, phenotypically, they were fully susceptible.

In the present study, *S. aureus* was chosen as a target organism for the development of a procedure that can be performed in clinical laboratories because it is easy to culture and because results are easy to check by conventional susceptibility testing. For economic reasons, array-based hybridization cannot replace methods such as agar dilution or disk diffusion for routine susceptibility testing of organisms such as staphylococci. But it appears to be very useful with regard to epidemiological questions concerning the prevalence and the spread of resistance genes and for the fast detection of staphylococcal toxins. More detailed studies concerning these topics are under way. Another promising application for DNA chip-based hybridization could be the screening for resistance determinants in organisms for which conventional testing is much more complicated, such as *Mycobacterium tuberculosis* [46].

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