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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 Nitrocefin 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 (Clondiag 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 (Clondiag) 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 (Clondiag).

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

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