INTRODUCTION

Invasive fungal infections (IFIs) continue to represent a significant problem in immunosuppressed individuals. It has been estimated that more than 90% of all IFIs are caused by *Aspergillus* and *Candida* species (Beck-Sague & Jarvis, 1993; Goodrich et al., 1991; Singh, 2001; Vincent et al., 1998). Early detection of the fungal pathogen is crucial for appropriate antifungal therapy and thus the clinical outcome of patients with IFI (Morace et al., 1997). Conventional diagnostic tests, including blood cultures (Duthie & Denning, 1995; Vincent et al., 1998) and serological detection of circulating fungal antigens (e.g. D-glucan or galactomannan) (Pfeiffer et al., 2006), have shown variable sensitivity and specificity. Histological analyses of computed tomography-guided biopsies are highly sensitive and specific, but are frequently associated with bleeding complications in patients with severe thrombocytopenia (Denning, 1998). Therefore, efforts are ongoing to develop less invasive, yet reliable, sensitive and specific diagnostic tests for IFIs to overcome the limitations of the traditional fungus detection methods. Recently, a variety of PCR-based methods have been developed for rapid and sensitive detection of fungal pathogens (Chryssanthou et al., 1994; Einsele et al., 1997; Jordan, 1994; Sandhu et al., 1995). Since non-albicans *Candida* spp. and non-fumigatus *Aspergillus* spp. are increasing in importance (Bille et al., 2005; Coleman et al., 1998; Torres et al., 2003), diagnostic approaches covering a large number of fungal species are required. Broad-spectrum PCR methods mostly exploit the highly conserved regions of the ribosomal multi-copy rDNA gene cluster to amplify numerous fungal strains by a universal primer set (Medlin et al., 1988).

A considerable amount of data is available on the detection of IFIs using real-time quantitative PCR (RQ-PCR)
approaches; however, a lot of published assays only permit detection of single fungal species (Costa et al., 2001; Loeffler et al., 2000; Spiess et al., 2003). To date, some TaqMan RQ-PCR methods covering either multiple Candida (Maaroufi et al., 2004; White et al., 2003) or Aspergillus species (Costa et al., 2002; Kami et al., 2001) are available. Earlier studies relying on LightCycler technology have demonstrated the principle feasibility of detecting moulds and yeasts in a single reaction (Jordândes et al., 2005; Klingspor & Jalal, 2006). Our assay, however, is based on TaqMan technology employing a short universal hydrolysis probe localized within a highly conserved fungal region, which minimizes the effect of point mutations on the detectability of the fungal species. Based on comprehensive sequence analysis of the fungal 28S rDNA genes, we have been able to establish an assay permitting the detection and monitoring of at least nine clinically relevant Candida species and six Aspergillus species. Our pan-Aspergillus and pan-Candida assay (Pan-AC assay; patent pending) permits economic fungus screening and accurate quantification of a broad range of pathogenic Aspergillus and Candida species in the clinical setting.

METHODS

Fungal strains, bacteria and virus isolates. Fungal strains for PCR testing were obtained from the American Type Culture Collection (ATCC) and from the German Collection of Micro-organisms (DSM, Germany): A. fumigatus (ATCC 36607), Aspergillus niger (ATCC 10535), C. albicans (ATCC 14053), Candida dubliniensis (ATCC MYA-646), Candida glabrata (ATCC 2001), Candida krusei (ATCC 6258), Candida parapsilosis (ATCC 22019) and Candida tropicalis (ATCC 750), and Aspergillus flavus (DSM 818), Aspergillus nidulans (DSM 820), Aspergillus terreus (DSM 826), Aspergillus versicolor (DSM 1943), Candida guilliermondii (DSM 70051), Candida kefyr (DSM 70073) and Candida lusitaniae (DSM 70102). Prior to DNA extraction, the fungal colonies of each fungus culture were homogenized in 500 µl lyticase lysis buffer (LLB) [50 mM Tris (pH 7.6), 1 mM EDTA (pH 8.0), 0.2% 2-mercaptoethanol, 10 U ml⁻¹ recombinant lyticase (Sigma)] and incubated at 37 °C for 1 h. After incubation, acid-washed glass beads 710–1180 µm in diameter (Sigma) were added and vortexed thoroughly for 2 min. A total of 400 µl supernatant were used for DNA extraction on a MagNA Pure compact instrument using the MagNA Pure compact nucleic acid isolation kit I (Roche Diagnostics) as described by the manufacturer. The DNA was eluted in a total volume of 100 µl elution buffer (Roche Diagnostics). DNA concentrations were determined by a PicoGreen dsDNA quantification kit (Molecular Probes) and fluorescence spectrophotometer F-2500 (Hitachi).

(ii) Blood specimens. After hypotonic lysis of the erythrocytes from 3 ml blood using red cells lysis buffer [10 mM Tris (pH 7.6), 5 mM MgCl₂, 10 mM NaCl], as described by Löffler et al. (1997), the leukocytes were pelleted and resuspended in 470 µl LLB. The subsequent procedure followed the extraction protocol described above.

(iii) Respiratory secretions. Samples were centrifuged at 5000 g for 10 min. The supernatant was removed and 430 µl LLB was added. The extraction was performed as described above.

(iv) Lung biopsies and cornea. Solid material was mechanically disrupted and homogenized in 1× PBS. The samples were centrifuged at 5000 g for 10 min, the supernatant was removed and 430 µl LLB was added. The subsequent steps were as described above.

(v) Bacteria and viruses. For the isolation of DNA from cultured bacteria and virus stocks, a commercially available kit (QIAamp DNA mini kit; Qiagen) was used as recommended by the manufacturer.

Target sequence analysis and primer/probe design. Conserved nucleotide sequences of the fungal ribosomal multi-copy genes (18S, 5.8S and 28S) of clinically relevant Aspergillus and Candida species were selected and aligned using the BLAST search software, freely accessible at http://www.ncbi.nlm.nih.gov/BLAST/. Within the 28S large ribosomal subunit [between nucleotides 146 and 311 based on the sequence of the 28S rDNA gene of C. albicans (NCBI accession no. Z48339)], a highly conserved region was identified that spans less than 150 bp in length, thereby optimally fitting the requirements of RQ-PCR analysis using a hydrolysis TaqMan probe. Sequences for the forward primer, the reverse primer, and the universal probe were selected using Primer Express software (version 2.0; Applied Biosystems) following the manufacturer’s guidelines (Table 2). The probe was labelled with FAM (6-carboxyfluorescein) as a reporter molecule at the 5'-end and TAMRA (6-carboxytetramethylrhodamine) as a quencher molecule at the 3'-end (Applied Biosystems). The optimal concentrations for the primers and the probe were assessed by serial analyses both from the functional and economic perspective, and were specified for 400 and 200 nM, respectively.

RQ-PCR. PCR reactions were set up in a total volume of 25 µl containing 12.5 µl universal master mix [2× concentration, including ROX-reference dye and uracil N'-glycosylase (UNG)] (Applied Biosystems), 1% formamide, a mixture of the forward and the reverse primer (400 nM each), 200 nM Pan-AC hydrolysis probe, and 5 µl genomic DNA (gDNA). The mixture was transferred to 96-well optical microtitre plates (Applied Biosystems). Amplification was performed on the ABI 7700 sequence detection system (Applied Biosystems) using the following cycling parameters: 2 min at 50 °C (degradation of potentially
TABLE 1. GenBank accession numbers of fungal species used for 28S rDNA alignment and selection of sequences targeted by the Pan-AC assay

<table>
<thead>
<tr>
<th>Name</th>
<th>NCBI accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus</td>
<td>AF027863, U28899</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>AF109335, U28460, Z48340</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>AF109337, U28856</td>
</tr>
<tr>
<td>A. niger</td>
<td>U28815, AF109344</td>
</tr>
<tr>
<td>A. terreus</td>
<td>U28841, AF109340</td>
</tr>
<tr>
<td>A. versicolor</td>
<td>AF433018, AF433059</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Z48339, X83717, L28817</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>U57685, AB031020</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>U44808, Z48341</td>
</tr>
<tr>
<td>C. guillermondii</td>
<td>AF374616, U45709</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>AF335978, Y15476</td>
</tr>
<tr>
<td>C. krusei</td>
<td>U76347, Z48567</td>
</tr>
<tr>
<td>C. lipolytica</td>
<td>AF335977</td>
</tr>
<tr>
<td>C. lusitaniae</td>
<td>U44817</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>AF374609, Z48343</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>AF267497, Z48346</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

In the present report, a RQ-PCR assay is described permitting the detection and quantification of a broad range of clinically relevant Aspergillus and Candida species (Table 1) in a single...
The Pan-AC primers and probe were evaluated for possible genera with similar sensitivity.

Specificity of the Pan-AC assay

The ability of the Pan-AC system to detect all fungus species of interest was determined by testing DNA derived from cultures of reference strains, including \textit{C. albicans}, \textit{C. glabrata}, \textit{C. krusei}, \textit{C. tropicalis}, \textit{C. parapsilosis}, \textit{C. guilliermondii}, \textit{C. kefyr}, \textit{Candida lipolytica}, \textit{C. lusitaniae}, \textit{C. dubliniensis}, \textit{A. fumigatus}, \textit{A. flavus}, \textit{N. niger}, \textit{A. terreus}, \textit{A. versicolor} and \textit{A. nidulans}. Target sequences of all species were amplified successfully and showed nearly identical amplification efficiencies (data not shown). Based on the sequence alignment of additional, less common \textit{Aspergillus} and \textit{Candida} species, the Pan-AC RQ-PCR assay can be expected to cover several other members of these two genera with similar sensitivity.

The Pan-AC primers and probe were evaluated for possible cross-reactivity with bacterial or viral pathogens, both by sequence alignment using the BLAST software, and experimentally by testing the Pan-AC assay against bacterial and viral DNA from different organisms (see Methods). No cross-reactivity between the Pan-AC primer/probe detection system and non-fungal pathogens was observed (data not shown). As indicated in Fig. 1, DNA sequences from different human genes show significant homology with the fungal DNA sequence targeted by the Pan-AC assay. Analysing human DNA extracted from peripheral blood of healthy volunteer donors initially revealed some degree of cross-reactivity. Different chemicals, including DMSO, glycerol and formamide, were tested in different concentrations (1–5 %) to increase the stringency of the PCR reaction without simultaneously compromising the overall amplification efficiency. DMSO and glycerol completely inhibited the PCR reaction even at low concentrations (data not shown). By contrast, formamide concentrations of 1 % abrogated the cross-reactivity with human DNA, while maintaining the high amplification efficiency of the PCR assay (Table 3). This concentration of formamide was, therefore, included both in the assessment of the detection limit and the testing of clinical specimens by the Pan-AC assay.

Detection limit of the assay

The detection limit of the Pan-AC assay was determined by testing serial dilutions of fungal gDNA derived from organisms representing yeasts (\textit{C. albicans}) and moulds (\textit{A. fumigatus}). The DNA concentration of the two fungal species was determined by fluorometric measurements, and serial logarithmic dilutions across a range of five logs (10 fg to 100 pg) were prepared. After PCR amplification, the cycle threshold (\(C_T\)) values of individual dilution steps were plotted against the template amount, leading to typical standard curves (Fig. 2). The y intercept, which corresponds to the theoretical limit of detection, was determined at \(C_T\) 38.9 for \textit{A. fumigatus} and \(C_T\) 37.9 for \textit{C. albicans}. In view of the inter-assay variability of the technique in the range of \(\pm 1\) \(C_T\), this difference can be regarded as minor. The amplification efficiencies of the \textit{Candida} and \textit{Aspergillus} species listed in Table 1 revealed only marginal differences (data not shown). The standard curves presented are, therefore, applicable in quantitative analysis of all fungal species covered by the Pan-AC assay. The lowest template amount permitting accurate and reproducible quantification of fungal DNA was in the order of 10 fg.

In order to investigate the applicability of the Pan-AC assay to the analysis of clinical specimens, peripheral blood from

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Target</th>
<th>NCBI accession no.</th>
<th>Nucleotide position*</th>
<th>Conc (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fw primer</td>
<td>TGGGTGGTAAATTTACATCTGGAAGCTA</td>
<td>\textit{C. albicans}</td>
<td>Z48339</td>
<td>185–210</td>
<td>400</td>
</tr>
<tr>
<td>Rev primer</td>
<td>CAAGTKCTTTCTCATTTTCSWTCAC</td>
<td>\textit{C. albicans}</td>
<td>Z48339</td>
<td>245–270</td>
<td>400</td>
</tr>
<tr>
<td>Probe (rev)</td>
<td>ACTGGTGGCCTATCGGGCTCYSGCC</td>
<td>\textit{C. albicans}</td>
<td>Z48339</td>
<td>217–241</td>
<td>200</td>
</tr>
</tbody>
</table>

*Nucleotide positions correspond to the NCBI sequence, accession no. Z48339.

Table 3. Influence of formamide concentration on the \(C_T\) and the fluorescence intensity of the reporter (\(\Delta Rn\))

The effect of formamide was tested for different fungal DNA template concentrations of both \textit{Candida} and \textit{Aspergillus} representatives ranging from 1 pg to 10 fg. The RQ-PCR results shown represent the effect of formamide observed at the lowest fungal template concentration tested (10 fg \textit{C. albicans} DNA). Although the overall fluorescence intensity decreased with rising concentrations of formamide, the concentration at which any cross-reactivity with human DNA was abrogated (1 %) generally revealed only slightly increased \(C_T\) values.

<table>
<thead>
<tr>
<th>Formamide concn (%)</th>
<th>(C_T) value</th>
<th>(\Delta Rn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33.60</td>
<td>2.41</td>
</tr>
<tr>
<td>0.5</td>
<td>34.17</td>
<td>1.72</td>
</tr>
<tr>
<td>1</td>
<td>34.12</td>
<td>1.55</td>
</tr>
<tr>
<td>1.5</td>
<td>35.46</td>
<td>1.10</td>
</tr>
<tr>
<td>2</td>
<td>36.61</td>
<td>0.95</td>
</tr>
<tr>
<td>2.5</td>
<td>40.86</td>
<td>0.50</td>
</tr>
</tbody>
</table>
healthy volunteer donors was spiked with tenfold serial
dilutions of *A. fumigatus* conidia and *C. albicans* cells,
covering a range between $10^5$ to 1 organism ml$^{-1}$. The
DNA was extracted and amplified as described in Methods.
In patients with IFI, the fungal load in peripheral blood
samples is generally low, often below 10 c.f.u. ml$^{-1}$
(Loeffler et al., 2000). Based on these observations, the
sensitivity of the PCR assay is crucial for the detection and
quantification of fungal pathogens. The detection limit of
the Pan-AC assay in spiking experiments was less than 10
organisms per PCR reaction; however, for reproducible
detection and quantitative analysis a higher volume of
peripheral blood providing larger overall number of fungal
organisms was beneficial (data not shown).

**Analysis of clinical specimens**

The Pan-AC assay was employed to investigate specimens
from 17 haematological or intensive care patients with
fungal infections diagnosed by other methodological
approaches, including primarily culture techniques and DNA
sequencing. According to the EORTC criteria, 4 patients were
classified as having a possible IFI and 12 patients a probable
IFI infection (Table 4, samples 1–16). Only for the patient
from whom the cornea specimen was derived, was no
EORTC classification available (Table 4, sample 17).

The Pan-AC assay revealed positive results in the clinical
specimens studied, including lung biopsies of patients with
pulmonary aspergillosis or candidiasis, peripheral blood
specimens from patients with different types of candidaemia,
and respiratory secretions positive for different *Aspergillus*
species (Table 4). In one of the latter specimens, co-infection
with *C. lusitaniae* was detected (Table 4, sample 13). The only
clinical sample that tested negative by the Pan-AC assay was a
cornea specimen, in which culture analysis revealed the
presence of *F. solani*. This specimen only served as a control
because this fungus is not within the detection spectrum of
the PCR assay presented (Table 4). Quantitative analysis of
the fungal load in specimens positive by RQ-PCR was
performed by using the appropriate standard curves, and the
amount of fungal DNA determined was translated to the
number of organisms on the basis of the estimated mean
genome masses of *Aspergillus* and *Candida* species of 32 and
37 fg, respectively.

**Prevention and control of contamination**

A major problem of fungal PCR assays is the high risk of
contamination (Loeffler et al., 1999), which is attributable
to the ubiquitous presence of airborne fungal spores, and
traces of fungal DNA in a variety of reagents and other
consumables. To avoid false-positive results, it is imper-
Table 4. Fungal species identified in clinical specimens

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>Diagnosis of IFI*</th>
<th>Microbiological evidence</th>
<th>Sequencing</th>
<th>Pan-AC PCR</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;</th>
<th>Fungal DNA (fg)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blood</td>
<td>Probable</td>
<td>C. glabrata</td>
<td>ND</td>
<td>Positive</td>
<td>34.8</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Blood</td>
<td>Possible</td>
<td>ND</td>
<td>C. albicans</td>
<td>Positive</td>
<td>28.1</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>Blood</td>
<td>Possible</td>
<td>ND</td>
<td>C. krusei</td>
<td>Positive</td>
<td>37.0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Lung biopsy</td>
<td>Possible</td>
<td>ND</td>
<td>A. fumigatus</td>
<td>Positive</td>
<td>35.7</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Lung biopsy</td>
<td>Possible</td>
<td>ND</td>
<td>C. lipovetica</td>
<td>Positive</td>
<td>30.7</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus, A. niger</td>
<td>ND</td>
<td>Positive</td>
<td>27.2</td>
<td>1429</td>
</tr>
<tr>
<td>7</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus</td>
<td>ND</td>
<td>Positive</td>
<td>32.4</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus</td>
<td>ND</td>
<td>Positive</td>
<td>31.6</td>
<td>69</td>
</tr>
<tr>
<td>9</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus</td>
<td>ND</td>
<td>Positive</td>
<td>26.1</td>
<td>2885</td>
</tr>
<tr>
<td>10</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus</td>
<td>ND</td>
<td>Positive</td>
<td>29.8</td>
<td>246</td>
</tr>
<tr>
<td>11</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. niger</td>
<td>ND</td>
<td>Positive</td>
<td>32.4</td>
<td>40</td>
</tr>
<tr>
<td>12</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus, A. flavus</td>
<td>ND</td>
<td>Positive</td>
<td>29.9</td>
<td>230</td>
</tr>
<tr>
<td>13</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus, C. lusitaniae</td>
<td>ND</td>
<td>Positive</td>
<td>35.7</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>Aspergillus spp.</td>
<td>ND</td>
<td>Positive</td>
<td>33.5</td>
<td>19</td>
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<tr>
<td>15</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus</td>
<td>ND</td>
<td>Positive</td>
<td>32.5</td>
<td>40</td>
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<tr>
<td>16</td>
<td>Respiratory tract</td>
<td>Probable</td>
<td>A. fumigatus</td>
<td>ND</td>
<td>Positive</td>
<td>31.5</td>
<td>78</td>
</tr>
<tr>
<td>17</td>
<td>Cornea</td>
<td>–</td>
<td>E. solani</td>
<td>ND</td>
<td>Negative</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ND, Not determined.

*The diagnosis of IFI was performed according to the EORTC criteria (http://www.doctorfungus.org/lecture/eortc_msg_rev06.htm).

†The numbers indicate the determined amount of fungal DNA at the beginning of the PCR-reactions. To calculate the fungal load in the clinical specimen investigated, the quantity of fungal DNA in fg assessed by RQ-PCR analysis can be translated to the number of fungal organisms on the basis of the estimated genome mass and the appropriate dilution factor of the sample analysed.

NOTE ADDED IN PROOF

While this paper was in press, a paper by Schabereiter-Gurtner et al. (2007) was published, in which a similar technique is described.

ACKNOWLEDGEMENTS

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REFERENCES


