

Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays

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ABSTRACT

This study evaluated a DNA oligonucleotide array that recognised 38 different *Staphylococcus aureus* targets, including all relevant resistance determinants and some toxins and species-specific controls. A new method for labelling sample DNA, based on a linear multiplex amplification that incorporated biotin-labelled dUTP into the amplicon, was established, and allowed detection of hybridisation of the amplicons to the array with an enzymic precipitation reaction. The whole assay was validated by hybridisations with a panel of reference strains and cloned specific PCR products of all targets. To evaluate performance under routine conditions, the assay was used to test 100 methicillin-resistant *S. aureus* (MRSA) isolates collected from a university hospital in Saxony, Germany. The results showed a high correlation with conventional susceptibility data. The *ermA* and *ermC* macrolide resistance genes were found in 40% and 32% of the isolates, respectively. The most prevalent aminoglycoside resistance gene was *aphA3* (57% of the isolates), followed by *aacA-aphD* (29%) and *aadD* (29%); *tet* genes, *mupR* and *dfrA* were rare. There were no isolates with *van* genes or genes involved in resistance to quinupristin-dalfopristin. Enterotoxins were detected in 27% of the isolates. Genes encoding Panton-Valentine leukocidin, toxic shock syndrome toxin and exfoliative toxins were not found. The DNA array facilitated rapid and reliable detection of resistance determinants and toxins under conditions used in a routine laboratory and has the potential to be used for array-based high-throughput screening.

Keywords Antimicrobial resistance, DNA array, epidemiology, microarray, MRSA, *Staphylococcus aureus*

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INTRODUCTION

Susceptibility testing of microbial pathogens is usually performed by measuring growth inhibition in the presence of defined amounts of antibiotics. However, such assays reveal only phenotypes, without determining the underlying molecular cause of resistance. The existence of inducible phenotypes and different protocols for testing, including varying compositions of growth media as well as different national breakpoints, complicate the interpretation of phenotypic susceptibility data. PCR-based methods that permit the detection of specific resistance genes have been introduced [1], but it is cumbersome to

screen high numbers of isolates, as it is often necessary to perform several PCRs to detect one resistance phenotype. DNA microarray technology allows the simultaneous detection of many targets [2]. This approach could facilitate studies of the epidemiology of resistance genes and the prediction of resistance properties, even in slow-growing or unculturable pathogens. DNA arrays could also facilitate a genotype-based assessment of the virulence of a given isolate by detecting virulence-associated genes.

The aim of the present study was to develop an array-based assay for use in a clinical laboratory without substantial investments in hardware and manpower. *Staphylococcus aureus* was selected as the target organism because of the complexity of its system of clinically important resistance determinants and toxins. Multidrug resistance in *S. aureus* causes major therapeutic problems [3,4], with some strains being resistant to most available antibiotics, including glycopeptides [5].

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Strains of *S. aureus* can also produce several potent toxins [6,7], which cause food poisoning, Ritter's disease and toxic shock syndrome.

An oligonucleotide array covering the genes encoding resistance determinants, toxins and species-specific sequences of *S. aureus* was designed. This was used to develop and optimise a complete assay procedure, including DNA preparation, amplification and labelling, hybridisation and detection. The assay was first validated by performing hybridisations with positive and negative control strains, as well as with cloned specific PCR products for all targets covered. Second, array-based genotypic data for clinical isolates were compared with phenotypic results from conventional susceptibility tests in order to evaluate the clinical relevance of the genotypic data. This kind of validation is necessary because non-functional, truncated or non-expressed genes might result in a false prediction of resistance, and because sequence variations might cause a false prediction of susceptibility.

MATERIALS AND METHODS

Isolates and culture

In total, 100 consecutive strains of methicillin-resistant *S. aureus* (MRSA) isolated at the University Hospital, Dresden, Germany in 2002–2003 were investigated. Duplicate isolates from the same patient were excluded. Twenty isolates came from intensive care units, three from the emergency department, 34 from inpatients, 22 from outpatients, 20 from a long-term rehabilitation centre, and one from a member of the medical staff. The highest number of isolates ($n = 34$) was obtained from diabetic foot ulcers. A further 24 isolates were from MRSA screens (mostly nasal swabs) performed as part of routine surveillance. Three isolates were from blood cultures and one was from the cerebrospinal fluid of a neurosurgical patient. The remaining isolates were from surgical swabs, respiratory specimens, stools or urine.

Specimens were spread on Columbia blood agar (Oxoid, Wesel, Germany) and incubated overnight at 37°C. Single colonies were used for further subculturing. Screening for clumping factor and coagulase was performed using Pastorex Staph-Plus (Bio-Rad, Munich, Germany) and rabbit plasma (Becton-Dickinson, Heidelberg, Germany). Routine susceptibility tests were performed using the VITEK I system (bioMérieux, Nürtingen, Germany) as recommended by the manufacturer. Methicillin resistance was confirmed by detection of penicillin-binding protein 2' (PBP2') using an agglutination assay (MRSA-screen; Innogenetics, Ghent, Belgium). Penicillinase activity was detected using the BBL DrySlide Nitrocefim test (Becton Dickinson).

MICs were determined by the standard agar dilution technique with Mueller–Hinton broth (Oxoid) for erythromycin, clindamycin, gentamicin, neomycin, tobramycin, amikacin, nurseothricin, ciprofloxacin, doxycycline, trimethoprim,

mupirocin and benzalconium chloride. Double-disk diffusion tests were performed with erythromycin and clindamycin (15 µg- and 2 µg-disks, respectively; Oxoid) as described previously [8,9] if erythromycin-resistant isolates were found to be susceptible to clindamycin. The presence of the enterotoxin genes *entA*, *entB* and *entC* was determined by PCR as described by Johnson *et al.* [10].

DNA preparation

Growth from a quarter of a standard plate of Columbia blood agar yielded sufficient bacterial cells after overnight incubation at 37°C. Harvested staphylococci were resuspended in 5 mL of isotonic saline and centrifuged at 2300 g for 10 min. The pellet was resuspended in 200 µL of lysis solution containing 0.1 mg lysostaphin (Sigma, Steinheim, Germany), 4 mg lysozyme (Sigma), 4 mg ribonuclease A (Sigma), 4 µL Tris-HCl (20 mM, pH 8.0), 4 µL EDTA (2 mM), and 2 µL Triton X-100. Following incubation on a shaker (45 min, 37°C, 300 rpm), 25 µL of proteinase K solution and 200 µL of buffer AL (both contained in the DNeasy kit; Qiagen, Hilden, Germany) were added, and this was followed by incubation for a further 45 min at 56°C. After the addition of 200 µL of ethanol, the extracted DNA was purified on a spin column (DNeasy kit) according to the manufacturer's instructions. The DNA concentration was determined spectrophotometrically at 260 nm. As fragmentation of DNA affects the overall sensitivity of the assay, gel electrophoresis and ethidium bromide staining were used to monitor aliquots of the DNA preparations.

Probe design and DNA array preparation

Probe sequences were designed from published target sequences using the Array Design software package (Clontech Chip Technologies, Jena, Germany). Targets, Genebank accession numbers for sequence data and probe sequences are listed in Table S1 (supplementary on-line material). Consensus regions in the alignments of all available sequences of each target were chosen for the probe design. Probe sequences were selected to be specific for the target and to have similar lengths, GC contents and melting temperatures in order to yield comparable signal intensities. The final probe sequences were compared with all available sequences in the Genebank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to exclude any theoretical false-positive reactions caused by cross-reactions, or false-negative reactions caused by sequence variations. Oligonucleotides (3'-amino-modified) were synthesised by Metabion (Martinsried, Germany), diluted in Spotting Buffer 1 (Schott Nexterion, Jena, Germany) to a concentration of 10 µM, and spotted on surface-coated glass (Clontech) using a Microgrid II spotting machine (Genomic Solutions, Huntingdon, UK) according to the procedure supplied by the manufacturer. Each probe was spotted five times on the array (Fig. 1). After production, arrays were inserted into ArrayTube reaction vials (Clontech).

Primer design

The primer set for the linear amplification procedure consisted of 39 antisense oligonucleotides (one primer for every target, but with two different primers for the 23S rRNA gene). A consensus region was identified for each target, situated up to 100 bp upstream of the probe-binding site. Sequences with

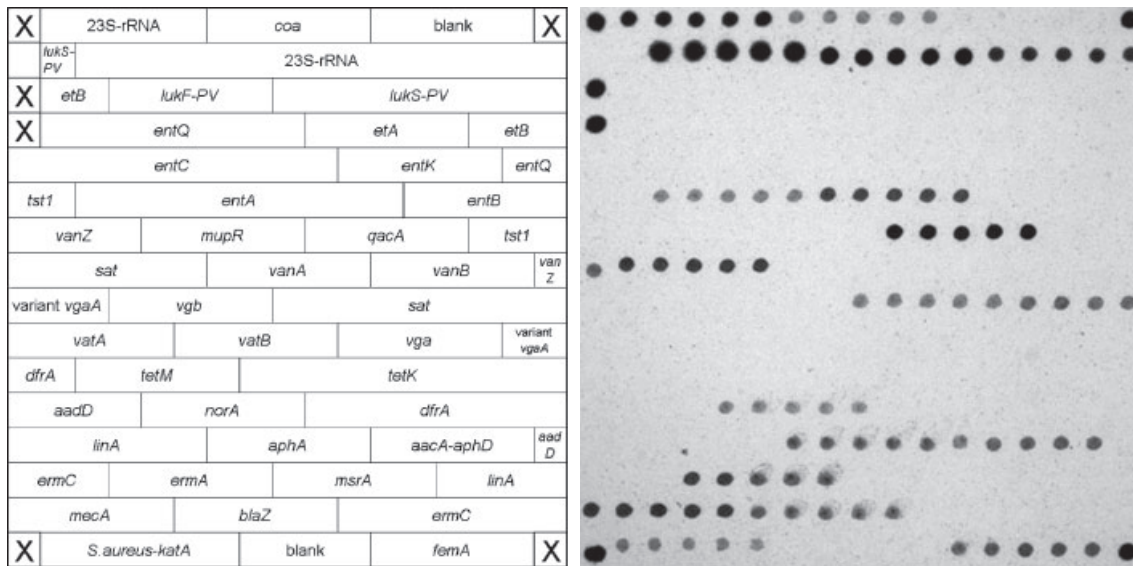


Fig. 1. Probe layout and microphotograph of a hybridised and stained array. The actual size of the array is 2.4×2.4 mm. Spots designated 'X' are positioning biotin markers whose asymmetrical distribution allows the correct alignment of the array to the grid.

similar physicochemical parameters (see 'Probe design') were chosen from these regions and used for primer design. Primer sequences (Table S1) were compared with the Genbank database to avoid theoretical cross-reactions or sequence variations. Oligonucleotides were synthesised by Metabion, and were used as a primer mixture containing each individual primer at a concentration of $0.135 \mu\text{mol/L}$.

Primer elongation reaction and labelling

The primer elongation reaction was performed using the primer mixture and a dNTP solution containing 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP and 0.35 mM biotin-16-dUTP (Roche, Penzberg, Germany). Each elongation reaction contained 0.3 μL Therminator polymerase (New England Biolabs, Frankfurt, Germany), 3 μL Therminator polymerase buffer (New England Biolabs), 3 μL primer solution, 3 μL dNTP stock solution, and 1–1.5 μg unfragmented *S. aureus* genomic DNA. Reaction conditions comprised 5 min at 96°C , followed by 45 cycles of 20 s at 62°C , 40 s at 72°C and 60 s at 96°C . Each reaction was then cooled to 4°C and used for hybridisation with the DNA array.

Hybridisation and detection

Each ArrayTube was first washed with 500 μL double-distilled water and 500 μL Hybridisation Buffer I (Schott Nexterion, Jena, Germany) using a thermomixing device (5 min at room temperature, 550 rpm). A 20- μL aliquot of the labelled sample was denatured in a separate microtube (2 min at 95°C) and then transferred into the ArrayTube and incubated for 60 min at 50°C (550 rpm). The sample was then removed from the tube and the array was washed three times (5 min at room temperature, 550 rpm), first with 500 μL $2 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}$, $0.015 \text{ M trisodium citrate}$, pH 7.0)

containing Triton X-100 0.01% v/v, then with 500 μL $2 \times \text{SSC}$, and finally with 500 μL $0.2 \times \text{SSC}$. This was followed by the addition of 100 μL of a 2% w/v solution of blocking reagent (Clondiag), dissolved in $6 \times \text{SSPE}$ ($1 \times \text{SSPE} = 150 \text{ mM NaCl}$, 10 mM sodium phosphate, 1 mM EDTA) containing Triton X-100 0.1% v/v, and incubation of the ArrayTube for 15 min at 30°C (550 rpm). Horseradish peroxidase-streptavidin conjugate (Clondiag) was diluted in $6 \times \text{SSPE}$ containing Triton X-100 0.1% v/v to a concentration of 100 $\mu\text{g}/\mu\text{L}$. The blocking solution was removed and 100 μL of the diluted conjugate was added to the ArrayTube; this was followed by incubation (15 min, 30°C , 550 rpm) and three washing steps as described above. The ArrayTube was then placed into the ATR 01 reading device (Clondiag) and 100 μL TMB peroxidase substrate (Clondiag) was added to start the staining reaction.

Measurements and data analysis

The ATR 01 reading device and IconoClust software (Clondiag) were used according to the manufacturer's instructions. After staining for 10 min at 25°C , an image of the array was recorded and analysed. Signal intensity and local background were measured for each probe position. Intensities of local backgrounds were subtracted from the intensities of the automatically recognised spots, and averages for all results of a given probe were calculated. Resulting values of <0.1 were considered negative, while those of >0.3 were considered positive. Values between 0.1 and 0.3 were considered to be equivocal [11].

Validation

Validation was performed using two different approaches. First, a positive and a negative control strain were defined for each target (Table S2) (supplementary on-line material) and

tested with the method described above. Second, PCRs were developed for every target included in the present study in order to verify the results from array-based experiments. The primers for these PCRs (Table S2) were designed to include both the probe-binding site and the primer-binding site of the array-based assay. PCRs comprised 19.6 μL distilled water, 2.5 μL $\times 10$ PCR buffer (containing 1.5 mM MgCl_2), 0.5 μL dNTPs (200 μM each dNTP), 0.5 μL each primer (10 μM), 0.4 μL *Taq* polymerase (5 U/ μL), and 1 μL sample DNA (all reagents except for the sample DNA supplied by Promega, Madison, WI, USA). PCR conditions were 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 20 s at 55°C and 2 min at 72°C. A PCR product was obtained from the corresponding positive control strain for every target represented on the array, and was demonstrated to have the predicted amplicon length by electrophoresis on 1% w/v non-denaturing gels. These PCR products were then used as templates instead of staphylococcal genomic DNA, i.e., they were subjected to the same labelling and hybridisation procedures as described above. Additionally, all amplicons were cloned using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Positive clones were picked and subcultured, after which plasmids were isolated using a Nucleo Spin Plasmid Kit (Macherey & Nagel, Düren, Germany) and digested with *EcoRI* (MBI Fermentas, Vilnius, Lithuania). The length of the inserts was checked by electrophoresis on 1% w/v non-denaturing gels. Additionally plasmids were partially sequenced. Plasmids with inserts were labelled as described above and then used in hybridisation experiments instead of genomic DNA, using an individual array for each target.

RESULTS

Assay sensitivity

A dilution series of genomic DNA from one MRSA isolate was analysed, with 1, 0.5, 0.2, 0.1, 0.05 and 0.01 μg of unfragmented genomic DNA being used in the labelling and subsequent hybridisation reactions. The detection limit of the assay was 0.05 μg , equivalent to *c.* 1.7×10^7 DNA copies, based on a genome size of 2.85×10^6 nucleotide residues corresponding to a molecular mass of 1.76×10^9 g/mol. However, in experiments with dilution series, if the DNA content approached the detection threshold, hybridisation signals of different probes sometimes had different intensities, so that the detection limits for different target genes might vary. This could result from steric effects [12–14], either from parameters that influence the stringency of binding (e.g., pH, ionic strength and hybridisation temperature), or from sequence variations within the binding regions of primers or probes. To overcome these effects, relatively large amounts of genomic DNA (1–1.5 μg) were used routinely to ensure total saturation of the binding capacity of each spot on the array.

Validation

All primer–probe combinations included in the definitive array gave clearly positive signals with the corresponding positive control strains, and unambiguously negative signals with negative control strains. These results were checked individually for each target by PCR, using the primers listed in Table S2. PCR products from positive control strains and plasmids derived from these PCR products were subjected to the entire amplification procedure with all 39 primers, followed by array hybridisations. Each product hybridised only with the corresponding probe on the array.

Genus- and species-specific markers

Reactions with 23S rRNA probes were always positive. *CoA*, *katA* and *femA* gave either positive or, in a few cases, ambiguous results (the *katA* primer and probe were designed to be specific for *S. aureus*, although coagulase-negative staphylococci also have a catalase gene). Results for *norA* were positive for 59 isolates, negative for 21 isolates, and ambiguous for 20 isolates. The results for *norA* showed no correlation with ciprofloxacin MICs; of 98 ciprofloxacin-resistant isolates (MIC > 4 mg/L), 21 were *norA*-negative, while both of the susceptible isolates (MIC \leq 1 mg/L) were positive for *norA*.

Ten reference strains and isolates of coagulase-negative staphylococci (*S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. warneri* and *S. lentus*) were tested as controls. All of these were negative for *S. aureus*-specific markers, although they gave positive signals with genus-specific 23S rRNA probes, as well as with some other probes, such as those for *mecA* or *blaZ*. In addition, DNA from *Escherichia coli*, *Yersinia enterocolitica*, *Enterococcus faecium* and *Bacillus cereus* was tested, but no positive reaction with any of the genus- or species-specific markers was observed.

The *mecA* and β -lactamase (*blaZ*) genes

All 100 MRSA isolates were positive for *mecA* in array hybridisations, as well as for PBP2' in an agglutination assay. Sixty-five *blaZ*-positive isolates, and 13 isolates with equivocal results for *blaZ*, had detectable penicillinase activity. Seven isolates displayed neither hybridisation signals for *blaZ* nor penicillinase activity. Thirteen

isolates were positive (and one was equivocal) for *blaZ*, but were negative in the nitrocephin assay. One isolate showed clearly negative results in repeated array hybridisations, although it had detectable penicillinase activity.

Genes determining resistance to macrolides, lincosamides and streptogramins

The most common macrolide resistance genes were *ermA* (40 isolates) and *ermC* (32 isolates). There were no isolates with multiple macrolide resistance determinants. Seventy-one isolates with *erm* genes had erythromycin MICs of >8 mg/L; one isolate with *ermC* had an MIC of 4 mg/L. The remaining 28 isolates were negative for both *ermA* and *ermC*, and had erythromycin MICs of ≤1 mg/L. The *msrA* gene was not detected.

The results for clindamycin were almost identical to those for erythromycin. Seventy-one *erm*-positive isolates had MICs of >8 mg/L. One *ermC*-positive isolate had a low clindamycin MIC (≤1 mg/L), but the double-disk diffusion test was positive, indicating an inducible resistance. Twenty-eight *erm*-negative isolates had MICs of ≤1 mg/L. No isolate contained *linA*.

The streptogramin resistance determinants *vatA*, *vatB*, *vga*, variant *vgaA* and *vgb* were not detected, and none of the isolates was resistant to quinupristin–dalfopristin.

Aminoglycoside resistance genes

No genes for aminoglycoside-modifying enzymes were detected in 18 isolates, while 30 harboured only *aphA3*, and 23 were positive only for *aadD*. The remaining 29 isolates harboured combinations of several genes for aminoglycoside-modifying enzymes (*aphA3* plus *aacA-aphD* in 23 isolates; *aadD* plus *aacA-aphD* in two isolates; and all three genes in four isolates). The 30 *aphA3*-positive isolates had neomycin MICs of 32–128 mg/L, while the 23 *aadD*-positive isolates had MICs of 16–64 mg/L. The 18 isolates with neither *aphA3* nor *aadD* had MICs of 0.5–1 mg/L. The remaining 29 isolates with combinations of several genes had neomycin MICs ranging from 16 to 64 mg/L.

Seventy-one isolates that did not harbour *aacA-aphD* were susceptible to gentamicin (MICs of 0.25–0.5 mg/L), even if they harboured *aphA3* or *aadD*, while 29 isolates that harboured

aacA-aphD in various combinations with other genes were resistant to gentamicin (MIC ≥ 8 mg/L). Isolates with only *aadD* had tobramycin MICs of ≥8 mg/L, while isolates with gene combinations including *aadD* and/or *aacA-aphD* had MICs of 4 to ≥8 mg/L. Isolates with either *aphA3* alone, or no genes for aminoglycoside-modifying enzymes, had tobramycin MICs of ≤1 mg/L. All the isolates had amikacin MICs of ≤4–8 mg/L.

The *sat* gene and nurseothricin resistance

The *sat* gene was present in 57 isolates, which had nurseothricin MICs of 8–64 mg/L; MICs for negative isolates were ≤2 mg/L. All *sat*-positive isolates also harboured *aphA3*.

Glycopeptide resistance

The glycopeptide resistance determinants *vanA*, *vanB* and *vanZ* were not detected, although two isolates showed reduced susceptibility to teicoplanin (MICs of 16 and 24 mg/L, respectively).

Resistance to tetracycline and trimethoprim

The *tetK* gene was detected in one isolate, and the *tetM* gene in three isolates. All *tet*-positive isolates had doxycycline MICs of ≥8 mg/L, while all *tet*-negative isolates had MICs of ≤1 mg/L. The *dfrA* gene was found in 12 isolates, including three isolates with equivocal hybridisation results, all of which had trimethoprim MICs of ≥64 mg/L. Of the *dfrA*-negative isolates, 75 had MICs of 1–4 mg/L, nine had an MIC of 8 mg/L, and four had MICs of 16 to >64 mg/L.

Mupirocin and benzalconium chloride resistance

Ninety-seven isolates were *mupR*-negative and were susceptible to mupirocin (MIC 4 mg/L). Three isolates were *mupR*-positive and had MICs of 256–1024 mg/L. Four isolates with a benzalconium chloride MIC of 8 mg/L were *qacA*-positive. Of 50 isolates with an MIC of 4 mg/L, 11 were *qacA*-positive and 39 were *qacA*-negative. Thirty-five of 36 isolates with an MIC of 2 mg/L, and all ten isolates with an MIC of ≤1 mg/L, were *qacA*-negative.

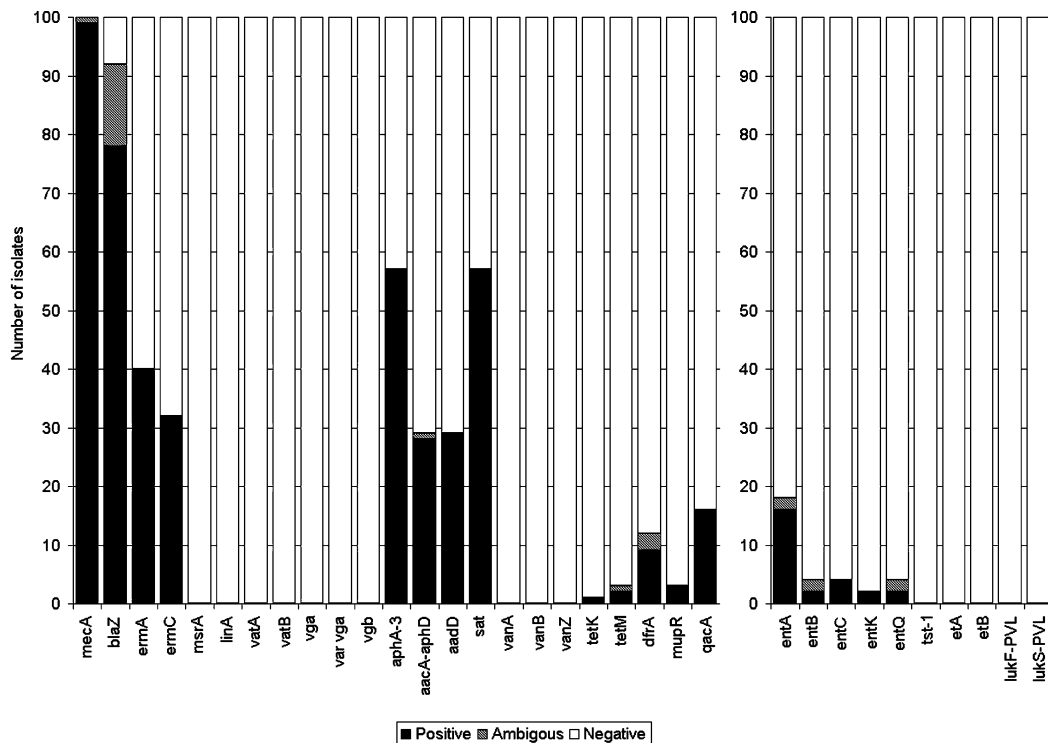


Fig. 2. Prevalences of resistance determinants and genes encoding toxins among 100 isolates of methicillin-resistant *Staphylococcus aureus*.

Staphylococcal toxins

The toxin-encoding genes *tst1*, *etA*, *etB*, *lukF-PV* and *lukS-PV* were not detected. Twenty-seven isolates harboured genes for enterotoxins. Sixteen isolates were positive for *entA*, while two gave ambiguous signals. The *entB* gene was detected in two isolates, and two others were equivocal. The *entC* gene was found in four isolates, and the *entK* gene in two isolates. Positive hybridisation signals for *entQ* were observed with two isolates, and two others gave equivocal results. Two isolates harboured multiple enterotoxins (*entA* + *entB* + *entK* + *entQ* and *entB* + *entK* + *entQ*, respectively).

Fig. 2 summarises the overall prevalences of the resistance and toxin genes investigated.

DISCUSSION

Several different DNA array-based assays are currently in use [2], but most systems are very expensive, time-consuming, technically demanding and difficult to adapt to the needs of high-throughput screening. This restricts their use to specialised research groups as opposed to diagnostic laboratories. In contrast, the assay

described in this article is relatively easy to perform, and the use of tube-integrated arrays with non-fluorescent labelling and rapid hybridisation protocols is time-saving, relatively inexpensive and allows large numbers of samples to be analysed [11,15,16]. An isolate, once cultured, can be processed within 1 day. The total cost per assay is estimated to be c. 15 euro (plus 5 euro for DNA preparation using the lysostaphin/Qiagen protocol described above). This is clearly less expensive than comparable genotyping methods (arrays or multiplex PCR), but is still greater than the cost of a phenotypic susceptibility test (c. 5 euro using an automated system such as the VITEK). However, the above estimate is based on small-scale experimental production rather than commercial mass-production.

For the present assay, a set of synthetic oligonucleotide primers was used to run a non-exponential, but linear, amplification. With the use of only one primer per target in an amplification protocol otherwise identical to a standard PCR protocol, a high rate of multiplexing with 39 possible reactions in one reaction mixture was achieved. Another advantage of this design is the high specificity, which resulted from two

consecutive site-specific hybridisations: first, between the primer and the target DNA; and second, between the labelled amplicon and the probe on the DNA array. A potential disadvantage of the single primer design is reduced sensitivity compared to a standard PCR, but this is not important, as the assay was developed for work with cultured bacteria rather than patient samples. The necessary number of cells can be obtained easily from an overnight plate culture, as is the case when performing conventional susceptibility tests.

Following validation of the array by hybridisations with control strains and PCR products/plasmids derived from these strains, as well as by bioinformatics analysis, the array-based data were evaluated by comparison with conventional susceptibility tests for a panel of clinical isolates. For 23S rRNA sequences, *coA*, *femA*, *katA*, *mecA*, macrolide and aminoglycoside resistance determinants, *tet* genes, *sat* and *mupR*, the correlation approached 100%, similar to that reported previously for multiplex PCR systems with *S. aureus* patient isolates [1].

Notable discrepancies between the genotypic and phenotypic results were observed only for *norA*, *blaZ*, *dfrA* and *qacA*. A high proportion of isolates showed no *norA* signal, or equivocal signals, even for samples with high DNA concentrations. This could indicate that some isolates lack *norA*, or that there were sequence polymorphisms, although the published sequences were accounted for by the present assay. A previous study [17] has mentioned divergent sequences in *norA*, which may render *norA* unsuitable as a species-specific marker for *S. aureus*. The absence of any correlation between the *norA* results and MICs of ciprofloxacin confirms that fluoroquinolone resistance is not related simply to the presence of *norA* [18].

Some isolates gave hybridisation signals for *blaZ*, although no β -lactamase activity was detected. However, the β -lactamase gene might not be expressed (or could even be lost) in MRSA isolates without disadvantage. One isolate was negative for *blaZ*, but had β -lactamase activity. This result may be caused by sequence variations. Similarly, the MICs for three *dfrA*-negative isolates were as high as the MICs for positive isolates, indicating either the presence of an alternative trimethoprim resistance mechanism or significant sequence variation within the

binding regions of the *dfrA* primer and/or probe. Benzalconium chloride was not a distinctive marker for *qacA*, and a previous study [19] also found only small differences in MICs between *qacA*-positive and *qacA*-negative isolates.

There was a high correlation between the array-based data for aminoglycoside-modifying enzymes and the substrate specificity of the corresponding enzymes [20]. The *aphA3* gene caused resistance to neomycin, but not to gentamicin or tobramycin. The *aacA-aphD* gene clearly conferred resistance to gentamicin, but all *aacA-aphD*-positive isolates also contained other aminoglycoside resistance genes. However, the MICs suggested that *aacA-aphD* had an influence on tobramycin, but not on neomycin resistance. The *aadD* gene determined resistance to tobramycin and neomycin, but did not affect gentamicin susceptibility. No isolates were resistant to amikacin, but as amikacin resistance has been reported in *aadD*- or *aacA-aphD*-positive strains [21], it would be interesting to investigate the expression of these genes.

Aminoglycoside resistance genes were found to be common, but their prevalences differed significantly from those found in a European survey of 191 MRSA isolates [22], which reported prevalence rates of 76% for *aacA-aphD*, 53% for *ant4'* (synonymous with *aadD*), and 7% for *aphA3*, with many isolates harbouring several aminoglycoside resistance genes. The *sat* gene appeared to be linked to *aphA3*, as also reported by Moon *et al.* [23], and Derbise *et al.* [24] sequenced a transposon, Tn5405, containing both genes (plus another *aad* aminoglycoside resistance determinant). This combination has also been observed in *S. intermedius* [25] and enterococci [26]. It was surprising to find *sat* in a majority of isolates, but this may be related to the historical use of streptomycin preparations contaminated with streptothricin (W. Witte, personal communication), or to the use of streptothricin in East German agriculture before 1989 [26,27].

The prevalences of the *ermA* and *ermC* erythromycin resistance genes were virtually identical to those found in another German survey [28], in which the rarity of *msrA* was also observed. The *linA* gene was not detected, and all observed cases of clindamycin resistance were attributable to *erm* genes. One *erm*-positive isolate showed inducible clindamycin resistance, which can cause treatment failure [29]. No resistance to quinu-

pristin–dalfopristin was found, and none of the responsible genes were detected. Quinupristin–dalfopristin had been used only occasionally in the hospital from which the isolates were collected, and none of the patients from whom these isolates were obtained had received this drug.

The *tetK* and *tetM* genes were detected rarely, but there was complete correlation between susceptibility tests and hybridisation results. None of the isolates harboured both genes, which is in contrast to a recent study [30] that found both genes in nearly one-third (21 of 66) of tetracycline-resistant strains sampled across Europe; however, there was only a small number of tetracycline-resistant isolates in the present study.

The toxin-encoding *tst1*, *etA* and *etB* genes were not found in the MRSA isolates tested in the present study, and none of the patients had a clinical condition which might have been related to these toxins. However, several isolates contained genes for enterotoxins, and two isolates had multiple enterotoxin genes corresponding to a pathogenicity island (*entB* + *entK* + *entQ*) described by Yarwood *et al.* [31]. The genes for Pantón–Valentine leukocidin (*lukF-PV* and *lukS-PV*) were not detected in any of the isolates investigated, but an emerging epidemic strain of community-acquired MRSA containing the Pantón–Valentine leukocidin genes [32,33] was detected with the array after the end of the study period (unpublished results).

The results of the present study demonstrate that the DNA array technology is a useful tool for the rapid detection of staphylococcal toxins and studies of the epidemiology of antimicrobial resistance genes. The proposed array can be expanded easily with additional target genes and can also be adapted for experiments with other pathogens. The proposed technical platform was suitable for use under routine conditions in a reference laboratory or in the laboratory of a tertiary-care facility.

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SUPPLEMENTARY MATERIAL

The following supplementary material for this article is available online:

Table S1. Sequences of primers and probes, and sources for sequence data

Table S2. Control primers and control strains

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