DNA Microarray for Genotyping Antibiotic Resistance Determinants in Acinetobacter baumannii Clinical Isolates

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In recent decades, Acinetobacter baumannii has emerged as an organism of great concern due to its ability to accumulate antibiotic resistance. In order to improve the diagnosis of resistance determinants in A. baumannii in terms of lead time and accuracy, we developed a microarray that can be used to detect 91 target sequences associated with antibiotic resistance within 4 h from bacterial culture to result. The array was validated with 60 multidrug-resistant strains of A. baumannii in a blinded, prospective study. The results were compared to phenotype results determined by the automated susceptibility testing system VITEK2. Antibiotics considered were piperacillin-tazobactam, ceftazidime, imipenem, meropenem, trimethoprim-sulfamethoxazole, amikacin, gentamicin, tobramycin, ciprofloxacin, and tigecycline. The average positive predictive value, negative predictive value, sensitivity, and specificity were 98, 98, 99, and 94%, respectively. For carbapenemase genes, the array results were compared to singleplex PCR results provided by the German National Reference Center for Gram-Negative Pathogens, and results were in complete concordance. The presented array is able to detect all relevant resistance determinants of A. baumannii in parallel. The short handling time of 4 h from culture to result helps to provide fast results in order to initiate adequate anti-infective therapy for critically ill patients. Another application would be data acquisition for epidemiologic surveillance.

The development of antibiotics at the beginning of the 20th century led to a considerable decrease in mortality caused by infectious diseases (1). However, a century of antibiotic usage initiated the evolution of a gigantic repertoire of bacterial resistance determinants (2).

Microarray technology is capable of detecting a huge number of different genes simultaneously in a short time. Several microarrays for resistance detection in different species, genera, or other groups of bacteria have been developed (3–8). One clinically relevant bacterial species for which no DNA microarray is yet available is Acinetobacter baumannii.

A. baumannii is an opportunistic pathogen which can cause severe infections, particularly pneumonia, sepsis, urinary tract, and wound infections. Although A. baumannii is not widespread in the community (9), it is one of the most frequently occurring pathogens causing outbreaks in intensive care units, and it is associated with high mortality and morbidity rates. Multidrug resistance in A. baumannii has emerged as a significant problem, leaving only a few anti-infective treatment options. The organism is characterized by an intrinsic resistance to various antimicrobial agents and has the ability to acquire transferable genetic resistance elements, as well as resistance-compromising mutations, to the point of pandrug resistance (10). A huge variety of acquired genetic resistance determinants have been described for A. baumannii (11), rendering the selection of an appropriate empirical anti-microbial treatment exceedingly difficult. Therefore, antibiotic susceptibility testing should be mandatory before treatment starts. However, an initial calculated therapy will always be called for in critically ill patients requiring immediate anti-infective support (12, 13). As a consequence, the availability of fast and reliable molecular methods for antibiotic resistance determination could pave the way for more adequate, or at least contemporaneous, therapy adjustments.

We developed a DNA microarray for genotyping genetic resistance determinants in A. baumannii isolates which allows the rapid prediction of antibiotic resistance. The microarray covers determinants for various mechanisms of resistance, like enzymatic inactivation of antibiotics, enzymatic modification, mutation of the target sites, gene overexpression by promoter alterations, and mutations in regulatory genes.

MATERIALS AND METHODS

Bacterial strains. The sequenced reference strain A. baumannii AYE was obtained from the American Type Culture Collection (ATCC). The strain HK302 was made available by the National Reference Laboratory for Gram-Negative Pathogens (NRZ), Bochum, Germany. These isolates were collected between 2009 and 2011 from patients all over Germany and originated from different body sites: respiratory tract (n = 22), urinary tract (n = 8), wound swab (n = 11), and others (n = 19). The inclusion criterion was a multiresistant phenotype. Two isolates were obtained from the Robert Bosch Hospital in Stuttgart, Germany. Typing of the strains, either by pulsed-field gel electrophoresis (PFGE) or the repetitive PCR (rep-PCR) (14)-based Diversilab typing system (bioMérieux), revealed that several different clonal lineages were included.

Received 25 April 2013 Returned for modification 11 May 2013 Accepted 11 July 2013

Published ahead of print 15 July 2013

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00863-13.

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Culture conditions. All bacterial strains were cultured either in Luria-Bertani broth or on Columbia blood agar (Heipha). Cells were grown at 37°C for approximately 20 h.

Strain characterization. Species identification was performed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) using a MALDI Bioanalyzer (Bruker). Additionally, the A. baumannii-specific chromosomally encoded blaOXAS-51-like gene was detected by PCR as described earlier (15).

The antibiotic susceptibility of each strain was determined by the automated VITEK2 system with AST-N248 cards (bioMérieux). This characterization included the determination of antibiotic susceptibility according to breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) against therapeutically relevant antimicrobial agents, such as piperacillin-tazobactam (TZP), ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), tigecycline (TGC), and colistin (CST). The system outputs the breakpoint of the MIC for each antibiotic, and the values are interpreted as resistant (R), intermediate (I), or susceptible (S).

DNA isolation. Total DNA from bacteria was purified with the DNeasy blood and tissue kit (Qiagen) according to the manufacturer’s instructions for Gram-negative bacterial cultures. The quality and quantity of the isolated nucleic acids were analyzed spectrophotometrically with the Nanodrop system (Thermo Scientific) by measuring the absorbance at 260 and 280 nm.

Target selection. The targets of the array were selected according to their association with antibiotic resistance. Targets included intrinsic and acquired genes, which have the potential to mediate resistance, resistance-relevant point mutations, and genetic alterations resulting in overexpression of resistance genes. The array was designed to detect genes for β-lactamases (blaOXA-23, blaOXA-37, blaOXA-40, blaOXA-48, blaOXA-58, blaJPS, blaRMP-1, blaRMP-2, blaTEM, blaSIM, blaGEO, blaVIM, blaTAX, blaADC, blaRTG, blaCTX-M-1, blaCTX-M-9, and blaDHQ), aminoglycoside-modifying enzymes [aph(3′)-I, aac(6′)-I, aac(3)-I, aac(3)-II, ant(2′)-I, and ant(3′)-I], efflux systems [adeB, adeR, adeS, adeM, qacE, tet(39), tetA, tetB, tetC, tetM, cmlA, floR, cfr, and tetrH], and others (parC, gyrA, armA, sulI, sulII, sulIII, catA, catB, strA, strB, strF, strH, strR, tetF, tetR, quinB, qnrB, ISAbA1, and IS1133). (blaOXA-23, blaOXA-37, blaOXA-40, blaOXA-48, blaOXA-58, blaJPS, blaRMP-1, blaRMP-2, blaTEM, blaSIM, blaGEO, blaTAX, blaADC, blaRTG, blaCTX-M-1, blaCTX-M-9, and blaDHQ) were used for the multiplex PCR amplification. The probe signals of the hybridized and washed probes were calculated. Signals with intensity values above 1,000 counts were considered positive, otherwise they were considered negative. The probe targeting the highest signal of each SNP position was considered the potential perfect match probe (PM), while probes specific for the particular SNP but showing lower signals were considered mismatch probes (MM). Hence, the MM/PM ratio ranges from 0 to 1. The classification as PM and MM was considered specific if this ratio did not exceed a value of 0.7 (16).

Microarray evaluation. The performance of the array in terms of resistance phenotype prediction was evaluated by the positive predictive value (PPV) (number of true positives × 100/number of true and false positives), negative predictive value (NPV) (number of true negatives × 100/number of true and false negatives), sensitivity (number of true positives × 100/number of true positives and false negatives), and specificity (number of true negatives × 100/number of true negatives and false positives). True positives were defined as isolates showing a resistant phenotype with a corresponding resistance gene, and true negatives were defined as isolates showing neither a phenotypic nor a genotypic resistance deter-

PCR target amplification. A set of three multiplex PCRs was set up to amplify and label the sequences of interest from the chosen genes. Each PCR mixture contained 0.02 U/μl Phusion polymerase (Thermo Scientific), 1× Phusion GC reaction buffer, 30 μM each deoxynucleoside triphosphate (dNTP) (40% of dCTP was Cy3 labeled), 1 ng/μl template DNA, and each primer (0.1 μM). The cycle reactions consisted of 35 cycles of 30 s at 97°C, 30 s at 60°C, and 45 s at 72°C.

Oligonucleotide chip construction. The microarray consisted of 322 probes, with each set of two forming a sense/antisense pair. The probes were diluted in spotting buffer (1:1 mixture of Nexterion Spot I and Nexterion Spot modified; Schott Nexterion) to a concentration of 50 μM and spotted in duplicates on an epoxy-functionalized Nexterion Slide E (Schott Nexterion) with a Microgrid II Spotter 610 arraying system (Bio Robotics) using SMP3 pins (Arrayit Corporation). The diameter of each spot was about 80 μm, and the distance between two spots was about 140 μm. The covalent coupling of the probes to the functionalized glass surface took place in a 60-min incubation step at 60°C directly after the printing process. Blocking of the slides was conducted immediately before use according to the manufacturer’s instructions.

Hybridization and washing. The hybridization mixture contained 10 μl of each of the 3 multiplex PCR pools and 15 μl hybridization buffer, resulting in a final concentration of 400 mM sodium chloride (NaCl), 40 mM trisodium citrate, and 0.1% sodium dodecyl sulfate (SDS). Neither purification nor digestion of the multiplex PCR amplification products was necessary before applying the hybridization mixture. Forty-five μl of the mixture was loaded on the array using a Lifterslip (Earlie Scientific Company), and it was incubated for 1 h at 58°C. After hybridization, the slides were washed with 2× sodium chloride sodium citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% SDS and then with 2× SSC, with a final wash in 0.2× SSC, each for 10 min at room temperature. After washing, the slides were dried with nitrogen.

Data acquisition. The probe signals of the hybridized and washed arrays were detected with an Axon GenePix 4300A fluorescence scanner (MDS Analytical Technologies GmbH), using a 532-nm laser for the excitation of the Cy3 fluorophore and the integrated filter (550 to 600 nm) for the purification of emitted light. The output of the scanner was a 16-bit TIFF file, which could be analyzed with the integrated GenePix Pro software. The software automatically identified probe spots and calculated the corresponding signal intensities, taking into account the local background signal. The signal value of each spot was exported to Microsoft Excel, and the means from replicates and their corresponding standard deviations were calculated. Signals with intensity values above 1,000 counts were considered positive, otherwise they were considered negative. The probe with the highest signal of each SNP position was considered the potential perfect match probe (PM), while probes specific for the particular SNP but showing lower signals were considered mismatch probes (MM).
TABLE 1 Number of resistant strains in the *A. baumannii* study collection

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of isolates by drug resistance group</th>
<th>MIC breakpoints (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Imipenem</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Meropenem</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0</td>
<td>58</td>
</tr>
<tr>
<td>Colistin</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Breakpoint according to CLSI.
<sup>b</sup> Breakpoint according to EUCAST.
<sup>c</sup> Concentration refers to trimethoprim.
<sup>d</sup> VITEK2 interpretation differs from those of EUCAST and CLSI.
<sup>e</sup> VITEK2 always interprets *A. baumannii* as at least having intermediate resistance to tigecycline regardless of the measured MIC. CLSI or EUCAST breakpoints are not available for tigecycline.
<sup>f</sup> Resistance of each strain against different antibiotics was measured by VITEK2 according to EUCAST breakpoints and, if none were available for the respective antibiotic by CLSI breakpoints. S, resistant; I, intermediate; R, resistant.

RESULTS

Antibiotic susceptibility testing. A collection of 60 *A. baumannii* strains was genotyped for array validation. PFGE-based genetic fingerprinting, as well as DiversiLab rep-PCR typing and clustering of the study isolates, revealed that the collection consisted of at least six different clonal complexes (data not shown). In order to compare the genotypic array results to the corresponding phenotype, all strains were phenotypically analyzed by the VITEK2 automated antibiotic susceptibility testing system (Table 1).

Multiplex PCR. All targets of the microarray were amplified and labeled in a multiplex PCR, which was split into three primer pools. To monitor the correct product amplification independently from the array, we analyzed the PCR product sizes generated from *A. baumannii* strain AYE as the template with an Agilent Bioanalyzer (Agilent Technologies) using an Agilent DNA-1000 kit. This strain harbored 29 targets of the array (17), putting the robustness of the PCR to the test. Since the dye Cy3 reduces the resolution of the Agilent Bioanalyzer, this experiment was conducted without labeling.

Most of the expected targets could be detected by the Agilent Bioanalyzer, although some targets were of the same or almost the same size and could not be distinguished by electrophoresis. Furthermore, the sensitivity of the Agilent Bioanalyzer was not sufficient to detect the products of *adeB*, *qacE*, and *bla<sub>ADC</sub>* even though they were generated to a low degree, as verified later by microarray analysis. The sensitivity of the Agilent Bioanalyzer, as stated by the manufacturer, was 0.1 ng DNA.

Microarray analysis. All 60 *A. baumannii* strains were analyzed by the developed microarray. The time to result for each analysis was about 4 h. As a representative example, the data for strain *A. baumannii* AYE are shown in detail (Fig. 1 and 2).

To evaluate the variability of the whole assay, the reference strain *A. baumannii* AYE was analyzed further. The variance (standard deviations × 100%/means) of the three signal intensities was calculated individually for every positive probe. The resulting values ranged from 4 to 54%. The mean variances (25%) served as an indicator for the reproducibility of the whole analyzing system.

Array analysis of the whole *A. baumannii* collection revealed a broad range of different resistance determinants (Table 2).

Phenotype-genotype correlation. The obtained microarray data for each strain were correlated with its phenotype data. In some cases, the correlation of genotype and phenotype was challenging. A distinct correlation could hardly be done when the detected genes conferred only a low level of resistance, conferred resistance only under certain circumstances, or conferred resistance when several subtypes of a gene existed which could not be distinguished by the array, but each resulted in a different phenotype (e.g., expanded-spectrum beta-lactamase [ESBL] and non-ESBL variants of the TEM β-lactamase). A false-negative result was regarded as more critical than a false-positive result, since in a clinical setting it may lead to therapeutic failure. Therefore, in case of doubt, such a gene was assumed to confer complete resistance. Thus, in the following analysis, we assessed a strain that carried a resistance determinant which results in phenotypic resistance at an elevated probability as a resistant strain.

Strains which were determined by the VITEK2 system to show intermediate resistance to an antibiotic were treated in the evaluation like fully resistant strains. This definition was also motivated by the high risk and the severe consequences of therapy failure. Since the VITEK2 system always interprets *A. baumannii* as at least having intermediate resistance to tigecycline regardless of the measured MIC, intermediate resistance against tigecycline was not counted as resistance in the analysis, unlike the case for other antibiotics.

Piperacillin-tazobactam. The concordance of genotype and phenotype regarding the piperacillin-tazobactam results was 100%. Unfortunately, the number of cases of susceptible strains was very low, influencing the statistical significance of the evaluation. Resistance against piperacillin-tazobactam was mainly caused by Ambler class B and C β-lactamas (18). The chromosomally encoded Ambler class C ADC β-lactamase conferred piperacillin-tazobactam resistance only when overexpressed, e.g., by integration of the insertion element ISAba1 or when it carried certain point mutations, such as the ADC V208A or N283S substitution. In the study collection, 41 out of 58 piperacillin-tazobactam-resistant strains showed an ISAba1 integration upstream of *bla<sub>ADC</sub>* 3 strains harbored base exchanges leading to the V208A mutation, and 7 strains harbored base exchanges leading to the N283S substitution. Although many Ambler class D β-lactamas do hydrolyze broad-spectrum cephalosporins only to a low degree, the OXA-23 enzyme is capable of conferring resistance against piperacillin-tazobactam. The corresponding gene was detected in 14 strains. Seven more strains harbored the Ambler class D metallo-β-lactamase genes *bla<sub>VM</sub>* (<i>n</i> = 1) and *bla<sub>NDM</sub>* (<i>n</i> = 7). The mentioned determinants were responsible for the resistant phenotype of 57 out of 58 resistant strains. For the remaining single strain, the genetic origin of resistance could not be deter-
mined with certainty. This strain harbored a gene for a GES β-lactamase, and although this enzyme hydrolyzes piperacillin, it is usually inhibited by tazobactam. However, inhibitor-resistant subtypes of GES have been described that may account for the observed resistant phenotype (19).

**Ceftazidime.** The genotype-phenotype correlation for ceftazidime was in complete concordance. Ceftazidime resistance was mainly mediated by the same determinants that accounted for the piperacillin-tazobactam resistance, except for the Ambler class D enzymes, which are poorly active against ceftazidime. Instead, the Ambler class A ESBLs conferred ceftazidime resistance. In the study collection, genes coding for the Ambler class A β-lactamases TEM (n = 15), GES (n = 8), and SHV (n = 1) were detected. Whereas all subtypes of GES are capable of hydrolyzing ceftazi-
dime, only some subtypes of the TEM and SHV enzymes possess ESBL properties (6). Since the array did not differentiate between these subtypes, strains that tested positive for bla TEM or bla SHV were assumed to be resistant to cefazidime with regard to the risk of therapy failure.

**Imipenem and meropenem.** Due to the preselection of the collection for a multiresistant phenotype, mostly accompanied by an elevated MIC for carbapenems, the number of detected carbapenemases was large. Besides the seven strains harboring genes for metallo-β-lactamas and the 14 strains harboring a bla OXA-40-like gene mentioned above, several other causes of carbapenem resistance were observed. These included 12 strains harboring a bla OXA-40-like gene, 10 strains with a dysregulated bla OXA-51-like gene due to ISAbl1 integration, 11 strains harboring a bla OXA-58-like gene, and 8 strains that positive for bla GES. Not all subtypes of GES possess carbapenem-hydrolyzing activity (20). In seven cases, the bla GES-positive strains also harbored another determinant which may account for the carbapenem resistance, but in one case it was the only detected resistance determinant. This indicated the presence of a GES subtype with the ability to hydrolyze carbapenems.

Most of the strains in the study collection were provided by the German National Reference Center for Gram-Negative Pathogens. These strains were screened for carbapenemases by singleplex PCR and partly sequenced. The screening results were compared to the results obtained by the developed microarray (Table 3).

**TABLE 2 Detected resistance determinants in 60 A. baumannii strains clustered by type and sorted by prevalence**

<table>
<thead>
<tr>
<th>Resistance determinants (no.) by cluster</th>
<th>AME</th>
<th>Other</th>
<th>Mobile element</th>
<th>Point mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactamase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bla OXA-23, 60; bla ADE, 59; bla TEM-15</td>
<td>strA, 42; strB, 42; apb(3')-Ia, 23; aph(3')-VI, 21; aac(3)-Ia 19; aac(6')-Ib, 15; ant(3')-Ia, 14; ant(2')-Ia, 13; aac(6')-Ib, 9; aac(3)-Iba, 2; ant(3')-Ib, 1</td>
<td>aadM, 60; ccaA, 60; gacE, 60; adeABC, 59; sul2, 39; tetR, 31; sul1, 29; armA, 8; dfrA7, 7; tetA, 4; catA, 4; floR, 3; arr, 3; cmIA1, 3; dfrA1, 3; tetG, 1; qnrB, 1; catB, 1; dfrA10, 1</td>
<td>ISAba1, 48; upstream of bla ADE, 41; upstream of bla OXA-23, 14; upstream of bla OXA-51, 10; integrated in adep, 2; upstream of bla OXA-58, 1; intII1, 32; ISI633, 1</td>
<td></td>
</tr>
<tr>
<td>bla OXA-40, 12; bla OXA-58, 11; bla GES, 8; bla OXA-23, 7; bla NDM, 7; bla TEM, 1; bla SHV, 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These data indicate that the detection of carbapenemases was always successful. However, one false-positive result for a bla OXA-58-positive, carbapenem-susceptible isolate was observed. The gene bla OXA-58 contributes to carbapenem resistance but may require additional factors, such as further genes for carbapenemases, modification of membrane permeability, or an altered expression profile of penicillin-binding proteins (21, 22). However, the detection of bla OXA-58 in this carbapenem-susceptible isolate was an important finding, since the isolate had an elevated chance of becoming resistant when treated with carbapenems. This should be taken into account when making therapy decisions.

**Trimethoprim-sulfamethoxazole.** Resistance against trimethoprim-sulfamethoxazole requires two different mechanisms of action, each affecting one of the two antibiotics. High-level trimethoprim resistance was conferred by, e.g., the acquired dfrA gene, which was detected 11 times in the study collection. Several further mechanisms exist, such as mutations in the dihydrofolate reductase, alterations of the outer membrane, or efflux, e.g., by abeM. The latter confers only low-level resistance but is intrinsic to A. baumannii. Therefore, the resistance against trimethoprim-sulfamethoxazole was mainly dependent on the presence of the sulfamethoxazole resistance determinants sul1 and sul2. Fifty-five strains were resistant to trimethoprim-sulfamethoxazole. Out of these, 16 were positive for sul1, 26 were positive for sul2, and 13 strains harbored both genes. Neither of the genes was detected in the five trimethoprim-sulfamethoxazole-susceptible strains.

**Amikacin, gentamicin, and tobramycin.** The prediction of phenotypic resistance on the basis of genotypic data was challenging for the aminoglycosides, especially amikacin. Many cases of aberrant phenotypic and genotypic data have been described so far (23, 24). Furthermore, most strains harbored several aminoglycoside resistance determinants in parallel (between 2 and 7), and the effects of all factors interact. Aminoglycoside resistance in A. baumannii is mostly mediated by aminoglycoside-modifying enzymes (AME), but efflux and ribosomal protection also occurs (25). Detected determinants associated with AMK, GEN, and TOB resistance were armA (n = 8) and the insertion of ISAba1 in the gene adep (n = 2). Furthermore, AMK resistance was conferred by the AME-coding genes aph(3')-VI (n = 21), aac(6')-Ib (n = 15), and aac(6')-Ib (n = 9), respectively. Each of the 37 AMK-resistant strains harbored at least one of these determinants, except for two strains for which the detection of a causative genetic origin of the AMK resistance failed. Additionally, two of the armA-positive and three of the aac(6')-Ib-positive strains were susceptible to AMK. The expression of the gene aac(6')-Ib usually leads to resistance against AMK and TOB, but single-nucleotide

**TABLE 3 Comparison of microarray and singleplex PCR results regarding carbapenemase detection**

<table>
<thead>
<tr>
<th>Microarray data</th>
<th>Singleplex PCR data from the NRZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detected gene</td>
<td>No. of cases</td>
</tr>
<tr>
<td>bla OXA-23, 14</td>
<td>14</td>
</tr>
<tr>
<td>bla OXA-40, 13</td>
<td>13</td>
</tr>
<tr>
<td>bla OXA-58, 11</td>
<td>11</td>
</tr>
<tr>
<td>ISAba1-bla OXA-51, 10</td>
<td>10</td>
</tr>
<tr>
<td>bla TEM, 1</td>
<td>1</td>
</tr>
<tr>
<td>bla GES, 8</td>
<td>8</td>
</tr>
<tr>
<td>bla NDM, 7</td>
<td>7</td>
</tr>
</tbody>
</table>

\[ a \] bla OXA-32 belongs to the bla OXA-40-like cluster.

\[ b \] bla OXA-164 belongs to the bla OXA-58-like cluster.

\[ c \] Carbapenem-resistant strains were screened for bla GES only if no other genes for carbapenemases were found.

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Table 4: Correlation of the phenotype with the corresponding genotype of each analyzed strain

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>True positive (n)</th>
<th>True negative (n)</th>
<th>False positive (n)</th>
<th>False negative (n)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TZP</td>
<td>58</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>CAZ</td>
<td>58</td>
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<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>IPM</td>
<td>54</td>
<td>5</td>
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<td>0</td>
<td>98</td>
<td>100</td>
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<td>MEM</td>
<td>54</td>
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<td>0</td>
<td>98</td>
<td>100</td>
<td>83</td>
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<tr>
<td>SXT</td>
<td>55</td>
<td>5</td>
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<td>0</td>
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<tr>
<td>AMK</td>
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<td>2</td>
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<td>90</td>
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<tr>
<td>GEN</td>
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<td>0</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>86</td>
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<tr>
<td>TOB</td>
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<td>0</td>
<td>98</td>
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<td>1</td>
<td>50</td>
<td>98</td>
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<td>98</td>
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</tbody>
</table>

Weighted mean: 98.3, 97.7, 99.5, 93.7

Absolute numbers of cases for true-positive, true-negative, false-positive, and false-negative array analysis for each tested antibiotic are listed. The derived PPV, NPV, sensitivity, and specificity, as well as their weighted means, are shown. Since the mutations causing colistin resistance are very diverse (36, 37), rendering them inappropriate for microarray detection, the developed array contains no probes specific for colistin resistance determinants.

Table 4: Overview of genotype-phenotype correlation. According to the detected resistance determinant repertoire of each strain, a prediction of the corresponding phenotype was made for each tested antibiotic. When the prediction matched the determined phenotype, the detection was stated as true positive or true negative. Otherwise, the array output was regarded as a false-positive or false-negative result (Table 4).

Microarray performance. The evaluation of microarray data is highly related to a precise discrimination of positive and negative signals. To achieve this goal, low background signals, as well as high positive signals, were inevitable. The overall mean of the signal intensity of all positive signals was 15,411 counts. The mean of the lowest positive signal of the different arrays was 1,995 counts. With an overall average of all-negative signals of 19 counts, the positive and negative signals were clearly distinguishable.

Discussion

A comparison between the observed phenotype and determined genotype was made for each tested A. baumannii strain. In most cases, the phenotype could be correlated with the corresponding genotype. In the following, discrepancies between array and VITEK2 output are discussed.

The concordance of genotype and phenotype regarding piperacillin-tazobactam and ceftazidime was 100%, although the array could not differentiate between genes coding for inhibitor-resistant and -susceptible GES subtypes and ESBL TEM and SHV variants. Some microarrays have already been designed to detect resistance determinants, including a subtyping of blaTEM and blaSHV variants (30, 31). However, these arrays were not specifically designed to analyze A. baumannii, sparing species-specific β-lactamas and other resistance determinants. Nevertheless, they provide more detailed information about ESBL genes, which could be important in clinical applications. An expansion of the developed array to include the blaTEM and blaSHV subtyping could improve its usefulness.

Regarding aminoglycoside resistance, the concordance of genotype and phenotype was not given in seven amikacin-, one gentamicin-, and one tobramycin-related case, respectively. According to a previous study (24), the two cases of armA-positive,

Exchanges can cause a change in the substrate spectrum of the encoded enzyme to GEN and TOB resistance (26). Since the probes of the microarray could not detect these mutations, a prediction of the resistance against AMK and GEN of aac(6’)-Ib-harboring isolates on the basis of the available genotypic data was not possible. Due to a strongly elevated chance of a resistant phenotype, these isolates were assumed to be resistant to TOB, AMK, and GEN, although one of them may still be potent. This causes an increased number of false-positive results of the array for AMK and GEN.

GEN resistance was mediated, aside from armA and the insertion of ISAbai in the gene adeS, by the AME coding genes aac(3)-Ia (n = 19), aac(3)-Iia (n = 2), aac(6’)-Ib (n = 15), and ant(‘2’)-Ia (n = 13). Every resistant strain harbored at least one of these genes, but one aac(6’)-Ib-positive strain was still susceptible to gentamicin.

Detected AME-coding genes responsible for TOB resistance were aac(3)-Iia (n = 2), aac(6’)-Ib (n = 15), aac(6’)-IIa (n = 9), and ant(‘2’)-Ia (n = 13). All TOB-resistant strains were positively tested for at least one of these genes, but one ant(‘2’)-Ia-harboring strain remained susceptible to TOB.

Ciprofloxacin. The most prevalent detected mechanism of ciprofloxacin resistance was a mutation in the gyrA gene, leading to the gyrA S83L substitution. This mutation accounts for ciprofloxacin resistance in all 58 isolates. In 46 strains, a further mutation in the parC gene was detected, leading in 45 cases to parC S80L and in one case to parC S80F. Additionally, one strain tested positive for qnrB.

Tigecycline. The identification of tigecycline resistance determinants was challenging. Many different mutations in the regulatory genes adeS and adeR, resulting in overexpression of the adeABC efflux system and resistance to tigecycline, have been described so far (27–29). These were only partially detectable by the developed array. The sole detected cause of tigecycline resistance was the insertion of ISAbai in the adeS gene (n = 2). However, only one of these two cases showed the respective phenotypic resistance, whereas the other strain had only an elevated MIC for tigecycline. In clinical practice, such an elevated MIC against tigecycline also may be relevant. Consequently, even this false-positive result of the array is noteworthy. For another resistant strain, no causative genetic origin was detected. The sensitivity and PPV regarding tigecycline analysis were poor due to the two cases of discrepancy between genotype and phenotype and the very low number of cases of tigecycline-resistant strains. In order to generate more reliable statistical data, it would be wise to test the microarray with a larger collection of tigecycline-resistant isolates.

Overview of genotype-phenotype correlation. According to the detected resistance determinant repertoire of each strain, a prediction of the corresponding phenotype was made for each tested antibiotic. When the prediction matched the determined phenotype, the detection was stated as true positive or true negative. Otherwise, the array output was regarded as a false-positive or false-negative result (Table 4).

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AMK-susceptible isolates very likely were related to a false-negative AMK susceptibility result provided by the VITEK2 assay rather than to a false-positive array signal. Taking this into account, the positive predictive value of the array for AMK would be increased from 88 to 93%. In four cases, the ambiguity was related to the aac(6′)-Ib gene and its point mutations, which may lead to a shift in the substrate spectrum of the encoded enzyme. Enhancing the array by the integration of probes specific for these single-nucleotide exchanges in aac(6′)-Ib may help to overcome this drawback and reduce false-positive results.

The present study gives insights into the distribution of resistance determinants occurring in clinical isolates of A. baumannii in Germany. The most prevalent carbapenemase-coding genes were blaOXA-23-like, blaOXA-40-like, and blaOXA-58-like, which corroborates previous observations (32). A blaNDM gene, which was described for the first time in Germany in 2007 (33, 34), was detected in 12% (n = 7) of the strains in the preselected study collection. PFGE fingerprinting clustered five of these blaNDM-positive isolates in the same clonal complex showing the same array pattern. The other two blaNDM-positive isolates differed in their genetic PFGE fingerprint pattern as well as in their detected array gene repertoire. Additionally, one of these two strains harbored another subtype of blaNDM along with blaNDM-2. These results indicate the occurrence of at least two independent blaNDM-harboring clonal complexes, one of which seems to be widely disseminated in Germany.

Although this prevalence is not representative for all A. baumannii strains in Germany, it indicates a considerable spread of blaNDM in recent years. Also noteworthy is the broad dispersion of the ISAba1 genetic element, which was detected in 80% of the strains. Depending on its integration site, it can affect susceptibility against every tested antibiotic.

The developed array provides therapy-relevant information significantly faster than culture-based methods and has several advantages over other molecular biological methods, such as next-generation sequencing (NGS). Despite considerable advances in NGS technologies in recent years, microarrays are much faster, cheaper, and more appropriate for high-throughput screenings (35). In particular, microarrays embedded in automated laboratory-on-a-chip systems, e.g., that provided by Unyvero system (Curetis AG), reduce the expenditure of human labor to a few minutes per analysis.

To summarize, we developed a DNA microarray for the simultaneous detection of 91 resistance determinants in A. baumannii. The array was validated with 60 multidrug-resistant A. baumannii strains. The assay protocol includes DNA extraction, multiplex PCR for target amplification, and sample hybridization on the array, respectively. The expenditure of time for the whole analysis did not exceed 4 h. In combination with its high average PPV, NPV, specificity, and sensitivity, the assay provides a fast and reliable tool to predict the resistance phenotype of clinical A. baumannii isolates based on the underlying genotype. With an average variance of probe signals of 23% between iterations with the same strain, the reproducibility was in an acceptable range. The established multiplex PCR has proven to be capable of amplifying at least 29 targets simultaneously, even enabling the analysis of pandrug-resistant isolates. Considering the short turnaround time, the array analysis could be especially favorable in cases of severe infections, such as pneumonia and sepsis. With its broad spectrum of detectable resistance determinants, the microarray not only allows a fast adaptation of an empirical antibiotic treatment but also serves as a tool for epidemiologic surveillance. Its ability to discriminate phenotypically indistinguishable isolates with different underlying genotypes may also serve to identify chains of infection.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 241446 (project Antiresdev).

We also thank our contributors of strains: Robert Bosch Hospital (Stuttgart, Germany), Animal Health and Veterinary Laboratories Agency (Waybridge, Great Britain), University of Berne (Berne, Switzerland), National Institute of Public Health (Prague, Czech Republic), and University College London (London, Great Britain). Furthermore, we thank Stefan Hartmann (Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart, Germany) for constructive proposals.

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