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Development of a DNA Microarray for Detection and Identification of Fungal Pathogens Involved in Invasive Mycoses

Dirk M. Leinberger,[1] Ulrike Schumacher,[2] Ingo B. Autenrieth,[2] and Till T. Bachmann[1]*

Institute of Technical Biochemistry, University of Stuttgart, Stuttgart, Germany,[1] and Institute of Medical Microbiology and Hygiene, Eberhard-Karls-University, Tübingen, Germany[2]

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Invasive fungal infections have emerged as a major cause of morbidity and mortality in immunocompromised patients. Conventional identification of pathogenic fungi in clinical microbiology laboratories is time-consuming and, therefore, often imperfect for the early initiation of an adequate antifungal therapy. We developed a diagnostic microarray for the rapid and simultaneous identification of the 12 most common pathogenic *Candida* and *Aspergillus* species. Oligonucleotide probes were designed by exploiting the sequence variations of the internal transcribed spacer (ITS) regions of the rRNA gene cassette to identify *Candida albicans*, *Candida dubliniensis*, *Candida krusei*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida lusitaniae*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus*. By using universal fungal primers (ITS1 and ITS4) directed toward conserved regions of the 18S and 28S rRNA genes, respectively, the fungal ITS target regions could be simultaneously amplified and fluorescently labeled. To establish the system, 12 precharacterized fungal strains were analyzed; and the method was validated by using 21 clinical isolates as blinded samples. As the microarray was able to detect and clearly identify the fungal pathogens within 4 h after DNA extraction, this system offers an interesting potential for clinical microbiology laboratories.

Invasive fungal infections have emerged as major causes of morbidity and mortality in immunocompromised patients. Candidiasis and aspergillosis are the most common invasive fungal infections (IFIs) in patients receiving immunosuppressive treatment for cancer or organ transplantation. An IFI incidence of up to 23% has been reported among high-risk patients, such as patients with prolonged neutropenia following chemotherapy for acute leukemia or allogeneic transplant recipients, especially following nonmyeloablative transplantation (16, 17, 47). In addition to the increasing incidence of IFIs, the number of fungal species which must be considered as potential fungal pathogens has also increased during the last few decades. Application of antifungal agents against common fungal pathogens has led to the emergence of resistant species, e.g., *Candida krusei*, *Aspergillus terreus*, and members of the class *Zygomycetes* (26, 36, 54). The conventional identification of pathogenic fungi in clinical microbiology laboratories based on phenotypic features and physiological tests is time-consuming and, therefore, often imperfect for the early initiation of an antifungal therapy. Several human fungal pathogens are characterized by high rates of intrinsic resistance. Therefore, identification of a fungal pathogen to the species level rather than antifungal drug susceptibility testing is presently the most important step in the selection of the adequate antifungal agent (39).

Until now, a variety of molecular methods have been applied for the detection and identification of fungal pathogens, including species or group discrimination with specific primers (35), identification of PCR products by gel or capillary electrophoresis (4, 8, 44), restriction fragment length polymorphism analysis (53), single-strand conformational polymorphism (48), and Southern or slot blot hybridization assays with oligonucleotide probes (10, 12, 21, 25, 40). Furthermore, a number of PCR-enzyme immunoassays (EIAs) were developed (11, 13, 30, 32, 41). One study showed the applicability of PCR-EIA for the resolution of discrepancies in phenotype-based identification between different institutions (6). This PCR-EIA (11) discriminates the different fungal species by applying the variability of internal transcribed spacer (ITS) region 2 (ITS2). More recently, real-time PCR assays have been described for the quantitative detection of either *Candida* or *Aspergillus* species (19) in serum (3, 7, 50) or other specimen types (51). Diaz and Fell applied the Luminex technology to the detection of pathogenic yeasts of the genus *Trichosporon* (9). These previously described methods feature only limited multiplexing capability, resulting in high costs if all relevant species must be considered. An economically more efficient approach would be the application of a protocol which is capable of identifying a panel of relevant species in a highly parallel fashion.

It was the aim of the present study to develop a species identification array by making use of the high multiplexing capacity of DNA microarrays in the field of molecular detection for identification of human fungal pathogens. As targets, 12 *Candida* and *Aspergillus* species representing the pathogens that are the most frequently isolated from invasive fungal infections as well as species with known intrinsic resistance to antifungal agents were chosen.

* Corresponding author. Mailing address: Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, Stuttgart 70569, Germany. Phone: 49 (0) 711 685 3197. Fax: 49 (0) 711 685 3196. E-mail: Till.Bachmann@itb.uni-stuttgart.de.

MATERIALS AND METHODS

Microorganisms. Ten reference strains, obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; *Candida albicans* DSM 1386, *Candida dubliniensis* DSM 13268, *Candida krusei* DSM 3433, *Candida parapsi-*

TABLE 1. Oligonucleotide probe sequences[a]

Melting temp (°C)[b]	Length	Name[c]	Position[d]	Probe sequence (5′-3′)[e]
52.7	24	Calb1S	ITS1	AACTTGTCACACCAGATTATTACT
52.5	21	Calb2S	ITS2	CTTGAAAGACGGTAGTGGTAA
51.7	23	Cdub1S	ITS1	AACTTGTCACGAGATTATTTTTA
54.2	18	Cdub2S	ITS2	CTTGAAAGACGGTAGTGGTAA
52.0	22	Cpar1S	ITS1	AACTTGTCACGAGATTATTTTTA
51.2	24	Cgla1S	ITS1	TTGCTAAGGCGGTCTCTG
53.6	21	Ckru1S	ITS1	GCGGAGTATAAACTAATGGATA
50.8	23	Ctro1S	ITS1	TTACTACACACAGTGGAGTTTACT
53.5	23	Cgui1S	ITS1	CGAAAACAACAACACCTAAAA
52.3	18	Clus1S	ITS2	AAACCAAACTTTTTATTTACAGT
54.1	20	Afla1S	ITS1	GGAGACACCACGAACTCTGT
51.5	18	Afla2S	ITS2	CGAACGCAAATCAATCTT
53.6	18	Anig1S	ITS1	ACCCCAACACGAACACTG
54.3	20	Anig2S	ITS1	CCAACACGAACACTGTCTGA
51.6	21	Ater1S	ITS1	AGTCTGAGTGTGATTCTTTGC
52.1	21	Ater2S	ITS1	GTCTGAGTTGTGATTCTTTGC
52.5	19	Afum1S	ITS2	CCGACACCCAACTTTATTT
52.3	17	Can1S	5.8S rRNA gene	TCTTGGTTCTCGCATCG
51.3	17	Can2S	5.8S rRNA gene	ATGCCTGTTTGAGCGTC
54.1	16	Asp1S	5.8S rRNA gene	ATGCCTGTCCGAGCGT
53.0	16	Asp2S	ITS1	CGGCTTGTGTGTTGGG
53.8	17	PC1S	18S rRNA gene	GTGAACCTGCGGAAGGA
51.8	17	PC2S	5.8S rRNA gene	GCATCGATGAAGAACGC
51.0	17	PC3S	18S rRNA gene	TCCGTAGGTGAACCTGC
48.9	18	Negative hybridization control		TCTAGACAGCCACTCATA
61.7	19	Positive hybridization control		GATTGGACGAGTCAGGAGC
48.9	18	Spotting control		TCTAGACAGCCACTCATA-Cy3

[a] For each species- or genus-specific probe or process control, only the sense version of the probe is shown.
[b] Calculated with Oligo (version 6.65; Molecular Biology Insights) and the parameters described.
[c] S, sense; the name of the corresponding "antisense"probe uses AS (e.g., Calb1AS).
[d] Position within the amplified target region.
[e] The sequence of the 14-thymidine spacer at the 5′ end is not shown.

losis DSM 5784, *Aspergillus fumigatus* DSM 819, and *Aspergillus niger* DSM 1988) and the American Type Culture Collection (ATCC; *Torulopsis glabrata* ATCC 90030, *Candida tropicalis* ATCC 750, *Candida guillermondii* ATCC 6260, and *Aspergillus flavus* ATCC 20043), as well as two clinical isolates from the Institute of Medical Microbiology and Hygiene, University of Tübingen (*Candida lusitaniae* VA 13910 and *Aspergillus terreus* H15), were used as precharacterized strains. Additionally, 21 clinical isolates, including 15 *Candida* strains belonging to 11 species, 4 *Aspergillus* strains belonging to 3 species, and 1 strain each of *Saccharomyces cerevisiae* and *Pseudallescheria boydii* from the Institute of Medical Microbiology and Hygiene, University of Tübingen, were included in this study (see Table 2) and were used as blinded samples. All clinical isolates were identified by standard laboratory procedures, including macro- and micromorphologies, germ tube formation, growth characteristics at different temperatures, and assimilation tests (ID 32C; Biomerieux).

DNA extraction. Prior to DNA extraction, the fungal strains were cultured on Sabouraud dextrose agar at 30°C for 42 h (yeasts) or 72 h (molds). *Candida* and other yeast colonies were inoculated in sterile saline. Mold cultures were washed with 10 ml of sterile saline (0.9%) to obtain conidia. The fungal suspensions were centrifuged at 3,000 × *g*, and then the pellets were incubated with 1 ml of leukocyte lysis buffer consisting of 10 mM Tris (pH 7.6), 10 mM EDTA (pH 8.0), 50 mM NaCl, 0.2% sodium dodecyl sulfate, and 200 μg of proteinase K (Roche Diagnostics, Penzberg, Germany) per ml at 65°C overnight. The suspensions were again centrifuged (3,000 × *g*), and the pellets were exposed to three cycles of freezing in liquid nitrogen for 30 s and heating at 95°C for 4 min to disrupt the fungal cells. Thereafter, the pellets were resuspended and incubated in 1,000 μl recombinant lyticase (L4276; Sigma) at 37°C for 45 min. After centrifugation at 1,600 × *g*, the DNA was extracted by using a QIAmp DNA mini kit (QIAGEN, Hilden, Germany).

Primers. Oligonucleotide primers were obtained from Sigma-Genosys, Steinheim, Germany. Amplification of the ITS1 and ITS2 regions was performed with universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′), as described by White et al. (52). The sequence of ITS1 is complementary to a conserved region at the end of the 18S rRNA gene, and ITS4 binds to a conserved region at the beginning of the 28S rRNA gene, leading to amplification of the ITS regions and the 5.8S rRNA gene, which is located between the noncoding ITS regions. ITS1- and ITS4-specific primers were also used for sequencing of the purified amplicon. Additionally, the primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) and ITS3 (5′-GCATCG ATGAAGAACGCAGC-3′) were used for sequencing, leading to overlapping fragments.

DNA sequencing. For DNA sequencing, the full ITS region was amplified by PCR in a final reaction volume of 50 μl. Each reaction mixture contained approximately 100 ng template DNA; 0.4 pmol (each) forward (ITS1) and reverse (ITS4) primers; 100 μM (each) dATP, dCTP, dGTP, and dTTP; 1× Expand High Fidelity reaction buffer containing 1.5 mM $MgCl_2$ (Roche); 2.5% dimethyl sulfoxide (Fluka Chemie, Buchs, Switzerland); and 2.6 U of Expand High Fidelity DNA polymerase (Roche). The amplification was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany). An initial denaturation step (94°C for 5 min) was followed by 35 cycles (with each cycle consisting of DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 1 min) and a final extension step at 72°C for 7 min. A no-template negative control was included in each PCR run. The PCR product was purified with a QIAquick Spin PCR purification kit (QIAGEN), according to the protocol of the manufacturer. The DNA was eluted in 30 μl of double-distilled H_2O (ddH_2O). The purified PCR products were sequenced with the primers ITS1, ITS2, ITS3, and ITS4 and the BigDye Terminator cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). Sequencing was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems). All fungal strains were sequenced in both directions with two primers each, resulting in four overlapping fragments. The sequence data were assembled by using SeqMan II software (version 5.00; DNAStar, Madison, Wis.). The sequences obtained have been submitted to GenBank.

Capture probe design. All species- or genus-specific oligonucleotide probes and process controls were designed from ITS sequences available in the GenBank database (EMBL and DDBJ databases). Multiple-sequence alignments were carried out by using ClustalX software (42) or SeqMan II software (version 5.00; DNAStar). By comparison of the sequences of the ITS1 and ITS2 regions of the target species, regions with interspecies variations could be identified and

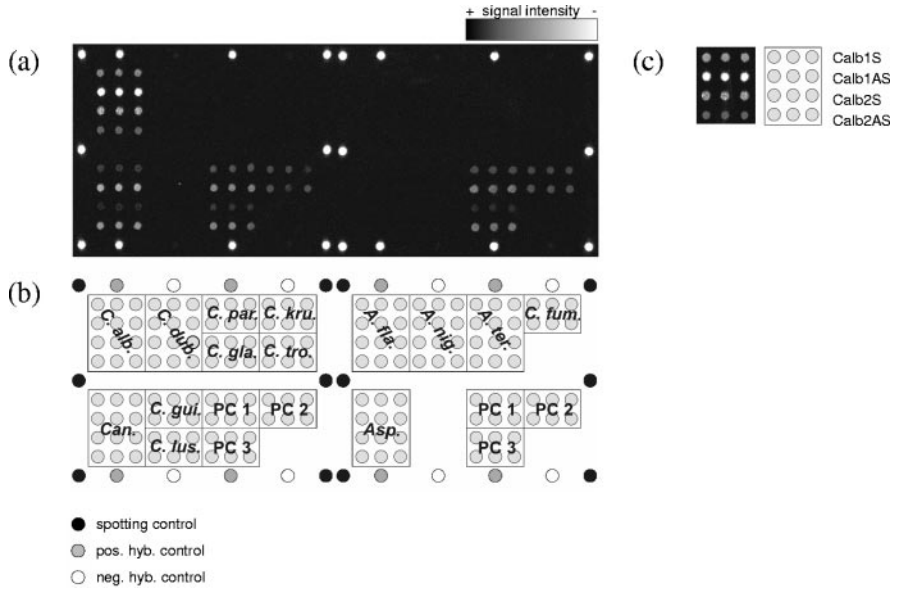


FIG. 1. (a) Fluorescence image after hybridization with 0.5 pmol of *C. albicans* isolate VA 115839-03 target DNA for 1 h at 53°C. The signal intensity is encoded in the 65,636 gray scales of the 16-bit TIF image. (b) Layout of the capture probes on the array. All species- and genus-specific probes and the process controls were spotted in triplicate. The other control probes are positioned at the corners of each subarray. Each subarray contains a set of process controls. pos. hyb., positive hybridization, neg. hyb., negative hybridization. (c) The enlarged results for the *C. albicans* probes describe the arrangement of different probes for the same species or genus within the indicated fields of the array.

were used to develop species-specific probes. Conserved regions served either as targets for probes which are able to discriminate between *Candida* and *Aspergillus* (genus-specific probes) or as process controls. In addition to the position of the probe within the target region, thermodynamic parameters were considered in order to get a set of probes with at least similar hybridization efficiencies. Oligonucleotide probes of various lengths (16 to 24 bases) but within a very narrow range of melting temperatures (T_ms; ±3.5°C) were designed. The T_ms of the probes (Table 1) and the stabilities of possible secondary structures were calculated with the software Oligo (version 6.65; Molecular Biology Insights, Cascade, Colo.) by using the following parameters and thresholds: 50 mM univalent ions, 1.5 mM free Mg^{2+}, and 250 pM DNA for T_m calculation; desired stability of hairpins, $\Delta G > 0$ kcal/mol (where G is Gibb's free energy); and desired stability of duplexes, $\Delta G > -4$ kcal/mol. All probes were designed as sense and antisense probes. To check the specificities of the probes, additional database searches were performed by using the BLAST and FASTA programs to compare the probe sequences with the sequences in the EMBL and GenBank DNA databases. The sequences of the probes developed are shown in Table 1.

Oligonucleotide array fabrication. Oligonucleotide arrays were constructed with 51 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 species- or genus-specific capture probe had a 14-thymidine spacer and an amino modification at the 5′ end and was spotted onto the microarray in triplicate. The array production process and the subsequent washing steps were carried out as described previously (15), with modifications. In brief, by using a MicroGrid II microarrayer with two MicroSpot 2500 pins (BioRobotics, Cambridge, United Kingdom), the oligonucleotide probes, which were dissolved in spotting buffer (160 mM Na_2SO_4, 130 mM Na_2HPO_4) to a final concentration of 20 μM (prelabeled spotting control; 10 μM), were spotted onto epoxy-coated glass slides (Elipsa, Berlin, Germany). Covalent immobilization was achieved by incubating the oligonucleotide array at 60°C for 30 min in a drying compartment (Memmert, Schwabach, Germany). After the probes were spotted, the slides were rinsed for 5 min in 0.1% (vol/vol) Triton X-100 in ddH_2O, 4 min in 0.5 μl of concentrated HCl per ml of ddH_2O, 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, 0.5 μl of concentrated HCl per ml of ddH_2O), with the spotted side facing upwards, at 50°C in a heating compartment (OV5; Biometra, Göttingen, Germany); rinsed in ddH_2O for 1 min; and finally, dried under a flow of nitrogen. The spot size and the spot-to-spot distance were estimated to be 160 μm and 320 μm, respectively. The processed slides were stored dry for a maximum of 20 days at room temperature in the dark until further use.

Controls. In addition to the species- and genus-specific capture probes, each array also included several controls: a prelabeled spotting control (5′-TTTTTTTTTTTTTTCTAGACAGCCACTCATA-cyanine3 [Cy3]-3′); a positive hybridization control (5′-TTTTTTTTTTTTTGATTGGACGAGTCAGGAGC-3′) complementary to a labeled oligonucleotide target (5′-Cy3-GCTCCTGACTCG TCCAATC-3′), which was spiked during hybridization; and a negative hybridization control (5′-TTTTTTTTTTTTTTCTAGACAGCCACTCATA-3′). All these control sequences are unrelated to sequences found in fungi. Three process controls (PC1 to PC3; Table 1) were designed by using universal fungal sequences. Additionally, the process controls were designed as sense and antisense probes. The spotting controls were set on the side positions of each subgrid spotted with the same pin. The positive and negative hybridization controls were spotted alternately at the upper and lower edges of each subarray. The process controls were spotted within both subgrids (Fig. 1a).

Amplification, labeling, and purification of target DNA. The target DNA used for hybridization on the oligonucleotide arrays was synthesized by PCR. For amplification and labeling, 100 ng of template DNA was supplemented with 0.4 pM (each) forward (ITS1) and reverse (ITS4) primers; 100 μM (each) dATP, dGTP, and dTTP; 50 μM dCTP; 50 μM Cy3-dCTP (ratio of unlabeled dCTP/labeled dCTP, 1:1; Amersham Biosciences, Freiburg, Germany); 1× Expand High Fidelity reaction buffer containing 1.5 mM $MgCl_2$ (Roche); 2.5% dimethyl sulfoxide (Fluka Chemie); and 2.6 U of Expand High Fidelity DNA polymerase (Eppendorf, Hamburg, Germany) in a total volume of 50 μl. The amplification was performed in a Mastercycler gradient (Eppendorf). The reaction profile was as follows: 5 min of initial denaturation at 94°C; 35 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 1 min; and a final extension step at 72°C for 4 min. A no-template negative control was included in each PCR run. After purification of the PCR product with a QIAquick Spin PCR purification kit (QIAGEN), the rate of incorporation of Cy3-dCTP, expressed as number of nucleotides/number of incorporated fluorescent dyes (NT/F), was determined by measurement of the optical density (ND-1000 spectrophotometer; NanoDrop Technologies, Rockland, Maine).

Hybridization. The purified target DNA (0.5 pmol) together with control DNA (Cy3-GCTCCTGACTCGTCCAATC; 0.05 pmol) was hybridized within a gene frame (15 by 16 mm) closed with a coverslip (Abgene House, Hamburg, Germany) in 70 μl of 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]). Prior to hybridization, the hybridization mixture was incubated for 10 min at 95°C, stored for 1 min on ice, and immediately used. For

hybridization with amplified target DNA from the 12 precharacterized strains, the DNA solution on the glass slide, within the gene frame, was incubated in a thermomixer with an exchangeable thermoblock for slides (Eppendorf) for 2 h at 50°C and at an agitation speed of 1,200 rpm. In order to improve the hybridization conditions, hybridizations with target DNA from a subset of target species were also carried out for 2 h at 53°C, 55°C, and 57°C. During the evaluation process, target DNA amplified from the 21 blinded samples was hybridized for 1 h at 53°C with agitation at 1,200 rpm. The reduction of the assay time was due to previous optimization experiments (data not shown). After hybridization, the slides were washed with 2× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate, then 2× SSC, and finally, 0.2× SSC for 10 min each time. The washing procedure was performed at room temperature with agitation in a glass container. The slides were subsequently dried with N_2.

Data acquisition and processing. After the hybridization reaction, the data from the oligonucleotide arrays were read by acquisition of the fluorescence signals by using a 418 Array Scanner (Affymetrix, Santa Clara, Calif.). The laser power and gain were adjusted to 100%. Image processing and calculation of signal intensities were performed with ImaGene (version 3.0; Biodiscovery, Los Angeles, Calif.). For calculation of the individual net signal intensities, the local background was subtracted from the raw spot intensity value. Further data processing was performed by using Microsoft Excel software (Microsoft, Richmond, Wash.). Only net signal intensities which were significantly higher than the background were considered. Therefore, a cutoff 1 of 300 intensity units (three times the detection limit of the array scanner) was applied. By definition, all values less than 300 were set equal to 0. For normalization, the net signal intensity of each spot was divided by the mean net signal intensity of the three replicates of the antisense version of process control 3 (referred to as "signal intensity" [I] of PC3 antisense) and multiplied by 100. All other process control probes were found to be unsuitable for data processing and were not used. The mean of the three normalized net signal intensities (referred to as the "relative signal intensity" [RI] of one probe) and the standard deviation of those values were calculated. Accordingly, the probe PC3 antisense always had an RI value of 100%. To discriminate between specific and unspecific hybridization signals, a cutoff 2 was defined. Only RI values which were significantly greater than 10% (RI > 10 + three times the standard deviation of the normalized net signal intensities of one probe) of the signal intensity of the process control 3 antisense were considered specific signals.

Nucleotide sequence accession numbers. The accession numbers of the sequences deposited in GenBank are AY939782 (*A. flavus* ATCC 20043), AY939783 (*A. niger* isolate ST 717-04), AY939784 (*A. niger* isolate VA 3590-04), AY939785 (*A. flavus* isolate VA 103936-04), AY939786 (*C. albicans* ATCC 10231), AY939787 (*A. niger* ATCC 16404), AY939788 (*A. terreus* isolate H15), AY939789 (*C. albicans* isolate ST 3477-03), AY939790 (*A. fumigatus* ATCC 9197), AY939791 (*C. albicans* isolate VA 115470-03), AY939792 (*C. guilliermondii* ATCC 6260), AY939793 (*C. glabrata* ATCC 90030), AY939794 (*C. glabrata* isolate VA 104009-04), AY939795 (*C. guilliermondii* isolate UR 9406-03), AY939796 (*C. krusei* isolate ST 3382-03), AY939797 (*C. lusitaniae* isolate VA 115231-03), AY939798 (*C. parapsilosis* ATCC 22019), AY939799 (*C. norvegensis* isolate ST 3481-03), AY939800 (*C. pelliculosa* isolate ST 3352/2-03), AY939801 (*C. tropicalis* isolate UR 9344-03), AY939802 (*P. boydii* isolate VA 103543-04), AY939803 (*C. parapsilosis* isolate VA 115230-03), AY939804 (*A. fumigatus* VA 104001-04), AY939805 (*C. dubliniensis* isolate ST 2792-03), AY939806 (*C. kefyr* isolate VA 116042-03), AY939807 (*C. albicans* isolate VA 115839-03), AY939808 (*C. krusei* ATCC 24210), AY939809 (*C. dubliniensis* isolate R2), AY939810 (*C. tropicalis* ATCC 750), AY939811 (*C. lusitaniae* VA 13910-03), AY939812 (*C. lusitaniae* isolate ST 3324-03), AY939813 (*C. dubliniensis* isolate VA 103469-04), AY939814 (*S. cerevisiae* isolate ST 3352/1-03), and DQ105856 (*C. dubliniensis* DSM 13268).

RESULTS

We constructed an oligonucleotide microarray for the rapid and simultaneous identification of the most common pathogenic *Candida* and *Aspergillus* species. By using the internal transcribed spacer regions of the rRNA gene complex as the diagnostic region, probes were designed against either the ITS1 region or the ITS2 region, or both (Table 1). By exploiting the full sequence of the amplicon obtained with primers ITS1 and ITS4, the sequence of the central 5.8S rRNA gene was used to design either genus-specific probes or process controls. The conserved region at the end of the 18S rRNA gene was used for the design of process controls. The size of the PCR product generated with those primers, using extracted genomic DNA as the template, varied, depending on the species, from 382 bp (*C. lusitaniae*) to 882 bp (*C. glabrata*). The ITS regions of *A. fumigatus*, *A. niger*, and *A. terreus* were amplified by adding 2.5% of dimethyl sulfoxide to resolve the secondary structures. The fluorescent label (Cy3) was incorporated during PCR. The NT/F ratios varied from 80 to 220, depending on the quality of the template DNA and on the species.

Setting up the system and proof of concept. To examine the specificities and hybridization efficiencies of the capture probes that were designed, to optimize hybridization conditions, and to establish the quantification and subsequent data processing, the array was tested by using 12 precharacterized strains, 1 strain of each target species. By hybridization for 2 h at 50°C, different hybridization patterns for the different species could clearly be seen on the microarray. Figure 1 shows the fluorescence intensities of the probes after hybridization with the *C. albicans* DSM 1386 target DNA. The hybridization efficiencies of the sense and antisense versions of one probe often showed big differences. At least one of the two versions showed a high hybridization efficiency, with fluorescent net signal intensities ranging from 21,000 to 55,400 intensity units (with species-specific probes) or 6,600 to 37,500 intensity units (with genus-specific probes). By using epoxy-coated glass slides in combination with the washing procedures described above, backgrounds which allowed the use of a low cutoff, 1 of 300 intensity units, could be achieved. Weak cross hybridizations (~1,000) were observed only between the *A. niger*-specific probe Anig1 antisense and the *A. fumigatus* or *A. terreus* target DNA. This was expected, as there is no sequence difference between *A. fumigatus* and *A. terreus* at the position within the ITS1 region to which the probe Anig1 antisense binds. The second *A. niger*-specific probe, Anig2 sense, showed weak but significant signals (I, ~400) only with *A. fumigatus*. Furthermore, we included in the array layout three process controls which were designed to be specific for universal fungal sequences with the aim of using one as an internal standard. The assumption was that the intensity of a process control would be similarly dependent on the parameters of the experiment (function of the labeling PCR, hybridization conditions), as was the case for the other probes on the array. To rate the suitability of the three different process controls as an internal standard, the relative signal intensities of certain probes calculated with different process controls on different slides were compared (data not shown). Due to the small deviations of the relative signal intensities on different slides with probe PC3 antisense, for calculation of the RI values it fulfilled the requirements for a normalization procedure and was chosen as the internal standard. Figure 2 shows the quantified data sets of all probes after hybridization with the labeled target DNA of the different precharacterized strains by using PC3 antisense for normalization and cutoff 2. This cutoff was chosen as a consequence of the weak cross hybridizations observed. By using this threshold, every target species could be unequivocally identified.

Optimization of hybridization conditions. To optimize the assay system, the performance of the probes during hybridiza-

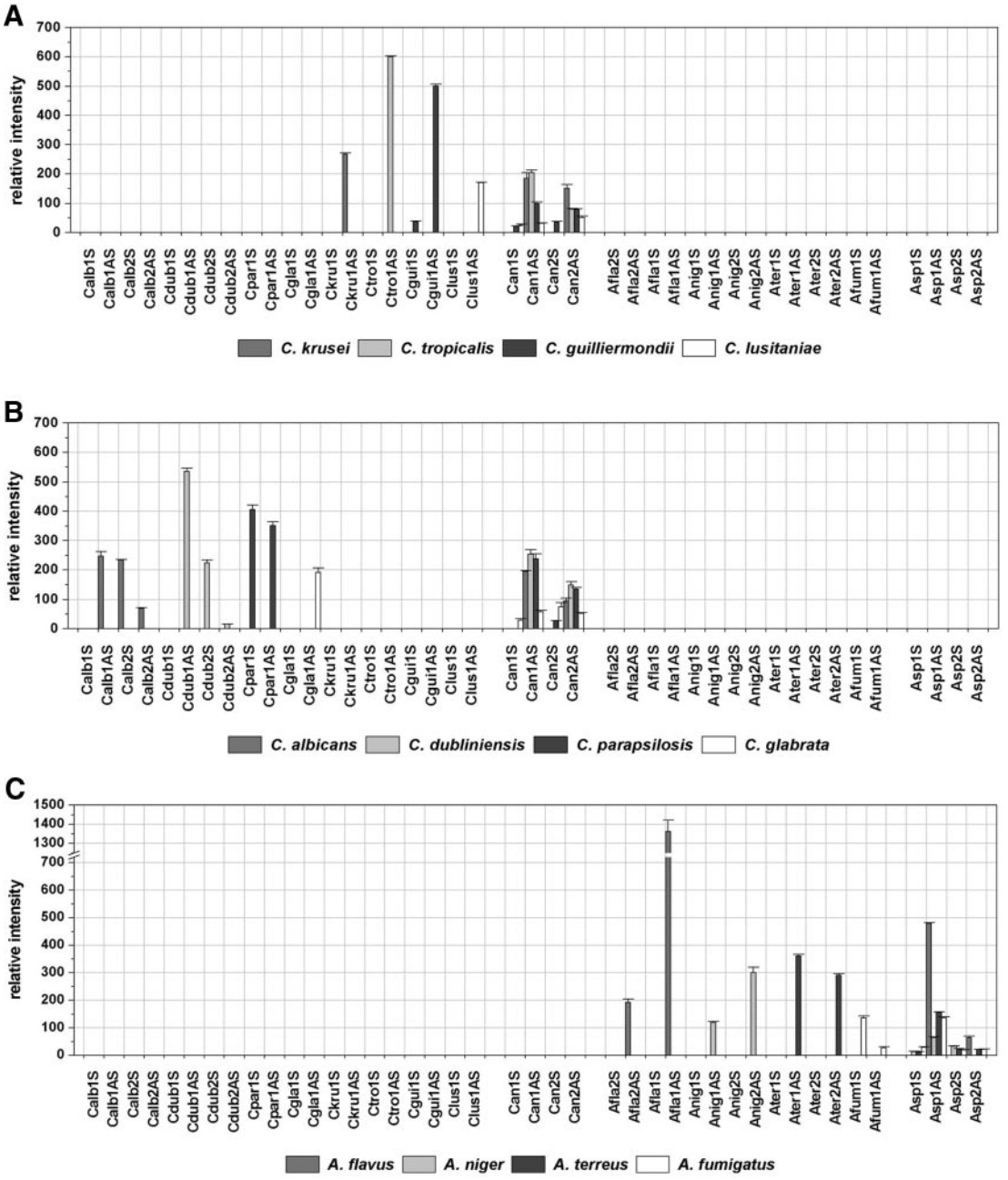


FIG. 2. Relative intensities after hybridization with labeled target DNA from the 12 target species. Cutoff 1, I = 300; cutoff 2, RI = 10. The mean RIs and their standard deviations are calculated for the triplicate spots on one slide (n = 3).

tion was examined under more stringent hybridization conditions. This was achieved by raising the hybridization temperature. The aim of these experiments was the optimization of the discrimination of target species by not loosing too much net signal intensity (and, therefore, sensitivity). *A. fumigatus* was representative in both aspects (the sole species which showed cross hybridization to other species-specific probes and which had relative low net signal intensities) and was chosen as the model target species. The increase in the temperature gradually resulted in a decrease of the net signal intensities of the specific signals (Fig. 3a) as well as of the unspecific signals (Fig. 3b). The most significant decrease was observed by raising the temperature from 50 to 53°C. The relative signal intensities of the species-specific probe Afum1 sense and the genus-specific probe Asp1 antisense had maxima at 53°C and 55°C, respectively (Fig. 3c). The highest unspecific signal had a minimum at 53°C (Fig. 3d). The concordance of the optimum of the specific signal of Afum1S and the minimum of the unspecific signal of Anig1AS at 53°C indicated that the assay would have the strongest discrimination power at this hybridization temperature. Consequently, all following hybridizations were performed at 53°C. Further experiments with *A. fumigatus*, *C.*

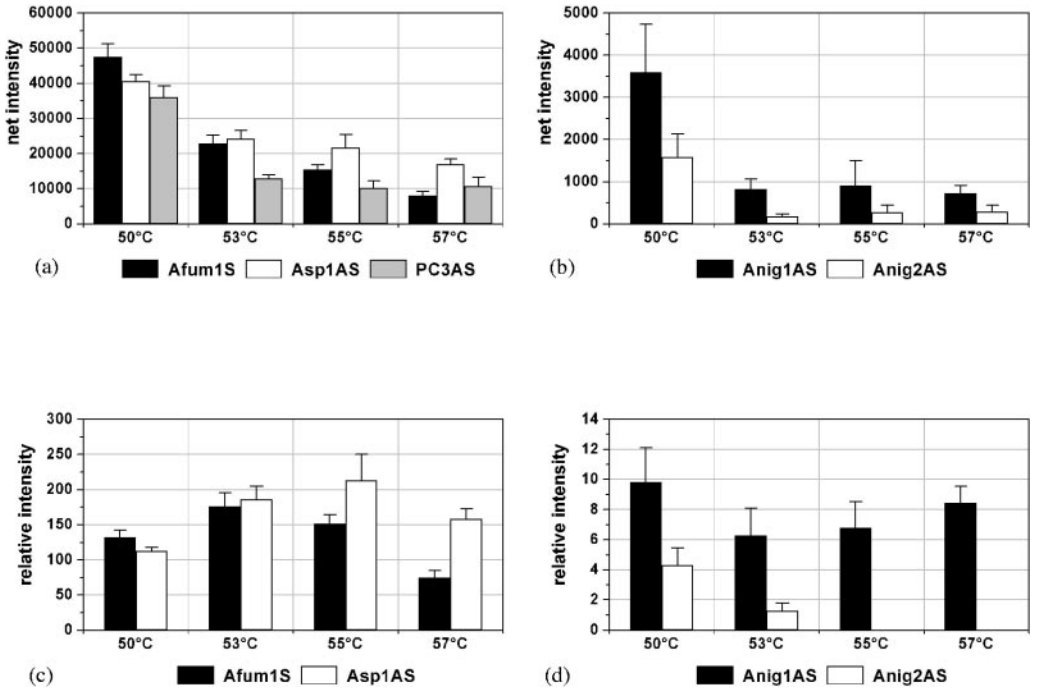


FIG. 3. Behaviors of I and RI of the species-specific probe Afum1 sense, the genus-specific probe Asp1 antisense, the process control PC3 antisense (a and c), and the *A. niger*-specific probes Anig1 antisense and 2 antisense (b and d) after hybridization with 0.5 pmol labeled *A. fumigatus* DSM 819 target DNA for 2 h at different hybridization temperatures. The columns depict the mean net signal intensities or the mean relative signal intensities and their standard deviations over three slides of the same experiment ($n = 9$).

lusitaniae, and *C. krusei* DNA showed the assay time could be reduced by reducing the hybridization duration from 2 to 1 h (data not shown).

Testing of clinical isolates. To examine the specificity of the designed probes and to assess their applicability for clinical isolates, the performance of the array was validated by using 21 blinded samples containing genomic DNA of clinical isolates of either the target species or fungal nontarget species. The application of nontarget species served as a cross-reactivity test. The array result was compared in each case to the result of the direct sequencing of the PCR product amplified out of the same sample in a separate PCR by using the same primers used for the labeling PCR. The identities of the sequences obtained were determined by performing searches of the sequences with the sequences in the GenBank DNA database by using the BLAST program. Additionally, the sequences were analyzed by comparison to the sequences used for probe design. By comparing the array results and the results of the sequencing to the previous identification obtained by standard methods, the array was able to unequivocally identify the contents of 16 samples (Table 2). Due to the absence of species-specific signals and the presence of genus-specific and control signals, four other isolates were identified as nontarget species by the array. In those cases the sequencing led to the identification of the fungal species and confirmed the array result. In the case of sample ST 3481-03, the species-specific probe for *C. krusei*, probe Ckru1AS, showed a weak hybridization signal with an RI value of 14 (Fig. 4e). Although this probe showed much higher RI values when with the *C. krusei* DSM 3433 strain (RI = 270; Fig. 2b) or isolate ST 3382-03 (RI = 511; Fig. 4a) was hybridized, we interpreted the result as specific, according to the global cutoff 2 described above. Sequencing identified the fungal content as *C. norvegensis*, which was concordant with the conventional identification results (Table 2). Accordingly, the sequence analysis revealed a 15-bp match between the *C. norvegensis* sequence and *C. krusei*-specific probe Ckru1AS. Regarding the full ITS amplicon, the sequences of the *C. krusei* DSM 3433 strain and the *C. norvegensis* isolate ST 3481-03 showed 81% homology, with high homology within the central 5.8S rRNA gene and sequence variations concentrating within the ITS regions. Other isolates also showed minor differences in sequence compared with those of the precharacterized strains either within regions not used for probe design (isolates ST 3382-03 and VA 104001-04) or within the diagnostic position (isolate VA 115231-03). Nevertheless, the correct identification of those isolates was possible, proving the robustness of the array (Fig. 4a, c, and d; Table 2). The fluorescence intensities of the probes quantified after hybridization with the labeled target region of the different isolates are shown in Fig. 4.

DISCUSSION

With the development of more aggressive therapeutic regimens, especially for the treatment of hematological malignancies, the incidence of invasive opportunistic yeast and mold infections has increased. The early initiation of antifungal therapy is critical in reducing the high mortality rate in patients with IFIs. Early and accurate identification of the fungal pathogen is the most important and critical step in providing ade-

TABLE 2. Evaluation of the array with blinded samples

No.	Isolate[b]	Isolate by conventional identification method	Array result	Sequencing result[a]
1	*C. albicans*	ST 3477-03	*C. albicans*	+
2	*C. albicans*	VA 115470-03	*C. albicans*	+
3	*C. albicans*	VA 115839-03	*C. albicans*	+
4	*C. dubliniensis*	ST 2792-03	*C. dubliniensis*	+
5	*C. dubliniensis*	VA 103469-04	*C. dubliniensis*	+
6	*C. glabrata*	VA 104009-04	*C. glabrata*	+
7	*C. guillermondii*	UR 9406-03	*C. guillermondii*	+
8	*C. krusei*	ST 3382-03	*C. krusei*	+
9	*C. lusitaniae*	ST 3324-03	*C. lusitaniae*	+
10	*C. lusitaniae*	VA 115231-03	*C. lusitaniae*	+
11	*C. parapsilosis*	VA 115230-03	*C. parapsilosis*	+
12	*C. tropicalis*	UR 9344-03	*C. tropicalis*	+
13	*Candida kefyr*	VA 116042-03	Nontarget species	*Candida kefyr*
14	*Candida norvegensis*	ST 3481-03	*C. krusei*	*Candida norvegensis*
15	*Candida pelliculosa*	ST 3352/2-03	Nontarget species	*Candida pelliculosa*
16	*A. fumigatus*	VA 104001-04	*A. fumigatus*	+
17	*A. niger*	ST 717-04	*A. niger*	+
18	*A. niger*	VA 3590-04	*A. niger*	+
19	*Aspergillus spp.*[c]	VA 103936-04	*A. flavus*	+
20	*Pseudallescheria boydii*	VA 103543-04	Nontarget species	*Pseudallescheria boydii*
21	*Saccharomyces cerevisiae*	ST 3352/1-03	Nontarget species	*Saccharomyces cerevisiae*

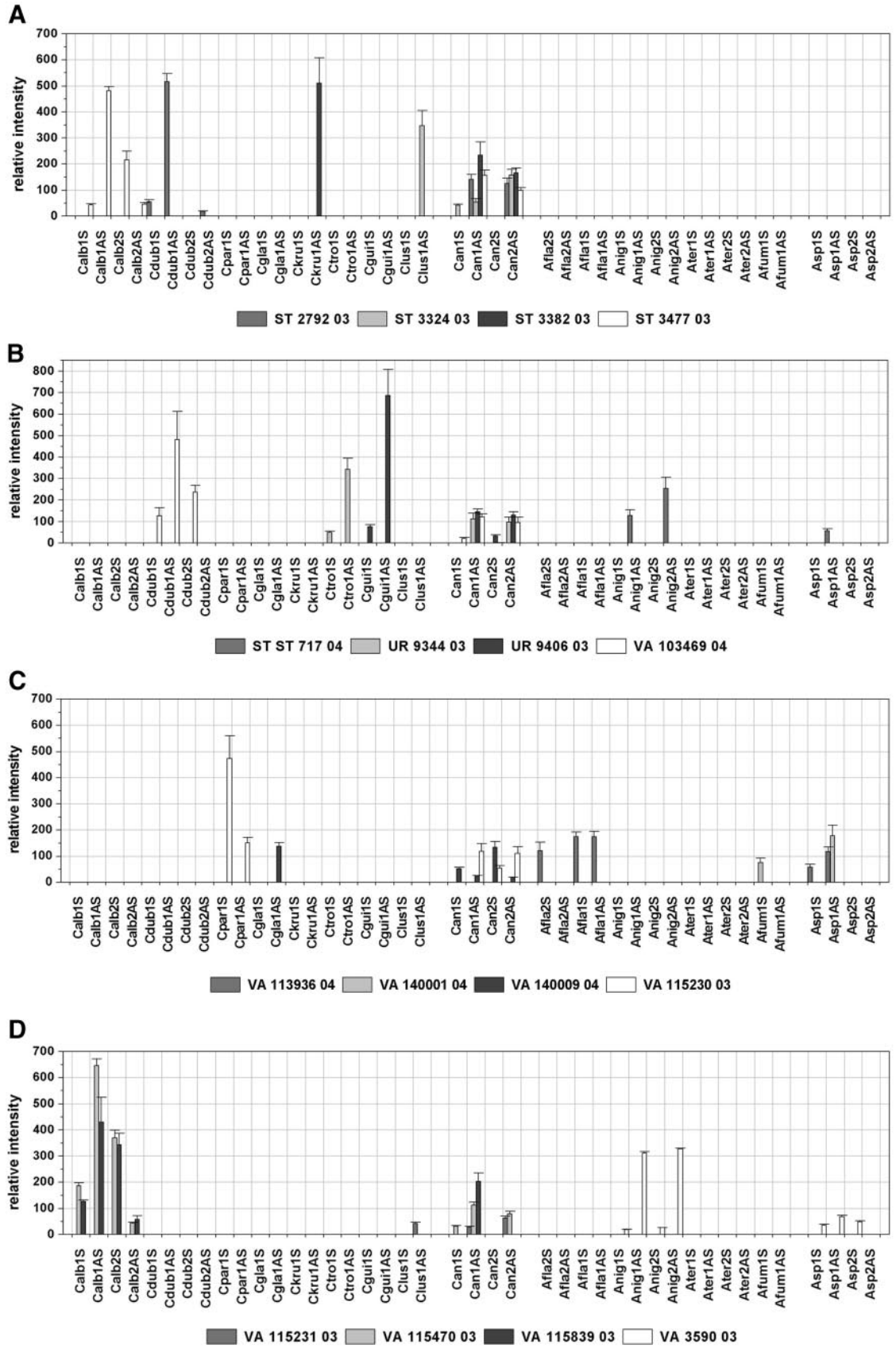
[a] +, concordance with the array result.
[b] Clinical isolates were identified by standard laboratory procedures.
[c] The content of this sample could not be identified to the species level by the described methods.

quate antifungal treatment in time. The conventional method of identification of pathogenic fungi used in clinical microbiology is based on phenotypic features and physiological tests and is therefore time-consuming. Instead, molecular genotyping methods could provide a rapid and specific means of identification of fungal pathogens. In view of the increasing number of fungal species isolated from clinical samples as potential pathogens, a broad-spectrum detection system as it is represented by the microarray technology would be desirable. Until today, diagnostic DNA microarrays were applied for the identification of viruses (5, 27–29, 56), bacteria (14, 24, 45, 46, 49), and mechanisms of resistance to certain antibiotics (15, 18, 55). To our knowledge, this study describes for the first time the application of the DNA microarray technology in combination with a quantification and data-processing method for the rapid and reliable detection of fungal pathogens. To achieve this, oligonucleotide capture probes were designed by exploiting the variations in the sequences of the ITS regions in a way similar to that described by other research groups (20, 23). The ITS regions were chosen as the target for several reasons: (i) as part of the rRNA gene complex, the ITS regions are present in numerous copies in the fungal genome; (ii) universal fungal primers based on the conserved regions of the rRNA genes are available; and (iii) the level of variability of the ITS regions of the target species is higher than that of the rRNA genes and is high enough for species resolution.

A frequent challenge in DNA microarray-based species detection studies is the discrimination between specific and unspecific signals, especially in the case of mixture analysis. Cross hybridization was observed in several identification microarray studies (1, 46), which made it necessary to design more than one probe per species (2). For this reason, we introduced a simple normalization procedure based on process controls. While the first cutoff in the study presented here was basically related to the background intensity, cutoff 2 was determined by the intensity value normalized on the basis of the process control for the most severe cross hybridization. The use of a global and very stringent cutoff indicates the possibility of misidentifications, as was found in the case of *C. krusei* and *C. norvegenis*. On the other hand, this low threshold left a lot of space between the cutoff 2 and the relative signals for the most efficient species-specific probes (well above 100) and for the genus-specific probes (from 50 to 250). For this reason, the use of an individual cutoff seems more appropriate for future studies, as this option would offer the possibility to take the sequence similarities of closely related species into account. Naturally, such an approach would require the analysis of larger numbers of samples in order to better reflect sequence variations in the clinical routine. On the other hand, the stringent cutoff offers the advantage of a higher resolution for samples with mixtures of microorganisms. Future studies will show at which ratios different species can be detected by using the present cutoff definition or if a modification will be necessary.

Two aspects of the specificity of the detection system can be discussed. First, the misidentification of sample ST 3481-03 as *C. krusei* due to sequence similarities between *C. krusei* and *C. norvergensis* suggests the need for the design of an additional *C. krusei*-specific probe which would be able to distinguish between these two species. Second, the big difference between the RI value of the perfect match and the cross hybridization would allow an individual cutoff 2 higher than 10 for *C. krusei*-specific probe Ckru1AS instead of a universal one. Nevertheless, a considerable robustness was demonstrated for an isolate which showed a point mutation within the diagnostic position (*C. lusitaniae* VA 115231-03).

With multiplexing capacity as one of the key features of DNA microarrays, the method developed in the present study should particularly be compared to methods which are able to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Cgla1AS
Ckru1S
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UR 9344 03
UR 9406 03
VA 103469 04
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VA 3590 03

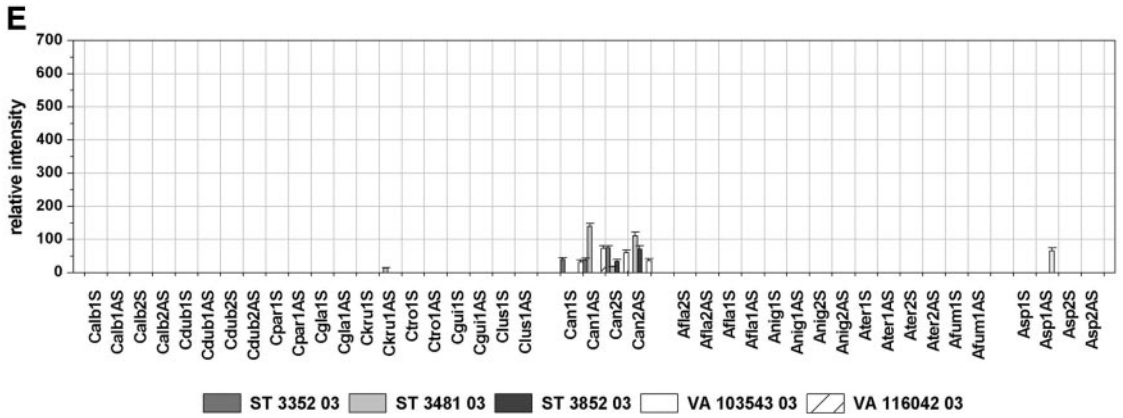

FIG. 4. Relative intensities after hybridization with labeled target DNA from blinded samples of clinical isolates. Cutoffs 1 and 2 are as described in the legend to Fig. 2. The columns depict mean relative signal intensities and their standard deviations over three slides of the same experiment ($n = 9$).

detect more than one parameter simultaneously. Using two multiplex liquid hybridization mixtures, Hendolin and colleagues needed 2 days for the detection of eight *Candida* and *Aspergillus* species from tissue samples (22). A line probe assay was designed for the simultaneous detection of 11 different *Candida* and *Aspergillus* species and *Cryptococcus neoformans* in spiked blood samples within 1 working day (38). However, the capacity of this format is nearly used up with this number of species (57). In contrast, microarrays have the potential to discriminate between thousands of targets, and principally, the design of specific probes which are able to discriminate between a set of species by using a particular target or targets is the only limiting factor. In this study a set of 12 target species was chosen, and the procedure could be performed within 4 h after DNA extraction. Additional time must be counted for DNA extraction, but optimized protocols for extraction in 4 h have been reported previously (31, 34). The opportunity to extend the amount of detectable species in combination with the fast assay procedure makes this array-based method an interesting tool for the rapid and parallel detection of fungal pathogens. The most time-saving effect from the introduction of molecular methods for the diagnosis of fungal infections could be expected if this microarray technique were successfully applied directly to clinical samples that are microscopically positive for fungal elements rather than fungal cultures. The quality of such a culture-independent diagnostic method is mainly dependent on the sensitivity of the DNA extraction method and the PCR protocol, as well as on the specificity of the target identification at the end of the detection process. All three steps of this diagnostic process are part of an ongoing study.

To have a diagnostic tool which can prevail in the routine clinical laboratory, the spectrum of detectable species should be expanded. In addition to IFIs due to non-*C. albicans Candida* species (43) and non-*A. fumigatus Aspergillus* species, the incidence of invasive mold infections caused by *Zygomycetes* (e.g., *Rhizopus*, *Mucor*, *Rhizomucor*, or *Absidia* spp.) and *Fusarium* spp. increased significantly in the late 1990s (37). It will be the aim of future studies to develop a more comprehensive array that includes these fungi as targets to improve the diagnosis of emerging yeast and mold infections. Also, yeasts like *Cryptococcus neoformans*, which particularly play a role in the care of AIDS patients, and representatives of the groups *Trichosporon* and *Malassezia* should be included as target species. As more and more molecular targets which are responsible for resistance or pathogenicity are currently identified (33), DNA microarrays also present a promising platform for the parallel species identification and the detection of pathogenicity factors or genes that confer resistance (18). The combined information about the species and its resistance would then lead to more efficient therapies while reducing the unwanted selection pressure of antifungal agents at the same time.

Although today only a limited number of laboratories that specialize in molecular biology are prepared with suitable technical equipment, a lot of work is under way, especially for the application of other, simpler readout systems for microarray slides. As the number of promising applications for microarrays targeting bacterial, parasitic, and fungal pathogens increases, it could be expected that commercial microarray systems will also be offered in the future at a reasonable price to a broader spectrum of customers.

Conclusions. The results presented in this paper showed the feasibility of the DNA microarray-based method for the detection and identification of 12 of the most common fungal pathogens belonging to the genus *Candida* or *Aspergillus*. The assay was demonstrated with isolated genomic DNA originating from precharacterized fungal strains and blinded clinical isolates. While mixture analysis and its application to primary specimens must be performed in the future, the microarray method can already bring great improvements to the laborious standard identification procedures and be a rapid tool for the identification of isolated fungal pathogens.

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