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Published Ahead of Print 9 December 2009.

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Integrated Detection of Extended-Spectrum-Beta-Lactam Resistance by DNA Microarray-Based Genotyping of TEM, SHV, and CTX-M Genes

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Received 15 April 2009/Returned for modification 21 June 2009/Accepted 30 November 2009

Extended-spectrum beta-lactamases (ESBL) of the TEM, SHV, or CTX-M type confer resistance to beta-lactam antibiotics in Gram-negative bacteria. The activity of these enzymes against beta-lactam antibiotics and their resistance against inhibitors can be influenced by genetic variation at the single-nucleotide level. Here, we describe the development and validation of an oligonucleotide microarray for the rapid identification of ESBLs in Gram-negative bacteria by simultaneously genotyping \textit{bla\textsubscript{TEM}}, \textit{bla\textsubscript{SHV}}, and \textit{bla\textsubscript{CTX-M}}. The array consists of 618 probes that cover mutations responsible for 156 amino acid substitutions. As this comprises unprecedented genotyping coverage, the ESBL array has a high potential for epidemiological studies and infection control. With an assay time of 5 h, the ESBL microarray also could be an attractive option for the development of rapid antimicrobial resistance tests in the future. The validity of the DNA microarray was demonstrated with 60 blinded clinical isolates, which were collected during clinical routines. Fifty-eight of them were characterized phenotypically as ESBL producers. The chip was characterized with regard to its resolution, phenotype-genotype correlation, and ability to resolve mixed genotypes. ESBL phenotypes could be correctly ascribed to ESBL variants of \textit{bla\textsubscript{CTX-M}} (76%), \textit{bla\textsubscript{SHV}} (22%), or both (2%), whereas no ESBL variant of \textit{bla\textsubscript{TEM}} was found. The most prevalent ESBLs identified were CTX-M-15 (57%) and SHV-12 (18%).

The resistance of widespread Gram-negative pathogens such as \textit{Escherichia coli}, \textit{Klebsiella} species, and other \textit{Enterobacteriaceae} against broad-spectrum beta-lactam antibiotics is a severe and growing issue in health care (29, 41, 42). In the beginning, the resistance to these antimicrobials, including oxyimino-cephalosporins (e.g., cefotaxime, cefpodoxime, and ceftazidime), was caused mainly by TEM- and SHV-type extended-spectrum beta-lactamases (ESBLs), which evolved via mutation from the classic TEM-1, TEM-2, and SHV-1 enzymes (18). Plasmid-mediated derivatives with an extended substrate spectrum have been known since the early 1980s (24, 44), and other mutational events that affect substrate affinity for beta-lactamase inhibitors, such as clavulanic acid, lead to an inhibitor-resistant (IRT) or complex mutant type (CMT) enzymes (8). During the last 10 years, other ESBL types have gained increased importance, especially the CTX-M enzymes (7, 27, 35, 40). The \textit{bla\textsubscript{CTX-M}} genes probably emerged through several mobilization events of their natural homologues in the genus \textit{Klyvera} (5, 21, 33, 34, 37, 39) and have undergone evolution since that time. They have intrinsic ESBL activity and are clustered in five subgroups, designated CTX-M1, CTX-M2, CTX-M8, CTX-M9, and CTX-M25 (5).

A fast and accurate identification of the antimicrobial resistance of the etiologic pathogen is decisive for the initiation of an appropriate antimicrobial therapy (36, 43). Routine culture-based phenotypic ESBL detection usually requires 2 days or more (48), and the reported sensitivities and specificities of different tests (50) leave room for improvement. In addition, as the required confirmatory tests are based on the synergy effect between antibiotics and inhibitors, the coexistence of the ESBL with other beta-lactamases can lead to misinterpreted results (14, 23). Phenotype-based resistance tests also do not provide gene variant information, which limits their usefulness for epidemiologic surveillance and refined antimicrobial therapy. Molecular ESBL detection has the potential to improve ESBL diagnosis, infection control, and epidemiological surveillance. In addition to standard PCR and gene sequencing, several molecular ESBL identification techniques have been developed during the past few years, such as PCR-restriction fragment length polymorphism (2), PCR-single strand conformation polymorphism (31), real-time PCR (4, 38), or pyrosequencing (22, 32) analysis. However, all of these techniques have an inherently limited multiplexing capacity. The genotyping of TEM, SHV, or CTX-M beta-lactamases, with more than 500 known variants altogether (http://www.lahey.org/studies/), technically would be excessive, very costly, and too time demanding to be used as a diagnostic aid. DNA microarrays, on

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† Supplemental material for this article may be found at http://jcm.asm.org/.
‡ Published ahead of print on 9 December 2009.
the other hand, are an increasingly used tool for multiplexed genotyping. Microarrays targeting resistance determinants have been described with focus on certain species (30, 45, 46, 49), specific resistance mechanisms (10, 53), or resistance in Enterobacteriaceae (3, 11, 17). Some examples covered beta-lactam resistance at the gene family level (25) or investigated six point mutations within blaSHV (54), the latter technically pointing toward the refined genotyping of beta-lactamase genes. To cover all known mutations in a beta-lactamase gene, we previously described a DNA microarray for the genotyping of TEM beta-lactamases (19).

In the present study, we describe an advanced DNA microarray that integrates an updated version of the TEM microarray (19) with two new modules for the genotyping of blaSHV and blbCTX-M genes. Because of its unparalleled high coverage of known variants and information depth, combined with its short time to result, this assay can be an enabling tool for epidemiological studies of ESBL outbreaks and infection control, and it offers an attractive way toward the development of the rapid molecular testing of ESBLs in the clinical routine.

MATERIALS AND METHODS

Bacterial strains and antibiotic susceptibility determination. As reference material for the system setup, we used a panel of reference strains and well-characterized clinical samples (the bla gene of interest was sequenced). Reference strains included E. coli DH5a (Clontech, Heidelberg, Germany) transformed with plasmid pUC19 (blaTEM-1,161 New England Biolabs, Frankfurt, Germany) or pCR9 (different strains with several blaSHV genes, including blaSHV-1, blaSHV-2, blaSHV-3, and blaSHV-12; provided by H. Hächler, Institute of Medical Microbiology, University of Zürich), European Antibiotic Resistance Surveillance System reference strain UA 1528 (blaTEM-1b), and additional E. coli reference strains provided by W. Witte, Robert Koch Institute, Wernigerode Branch, Wernigerode, Germany (blaTEM-2 and blaTEM-5). Clinical samples additionally harbored blaTEM-1 (several species), blaCTX-M-3 (K pneumoniae), blaCTX-M-8 (Salmonella enterica serovar Typhimurium), blaCTX-M-14 (Enterobacter sp.), blaCTX-M-15 (E. coli), and blaCTX-M-18 (E. coli) and were provided by our partners in Moscow and Frankfurt am Main.

For the evaluation of the ESBL array, isolates were collected from the Department of Laboratory Medicine at the Robert Bosch Hospital, Stuttgart, Germany (n = 22), and the Institute of Medical Microbiology and Infection Control, J. W. Goethe University, Frankfurt am Main, Germany (n = 38). They were recovered from respiratory samples (n = 8), swabs (n = 27), urine (n = 18), and other sites (n = 7). Species identification was carried out with the API 20NE system (bioMérieux, Marcy l’Etiole, France). For the Gram-negative identification card GN on a Vitek 2XL system, all bacterial strains were routinely cultured at 37°C on Columbia blood agar (Heipha Diagnostika) or grown in Schaedler-Bouillon (bioMérieux, Marcy l’Etoile, France). Plasmid DNA from bacteria was extracted with the QiAprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. Antibiotic susceptibility was determined by disc diffusion (Kirby-Bauer method) on Mueller-Hinton agar (bioMérieux, Marcy l’Etoile, France) and was determined by the Gram-negative identification card GN on a Vitek 2XL system. All bacterial strains were routinely cultured at 37°C on Columbia blood agar (Heipha Diagnostika) or grown in Schaedler-Bouillon (bioMérieux, Marcy l’Etoile, France).

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Probe design. Multiple sequence alignments of blaTEM, blbSHV, and blbCTX-M nucleotide sequences were performed to identify sequence variations, mainly single-nucleotide polymorphisms (SNPs), leading to amino acid exchanges (http://www.lahey.org/studies/). Sequence alignments were carried out using ClustalX (version 1.83) (47), the BioEdit sequence alignment editor (version 7.0.5.3) (20), or Sequencher II software (version 5.0.0; DNAStar). This study includes the integration of previously designed probes, redesigned probes, and de novo-designed probes for additional SNPs to update the ESBL microarray. For the current version of the microarray, 150 probe sets were characterized for the demonstration of polymorphisms leading to 59 amino acid substitutions known for TEM, 53 known for SHV, and 44 known for the different CTX-M beta-lactamases were developed. Furthermore, four probe sets per CTX-M group identification were included. The subgroups for CTX-M-8-like and CTX-M-25-like genes were examined together and labeled group M8/M25. The standard probe design was carried out as described previously (19), with minor modifications. In brief, oligonucleotide probes of various lengths (14 to 27 bases) were designed to detect single-nucleotide substitutions within the blbTEM, blbSHV, and blbCTX-M genes, leading to amino acid exchanges. For each SNP, a probe set of four probes was designed with identical sequences except the investigated mutation site, which was either A, G, C, or T. Ideally, the probes were designed with the mutation at the central position within the probe sequence to maximize perfect match (PM)-to-mismatch (MM) discrimination. The probe length, melting temperature (Tm), and free energies of possible secondary structures (e.g., hairpins [DGhairpin] and dimers [DGdimer]) were calculated with the online tool OligoAnalyzer (http://eu.ittdna.com/analyzer/Applications/OligoAnalyzer/) using the default settings for monovalent ions and DNA concentration (50 mM and 0.25 μM). The following values and thresholds were regarded as optimal: Tm = 53 ± 3.5°C; DGhairpin ≥ 0 kcal/mol; and DGdimer ≥ –6 kcal/mol. All probes were designed as sense probes. The probe sets are named after the position of the amino acid substitution within the blbTEM, blbSHV, or blbCTX-M sequence. Unless otherwise noted, amino acid residue numbering followed the standard numbering scheme (25). A beta-lactamase gene was regarded as a positive control, and it offers an attractive way toward the development of the rapid molecular testing of ESBLs in the clinical routine.

Oligonucleotide array fabrication. Oligonucleotide arrays consisted of three stripes of subarrays, each representing a chip module for the detection of TEM, SHV, or CTX-M beta-lactamases. Each array was manufactured with 618 different oligonucleotide capture and control probes. The oligonucleotides were purchased from Metabion (Planegg-Martinsried, Germany) in desalted purity and quality controlled by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Each oligonucleotide was equipped with a 13-thymidine spacer and an amino modification at the 5′ end. Every array included 5′-end dimer and quality controlled by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Each oligonucleotide was included in the chip assembly, to ensure that the hybridization event to compensate for the loss of hybridization efficiency associated with such a method, the probe length usually had to be elongated to match a Tm mismatch (also calculated with OligoAnalyzer) within the desired Tm range (as described above). Where required, different versions of a probe set were tested, and the one showing the best PM-MM discrimination and hybridization efficiency using the applied hybridization conditions was selected. Sequences of all probes included on the chip are shown in Table S1 in the supplemental material.
print the oligonucleotide probes, which were dissolved in spotting buffer (Nexterion Spot with 0.0075% Triton X-100 [final concentration]; Schott, Jena, Germany) to a final concentration of 20 μM (except for the spotting control [5 μM] and 11 different capture probes of the CTX-M chip module [12 to 50 μM]) onto epoxy-coated glass slides (Nexterion Slide E; Schott, Jena, Germany). For covalent immobilization, the oligonucleotide array was incubated at 60°C for 30 min in a drying compartment (Memmert, Schwabach, Germany). After being spotted, the slides were rinsed for 5 min in 0.1% (vol/vol) Triton X-100 in double-distilled H₂O (ddH₂O), 4 min in 0.5 μL of concentrated HCl per mL of ddH₂O, and 10 min in a 100 mM KCl solution with constant stirring. Subsequently, the slides were incubated in blocking solution (25% [vol/vol] ethylene glycol in ddH₂O, 0.5 μL of concentrated HCl per mL of blocking solution) with the spotted side upwards at 50°C in a heating compartment (OV5; Biometra, Göttingen, Germany), rinsed in ddH₂O for 1 min, and finally dried under a flow of nitrogen. The average spot size was 130 μm, and the spot-to-spot distance was 320 μm. The processed slides were stored dry at room temperature in the dark for a maximum of 20 days until further use.

Controls. In addition to the beta-lactamase gene-specific probes, each array contained several controls: a prelabelled spotting control (5′-TTTTTTTTTTTTTTTAGACAGCACTACATA-cyana3 [Cy3]-3′), a positive hybridization control (5′-TTTTTTTTTTTTTGAAGGTAGTTCACTGGTGCTGC-3′) complementary to a labeled oligonucleotide target (5′-C5′-GCTCCTGACTGCTAATGCTTAATCAGTGA-3′), which was spiked during hybridization, and a negative hybridization control (5′-TTTTTTTTTTTTTTTTTTTAGACAGCACTACATA-cyana3-3′). All of these control sequences are unrelated to sequences found in bacterial species. The spotting controls were set at the corner positions of each of the 14 subarrays. The positive and negative hybridization controls were spotted alternately at the side borders of oligonucleotide arrays (TEM, SHV, and TEM/SHV). A spotting control (5′-TTTTTTTTTTTTTTTTTTTAGACAGCACTACATA-cyana3-3′), and a positive hybridization control (5′-TTTTTTTTTTTTTTTTTTTAGACAGCACTACATA-cyana3-3′) corresponded to sequences conserved within the blaTEM, blasIV, and blaCTX-M gene families. Within the subarrays belonging to a certain chip module, the corresponding process control was spotted in two lines bordering the central probe sets (Fig. 1; also see Fig. S1 in the supplemental material).

Amplification, labelling, and purification. Target DNA for the hybridization on the oligonucleotide arrays was amplified by PCR. The blaTEM, blasIV, and blaCTX-M gene families. Within the subarrays belonging to a certain chip module, the corresponding process control was spotted in two lines bordering the central probe sets (Fig. 1; also see Fig. S1 in the supplemental material).

Amplification, labelling, and purification. Target DNA for the hybridization on two chips (duplicate) was amplified with 0.4 μL DNAase I (Promega, Mannheim, Germany) per ng labeled target DNA to increase the efficiency of hybridization and avoid denaturation. All target DNA amplified from one isolate were included in the same reaction. The fragmentation was carried out in 1× reaction buffer (40 mM Tris-HCl [pH 8], 10 mM MgSO₄, 1 mM CaCl₂) at room temperature for 5 min in a total volume of 20 to 30 μL depending on the concentrations of the purified PCR products. The reaction was stopped by the addition of 2 mM EGTA and incubation at 65°C for 10 min. Fragment sizes were estimated to be in the range of 15 to 150 bp using lab-on-a-chip electrophoresis (Bioanalyzer 2100, DNA 1000 LabChip kit; Agilent, Böblingen, Germany).

Hybridization. Depending on the presence of either one or both genes, 150 or 200 ng TEM/SHV target DNA was hybridized. For CTX-M, 150 ng target DNA was used. As standard conditions, the fragmented target DNA for one slide together with DNA complementary to the positive control (Cy3-GCTCCTGACAGCACTACATA-cyana3-3′) was hybridized in 65 μL of 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]) containing 0.1% sodium dodecyl sulfate (SDS). For hybridization, an HS400 automated hybridization station (Tecan, Crailsheim, Germany) was used with an initial wash step at 54°C for 30 s with 2× SSPE (the same concentration as that used for the hybridization solution) followed by the injection of 65 μL hybridization solution and hybridization at 47°C for 1 h using a high-agitation frequency. After hybridization, the slides were washed twice at room temperature: 1.5 min with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), and 1 min with 0.2× SSC. The slides subsequently were dried with N₂ at 30°C for 4 min. The duration of the whole procedure was 90 min. Different SSPE and DSS concentrations or hybridization temperatures were tested during array optimization.

Data acquisition and processing. After the hybridization reaction, the data from the oligonucleotide arrays were read out by the acquisition of the fluorescence signals using a ScanArray Express microarray scanner (Perkin-Elmer, Waltham, MA). The laser power setting was 100%. Each slide was scanned with multiple photomultiplier tube (PMT) gain settings, including 55, 60, 65, and 70% and a 10-μm resolution. The image processing and calculation of signal intensities were performed with ScanArray Express analysis software, version 3.0 (Perkin-Elmer, Waltham, MA). Individual net signal intensities were calculated by the subtraction of the local background from the raw spot intensity value. Further data processing was performed using Microsoft Excel (Microsoft, Redmond, WA). Unless otherwise stated, the standard data processing procedure as follows. Net signal intensities were extracted from the PMT 60% scans, 55, 60, 65, and 70% and a 10-μm resolution. The image processing and calculation of signal intensities were performed with ScanArray Express analysis software, version 3.0 (Perkin-Elmer, Waltham, MA). Individual net signal intensities were calculated by the subtraction of the local background from the raw spot intensity value. Further data processing was performed using Microsoft Excel (Microsoft, Redmond, WA). Unless otherwise stated, the standard data processing procedure as follows. Net signal intensities were extracted from the PMT 60% scans. The calculation of the mean net signal intensities, referred to as signal intensity, the six spot replicates of a certain oligonucleotide probe on two identically processed microarrays (three replicates each) were used. Standard deviations also were calculated. Within each oligonucleotide probe set related to a certain mutation, the probe...
showing the highest signal intensity was considered the potential perfect match (PM). The remaining ones with lower signal intensities were considered mismatches (MM). The quality of the discrimination between single nucleotides within the target using such a probe set is reflected by the difference between the perfect match signal and mismatch signals. Therefore, the ratio between the individual net signal intensity of each spot replicate of a certain probe within the probe set ($N_{\text{ISpot}}$) and the mean signal intensity of the perfect match signal ($I_{\text{PM}}$) was calculated ($N_{\text{ISpot}}/I_{\text{PM}}$). The mean of those values is referred to as the relative intensity (RI or $RI_{\text{MEAN}}$) of a certain oligonucleotide probe. Accordingly, the perfect matches always had RI values of 1 ($RI_{\text{PM}} = 1$), and the relative

FIG. 1. Chip layout. (a) Fluorescence image after hybridization with 200 ng labeled TEM-1/SHV-1 (NT/F = 97) target DNA (Iso-37; PMT, 60%). Box 1, fluorescence signals of some process controls; box 2, probe sets for CTX-M group determination; box 3, signal pattern of probe set TEM 39. The signal intensity is encoded in the 65,636 gray scales of the 16-bit TIF image. (b) Schematic representation of the modular layout of the oligonucleotide microarray. The areas specific for the different beta-lactamase genes and the different subgroups of the CTX-M type genes are labeled. The enlarged part describes in detail the arrangement of the A, G, C, and T probes of each probe set. The probe sets are named as described in Materials and Methods, and numbers represent interrogated amino acid positions. Theoretical perfect matches after hybridization with TEM-1, SHV-1, CTX-M-1, CTX-M-2, CTX-M-8, and CTX-M-9 are highlighted in black. An enlarged version of this figure is shown in Fig. S1 in the supplemental material.
intensities of the mismatches have values below 1 \( (R_I_{\text{TEM}} < 1) \). Standard deviations also were calculated.

For the evaluation of the performance of each probe set, the median of the relative intensities \( (N_{\text{rel}}/N_{\text{tr}}) \) of the six technical replicates (three spots on two arrays each) were calculated and referred to as \( R_I_{\text{MEDIAN}} \). The means and standard deviations from the \( R_I_{\text{MEDIAN}} \) values of repeated experiments (biological replicates) were calculated, and the means again were referred to as relative intensity \( (R_I) \). The probe sets of the TEM module were analyzed using data of all clinical isolates harboring a TEM-1 gene \( (n = 38) \). For the SHV module, all SHV-1-positive isolates \( (n = 10) \) were used, and for the CTX-M module, all CTX-M-15 \( (n = 34) \) or CTX-M-14/M-9 \( (n = 3 \text{ to } 8) \)-positive isolates were used. Signals obtained from mixed genotypes were evaluated calculating the 95% confidence intervals in addition to standard deviations. Standard \( t \) testing was applied where necessary.

**Nucleotide sequence accession numbers.** The sequences obtained by sequencing have been submitted to GenBank under accession numbers FJ668714 to FJ668818.

**RESULTS**

In the present study, we describe a diagnostic oligonucleotide microarray for the rapid identification of ESBLs in *Enterobacteriaceae* by the simultaneous genotyping of TEM, SHV, and CTX-M beta-lactamase genes. The performance of this integrated chip and its applicability as an identification tool was validated using 60 blinded clinical samples collected in two clinical laboratories. The current version of the chip includes probe sets for the identification of 99% of all TEM variants and 89% of all SHV variants published (http://www.lahey.org/studies/; as of 2008). All positions known to be associated with ESBL activity \( (18) \) and inhibitor resistance \( (8) \) were included. Probes within the CTX-M chip module cover CTX-M group determination and the identification of 82% of all known CTX-M group M1 beta-lactamases as well as 83, 50, and 50% of all group M2, group M9, and group M8/M25 beta-lactamases.

**ESBL chip development.** The development of the described chip included the integration of previously designed chips for different beta-lactamase genes as well as the integration of newly designed and redesigned probe sets. An easily expandable modular organization was chosen as the layout (Fig. 1; also see Fig. S1 in the supplemental material). During the integration process, reference strains with known gene variants were used and several adjustments to hybridization parameters were made compared to those of our previous studies \( (19) \). As standard conditions, the hybridization of fragmented target DNA was carried out for 1 h at 47°C in a solution containing 2× SSPE and 0.1% SDS. The application of the automated hybridization station resulted in a hybridization procedure of only 90 min, including washing and drying steps. The data acquisition was possible immediately afterwards.

For the production of target DNA, plasmid DNA isolated from clinical samples or from reference material was amplified by PCR. For the simultaneous genotyping of different gene targets, a multiplexed amplification and labeling strategy is desirable. TEM and SHV genes were amplified and labeled with Cy3 using a robust dual-plex PCR. Amplification product sizes were 867 and 927 bp, respectively. The integration of the CTX-M amplification into a multiplexed format, which would simultaneously amplify and label all of the TEM, SHV, and CTX-M genes, was dropped due to the formation of heterodimers between primers specific for the different CTX-M groups. Hence, CTX-M target DNA was amplified with group-specific primers in a singleplex PCR after clarifying the allocation to a certain CTX-M group by using a multiplex screening PCR. The PCR product sizes for CTX-M1 and CTX-M9 group members were 864 and 870 bp, respectively. The Cy3 incorporation rates were similar for all labeling PCRs applied. The mean NT/F ratios (variation depending on template quality) of the TEM/SHV PCR products were 84 \( (45 \text{ to } 170) \) and 97 \( (56 \text{ to } 173) \), respectively, for the CTX-M group-specific PCR products.

The performance of an individual probe set was assessed with regard to its discrimination power for various DNA targets and expressed as the mismatch-to-perfect match ratio \( (MM/PM) \). For allele-specific hybridization, which was chosen as a format in the presented microarray, the probe position relative to that of the target is virtually fixed if single-nucleotide polymorphisms are targeted. However, at certain positions it was impossible to select probes, because stable secondary structures would have formed within the probe themselves, rendering a meaningful detection impossible. We therefore chose to incorporate artificial mismatches at positions involved in the undesired secondary structure formation at difficult target positions. Figure 2 compares the standard and alternative probe designs for probe sets SHV 61 and TEM 224. In both cases, the probe containing a cytosine represents the perfect match when hybridizing SHV-1 and TEM-116 target DNA. For SHV 61, the replacement of a cytosine by thymine at a position significantly involved in dimer formation shifted the highest hybridization signal from the wrong A probe to the correct C probe. The highest mismatch signal of the resulting probe set showed only 52% of the perfect match signal. In another case, the performance of the already correct working probe set TEM 224 was further improved by the same method (Fig. 2). Here, the average \( R_I_{\text{MM}} \) value was decreased from 0.32 to 0.11. For a complete list of all probes used for the current version of the chip, see Table S1 in the supplemental material.

**Testing of clinical isolates.** The performance of the array was validated using 60 blinded samples containing DNA isolated from clinical isolates collected in clinical routines of the Department of Clinical Chemistry and Laboratory Medicine at the Robert Bosch Hospital (RBK) in Stuttgart and the Institute of Medical Microbiology and Infection Control of the J. W. Goethe-Universität in Frankfurt am Main. Fifty-eight were phenotypically characterized as ESBL positive using standard laboratory procedures for susceptibility testing (agar diffusion using CLSI breakpoints; Vitek 2). Two samples showed an ambiguous or ESBL-negative phenotype \( (\text{ISO-23 and ISO-62}) \). The identified species were mainly *E. coli* and different *Klebsiella* species, but also one isolate each of *Enterobacter sakazakii, E. intermedius, Proteus vulgaris, C. freundii*, and *Rahnella aquatilis* (Table 1).

Prior to the genotyping with the integrated ESBL chip, all samples were screened by the described amplification procedures for the presence of the target genes. As a result, 63% of all 60 isolates were positive for \( \text{bla}_{\text{TEM}} \) 38% for \( \text{bla}_{\text{SHV}} \), and 70% for \( \text{bla}_{\text{CTX-M}} \); 3% were negative for all targets. From all isolates found to be *E. coli*, 55% harbored \( \text{bla}_{\text{TEM}} \) 3% \( \text{bla}_{\text{SHV}} \), and 94% \( \text{bla}_{\text{CTX-M}} \). For *Klebsiella* spp., 69% were positive for \( \text{bla}_{\text{TEM}} \) 81% for \( \text{bla}_{\text{SHV}} \), and 58% for \( \text{bla}_{\text{CTX-M}} \). With the applied multiplex screening PCR for \( \text{bla}_{\text{CTX-M}} \) it was possible
to distinguish between different CTX-M groups. The highest prevalence within the CTX-M-positive isolates was found for group M1 (81%), followed by 14% for group M9 and 4% for group M1 and M9 together.

The further identification of the isolates’ beta-lactamase genotype was based on a hierarchical readout of the DNA microarray signals after labeling PCR and hybridization to the ESBL chip. As an example, Fig. 1a shows the hybridization result for Iso-37. (i) The gene family was determined by the process controls (PC), which act as gene family-specific probes. For Iso-37, the PCs detected the presence of a TEM, SHV, and CTX-M gene (box 1). (ii) The classification of detected CTX-M genes into subgroups (M1, M2, M9, and M8/25) was done by four probe sets (see the layout section labeled CTX-M group in Fig. 1b). The Iso-37 CTX-M gene showed the characteristic hybridization signal pattern for a \(\text{bla}_{\text{CTX-M-1}}\)-like gene as indicated by the identified adenosine (A) for probe set 73.M1/2, the absent signal for probe set 73.M9, and the identified guanosines (G) for probe sets 132 and 267 (box 2). (iii) Finally, the beta-lactamase gene variant was identified by signal patterns exhibited by the individual probe sets corresponding to the different interrogated mutation positions. For example, when hybridizing the Iso-37 TEM gene, the signal pattern of probe set 39 (box 3) identified the presence of cytosine (C) and therefore a TEM-1 (wild-type) codon at this position (box3). None of the other TEM probe sets identified a point mutation. Hence, the gene was identified as \(\text{bla}_{\text{TEM-1}}\). The SHV gene also was identified as the parental \(\text{bla}_{\text{SHV-1}}\) gene, as no mutation was identified. The CTX-M gene was identified as \(\text{bla}_{\text{CTX-M-15}}\) due to the specific mutations identified with the probe sets 77, 114, 140, 240, and 288 (compare theoretical perfect matches after hybridization with \(\text{bla}_{\text{CTX-M-1}}\) and the actual hybridization signals for Iso-37). The results for all 60 isolates are summarized in Table 1. In 54 isolates an ESBL gene variant was detected, whereas 6 isolates contained no TEM-, SHV-, or CTX-M-derived ESBL. All \(\text{bla}_{\text{TEM}}\) genes coded for TEM-1, meaning that no ESBL phenotype could be assigned to TEM. For SHV, a range of gene variants was detected covering SHV-1, SHV-12, and SHV-5. CTX-M-15 and CTX-M-3 were found as members of CTX-M group M1 and CTX-M-9, CTX-M-14, and CTX-M-14b as members of the group M9. SHV- and CTX-M ESBLs were detected in the following combinations: CTX-M (72.2%, 39/54), SHV (22.2%, 12/54), 2/SHV (3.7%, 2/54), and SHV plus CTX-M (1.9%, 1/54). SHV-12 was the most prevalent SHV-ESBL (84.6%, 11/13), CTX-M-15 the most frequently detected CTX-M-ESBL (81%, 34/42). SHV-ESBLs were found mainly in Klebsiella species (84%), CTX-M group M9 ESBLs in E. coli (87%), and CTX-M group M1 ESBLs in E. coli and Klebsiella at similar frequencies (55 and 41%). All DNA array results were confirmed by Sanger sequencing. The three E. coli isolates Iso-34, Iso-44, and Iso-53 did not harbor a TEM-, SHV-, or CTX-M ESBL gene and also were found to be negative in a separate PCR screening for the presence of a \(\text{bla}_{\text{CMY-2}}\)-like AmpC gene.

Table 1 also includes the ceftazidime (CAZ) and cefotaxime (CTX) MICs for the different clinical samples obtained by measurements with the Vitek 2 Gram-negative susceptibility card (AST-N062). SHV-5 and SHV-12 both feature the Glu240Lys mutation in combination with Gly238Ser, which is
TABLE 1. Genotyping of clinical isolates

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<th>No.</th>
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<th>bl$_{SHV}$</th>
<th>bl$_{CTX-M}$</th>
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<th>CTX</th>
<th>CAZ</th>
<th>CTX/CAZ</th>
<th>ESBL test</th>
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a Data were sorted first by the different genotypes detected and afterwards were clustered by species. Isolate numbers were used for labeling the blinded plasmid isolates; *, samples originally collected in Stuttgart; **, samples originally collected in Frankfurt. Samples Iso-27 and Iso-59 originated from the same patient but different specimens (Iso-27, urine; Iso-59, respiratory sample).

b Species were identified as described in Materials and Methods.

c Array results were validated by standard the DNA sequencing of the PCR product received.

d The Vitck ESBL test is FDA approved only for E. coli, K. pneumoniae, and K. oxytoca. Pos, positive; Neg, negative.
known to shift the substrate specificity toward ceftazidime (18).
Generally, a CTX/CAZ MIC ratio of <1 was observed for the
samples harboring those ESBLs. Typically, CTX-M enzymes
favor cefotaxime over ceftazidime as a substrate. This leads to
the observed CTX/CAZ ratio of >1 for the CTX-M ESBLs.
CTX-M-15 features the Asp240Gly mutation, which is thought
to increase activity against ceftazidime compared to that of
CTX-M-3,-M-9, or -M-14 (4, 5). This trend also was observed.
Only sample Iso-46 showed a deviant behavior with a CTX-
M-9 gene present and a CTX/CAZ ratio of <1. For the full
Vitek data set as well as standard susceptibility testing results,
see Table S2 in the supplemental material.
Chip performance. The performance of the ESBL chip was
assessed according to the distribution of RI\text{MM} values across
defined RI\text{MM} ranges, as displayed in Fig. 3. For the performance
analysis, all experiments leading to the same perfect
match signals were subsumed as replications. For the whole
ESBL chip, 94% of all MM signals had an RIMM of
match signals were subsumed as replications. For the whole
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FIG. 3. Performance of the whole chip (a) and individual chip modules (b). The distribution of RI\text{MM} values across different RI\text{MM} ranges is
displayed. The total amount of mismatch probes is 394 for the whole chip and 154, 135, and 105 for the TEM, SHV, and CTX-M chip modules,
respectively. Relative intensities of the probe sets are averaged from data of all clinical isolates harboring the TEM-1 (n = 38), SHV-1 (n = 10),
CTX-M-15 (n = 34), and CTX-M-14/M-9 (n = 3 to 8) genes.

With an increased prevalence of ESBL-producing pathogens,
the fast and accurate identification of such isolates becomes
increasingly clinically important. Despite a variety of
available methods, the identification of ESBLs by conventional
phenotypic methods remains difficult in practice due to the
large number of beta-lactamase variants, the association of
these enzymes with other antibiotics, and the change of outer-membrane permeability (28). Hence, molecular
techniques that have the potential to disentangle this com-
plicated resistance etiology and translate it into clinically useful
information have the potential to improve the current diag-
nostic practice. Multiplexing is a key requisite to cover this
task, and microarray platforms have been used repeatedly for
such purposes. Several studies described DNA microarrays
for the detection of various resistance gene panels, including some
beta-lactam resistance genes (11, 12, 25). These publications
used PCR products (200 to 800 bp) or long oligonucleotide
(70mer) probes (6, 17), which are not suitable for gene variant
discrimination at the single-nucleotide level. A more refined
chip was described by Batchelor and colleagues, who developed an oligonucleotide microarray that covered beta-lactam resistance genes with 14 probes (3). Most of their targets, including \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \), were detected on a family level using group-specific probes. The \( \text{bla}_{\text{CTX-M}} \) genes were detected on a subgroup level with three probes specific for CTX-M-1-like, CTX-M-2-like, and CTX-M-9-like genes. Another similar study recently reported a microarray-based method to detect...
bla<sub>TEM</sub>, bla<sub>SHV</sub>, and bla<sub>CTX-M</sub> in combination with plasmid-encoded AmpC beta-lactamase genes (54). The level of detection was also on the gene family level, plus six point mutations in the bla<sub>SHV</sub> gene. These studies all demonstrate strategies to set up diagnostic systems that quickly give a deeper insight into the genetic background of the beta-lactam resistance of a clinical isolate. In this study, we aimed to further extend these activities and cover all known gene variants of the three selected beta-lactamase families. As a result, the DNA microarray was able to genotype mutations within bla<sub>TEM</sub>, bla<sub>SHV</sub>, and bla<sub>CTX-M</sub> genes, which account for their classification into different variants and define their resistance spectra. As an example, the array was able to detect SHV-12 and SHV-5 in different Klebsiella and E. coli isolates. In a study that retrospectively correlated ESBL types, including SHV-12 and SHV-5, with treatment outcomes in bloodstream infections, it was found that the therapy failed when ESBL-producing strains were treated empirically with ceftazidime (51). If the respective clinician had had the right information before choosing this extended-spectrum cephalosporin, i.e., from a hospital infection control, knowing that this type of organism was present in the hospital, the type of empirical therapy could

![FIG. 5. Detection of mixed SHV and CTX-M genotypes. (a) Relative signals for probe sets SHV 35 and SHV 238/240. The displayed intensities were calculated by analyzing data for all isolates harboring SHV-1 (n = 7), SHV-12 (n = 4), or both (n = 7). Error bars represent the 95% confidence interval (CI) scores calculated. (b) Sequencing results at the relevant nucleotide positions for isolates containing bla<sub>SHV-1</sub> (Iso-37), bla<sub>SHV-12</sub> (Iso-24), or a mixture of both (Iso-2). Depending on the different sequences, the potential perfect match probes are indicated (PM). (c) Relative signals for group discrimination probe set CTX-M 267. The displayed intensities were calculated by analyzing data for all isolates harboring a CTX-M group M1 gene (n = 35), a CTX-M group M9 gene (n = 6), or both (n = 2). Error bars also represent the 95% confidence interval scores calculated.](#)
have been based on the molecular surveillance data obtained by the hospital, leading to alternative drug choices (e.g., imipenem [36]). With its short assay time, the ESBL array described in this study has the potential to provide such information faster than conventional susceptibility tests. At the time of design, the ESBL chip could identify all known mutations in the target genes. Naturally, such a DNA microarray would have to be updated as new mutations emerge and are described, but as we have experienced since our first study (19), this can be done with a reasonable amount of time and effort. With regard to the published literature, this progression currently is faster with the CTX-M type-beta-lactamases than the TEM- and SHV-type beta-lactamases. CTX-M-type enzymes are now the most prevalent beta-lactamase species worldwide (40).

The performance of the new integrated chip was tested with 60 blinded clinical samples, which were phenotypically pre-characterized mainly as ESBL positive (58 isolates). Using the phenotypic characterization as the gold standard, our assay showed 93% sensitivity (54 of 58) for the detection of ESBLs. DNA microarray results and DNA sequencing were 100% concordant. For four isolates, the resistance phenotype could not be explained by the presence of a TEM, SHV, or CTX-M ESBL gene. When calculating the sensitivity, it has to be considered that the phenotypic gold standard is contentious and also error prone for ESBL detection. For example, the MICs for CTX-M-15-positive E. coli isolate 33 were identical to those for other CTX-M-15-positive E. coli isolates (see Table S2 in the supplemental material), but a synergy-based Vitek ESBL test was negative. Additionally, CLSI recommendations and the Vitek ESBL test are valid only for E. coli, K. pneumoniae, K. oxytoca, and Proteus mirabilis, but not for other Gram-negative species like C. freundii or R. aquatilis. As a consequence, the sensitivity could be underestimated. Molecular methods like the described DNA microarray assay can be used for a wide range of species, including AmpC producers like C. freundii. As possible explanations for the phenotypic data from the three E. coli isolates 31, 44, and 53, we anticipate the presence of a plasmid-carried ampC gene (other than C. freundii-derived blaCMY genes, as they could be experimentally excluded), promoter mutations within the chromosomal ampC gene, metallo-beta-lactamases, alterations of the membrane composition, or rare beta-lactamases. The fourth false-negative sample contained a C. freundii strain that could harbor a derepressed chromosomal ampC gene. For all positive isolates, the substrate spectra connected to the identified gene variants and the phenotypically measured MIC data correlated very well. The increased hydrolytic activity against ceftazidime of the CTX-M-15 variant was especially apparent compared to that of most other CTX-M variants, such as CTX-M-3, which differs in only one point mutation from CTX-M-15. Since only a small number of ESBL-negative isolates were included, the DNA array achieved a theoretic specificity of 100%. However, this value needs to be seen in context and the performance to be confirmed in future studies including a larger number of negative isolates.

A comparison of the prevalence and spread of ESBL-producing Enterobacteriaceae in Europe (8) with the ESBL genes identified in this study revealed a high concordance with the current resistance situation. CTX-M enzymes have become more and more predominant, and the blaCTX-M genes also were by far the most prevalent ESBL genes found among the tested isolates. CTX-M-15 has risen to prominence all over Europe (27) and also was the most frequent CTX-M ESBL in our study. CTX-M-15 was detected in E. coli as well as in Klebsiella isolates. Within the SHV family, SHV-12 is one of the most prevalent enzymes and has been reported all over the world for K. pneumoniae (9). In our study, SHV-12 also was the most prevalent SHV ESBL and was detected exclusively in Klebsiella isolates. Interestingly, no TEM ESBL was identified. More than 60% of the tested isolates harbored a blaTEM gene, but all were identified as TEM-1 and thus not an ESBL. These findings for TEM and SHV indicate that the detection of a resistance gene family alone is not sufficient as a diagnostic parameter.

Conclusions. We described an integrated oligonucleotide microarray for the genotyping of TEM, SHV, and CTX-M beta-lactamase variants at the single-nucleotide level within 5 h and with a sensitivity of 93%. As it is, the DNA microarray provides an interesting tool for epidemiological studies or surveillance with unparalleled coverage. The integration of other resistance genes is under way and will further increase its information value. In addition, this study provides a key building block for the development of advanced antibiotic resistance diagnostics, such as microfluidic sample-to-answer systems, which integrate the microarray analysis with preanalytics and readout. Their introduction into the clinical routine could contribute to the prudent use of antibiotics and optimal treatment outcome.

Acknowledgments

This work was financed by the German Federal Ministry of Education and Research (BMBF) within the GenoMik (Genome Research on Microorganisms) project. We also thank our scientific partners within the project for helpful discussions, W. Witte and H. Höchler for supply of isolates, and our industry partner Eppendorf AG (Hamburg, Germany) for technical support and equipment.

References

9. Cantón, R., A. Novais, A. Valverde, E. Machado, L. Peixe, F. Baquero, and...


