

The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of *Aspergillus* and *Candida* species

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In view of the growing incidence and the high mortality of invasive aspergillosis and candidiasis, adequate diagnostic techniques permitting timely onset of treatment are of paramount importance. More than 90 % of all invasive fungal infections in immunocompromised individuals can be attributed to *Candida* and *Aspergillus* species. To date, standardized techniques permitting rapid, sensitive and, no less importantly, economic screening for the clinically most relevant fungi are lacking. In the present report, a real-time quantitative PCR assay, developed for the detection of the most common pathogenic *Candida* and *Aspergillus* species, is described. The single-reaction PCR assay targets a judiciously selected region of the 28S subunit of the fungal rDNA gene. The unique design of the universal primer/probe system, including a pan-*Aspergillus* and pan-*Candida* (Pan-AC) hydrolysis probe, facilitates the detection of numerous *Aspergillus* species (e.g. *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus versicolor* and *Aspergillus nidulans*) and *Candida* species (e.g. *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, *Candida kefyr*, *Candida guilliermondii*, *Candida lusitanae* and *Candida dubliniensis*). The assay permits highly reproducible detection of 10 fg fungal DNA, which corresponds to a fraction of a fungal genome, and facilitates accurate quantification of fungal load across a range of at least five logs. Upon standardization of the technique using cultured fungal strains, the applicability in the clinical setting was assessed by investigating a series of clinical specimens from patients with documented fungal infections ($n=17$). The Pan-AC assay provides an attractive and economic approach to the screening and monitoring of invasive aspergillosis and candidiasis, which is readily applicable to routine clinical diagnosis.

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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-Sagué & 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 (Chrysanthou *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)

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Abbreviations: C_T, cycle threshold; EORTC, European Organization for Research and Treatment of Cancer; gDNA, genomic DNA; IFI, invasive fungal infection; Pan-AC, pan-*Aspergillus* and pan-*Candida*; RQ-PCR, real-time quantitative PCR; UNG, uracil N'-glycosylase.

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 (Jordanides *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 lusitanae* (DSM 70102). Prior to DNA extraction, the fungal isolates were cultured on Sabouraud dextrose agar at 30 °C; *Candida* isolates were cultured for 48 h and *Aspergillus* isolates for 72 h. Suspensions of all fungi (*Candida* species, cell suspension; *Aspergillus* species, conidia suspension) were prepared using an aliquot of cultured fungus resuspended in sterile 0.9% NaCl solution.

In addition, a panel of bacterial and viral micro-organisms were selected for testing of cross-reactivity. The panel included *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Proteus vulgaris*, human adenoviruses, Epstein-Barr virus, cytomegalovirus, varicella-zoster virus, herpes simplex virus types 1 and 2, and parvovirus B19 (PVB19).

Clinical materials. The clinical specimens from patients with documented fungus infections were obtained upon informed consent. The specimens included biopsies of pulmonary infiltrations ($n=2$) and peripheral blood ($n=3$) from haemato-oncological patients, bronchotracheal secretions ($n=11$) from intensive care patients, and a cornea control specimen with a culture-documented infection by *Fusarium solanii*, which is outside the detection spectrum of the Pan-AC assay (kindly provided by St Anna Children's Hospital, Vienna, Austria and the Institute of Hygiene and Medical Microbiology, Medical University of Vienna, Austria). Peripheral blood specimens from healthy volunteer donors were used to test for cross-reactivity with human DNA.

DNA extraction. All steps were performed in a laminar flow hood. Reagents used for extraction were filtered through 0.2 µm sterile filters.

(i) Fungal strains. A colony of each fungus culture was 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 Loeffler *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 at 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

Name	NCBI accession no.
<i>A. flavus</i>	AF027863, U28899
<i>A. fumigatus</i>	AF109335, U28460, Z48340
<i>A. nidulans</i>	AF109337, U29856
<i>A. niger</i>	U28815, AF109344
<i>A. terreus</i>	U28841, AF109340
<i>A. versicolor</i>	AF433108, AF433059
<i>C. albicans</i>	Z48339, X83717, L28817
<i>C. dubliniensis</i>	U57685, AB031020
<i>C. glabrata</i>	U44808, Z48341
<i>C. guilliermondii</i>	AF374616, U45709
<i>C. kefir</i>	AF335978, Y15476
<i>C. krusei</i>	U76347, Z48567
<i>C. lipolytica</i>	AF335977
<i>C. lusitaniae</i>	U44817
<i>C. parapsilosis</i>	AF374609, Z48343
<i>C. tropicalis</i>	AF267497, Z48346

present contaminating dUTP-containing amplicons by UNG), 10 min at 95 °C (inactivation of UNG and activation of AmpliTaq Gold DNA polymerase), followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C for target amplification. For absolute quantification of the fungal DNA, standard curves were prepared using serial logarithmic

dilutions covering a range of five logs (10 fg–100 pg) gDNA derived from *C. albicans* and *A. fumigatus* as positive control samples.

Assessment of the detection limit. To determine the detection limit of fungal pathogens in peripheral blood, 1 ml EDTA-anti-coagulated whole blood from healthy volunteer donors was spiked with tenfold serial dilutions of *A. fumigatus* conidia and *C. albicans* cells (10^5 to 1 cell). The number of fungal particles was determined microscopically using a counting chamber. DNA extraction was performed according to the protocol outlined above.

Controls. A number of precautions were undertaken to control the occurrence of false-positive results. Multiple no-template and non-homologous template controls were processed together with the specimens tested, as described previously (Watzinger *et al.*, 2004). To document the efficiency of DNA extraction and amplification, various positive controls were included, as already published (Lion, 2001). In clinical samples containing human cells, a single-copy housekeeping gene (β 2-microglobulin) was co-amplified in parallel with the fungal targets (Lion, 2001). For largely cell-free human specimens, defined concentrations of a seal herpes virus were spiked into the sample prior to DNA extraction and analysed by RQ-PCR to control for the potential occurrence of inhibitory effects (Watzinger *et al.*, 2004).

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

NCBI acc.no.	Species	Position related to <i>C. albicans</i> Z48339
		178 277
U28899	<i>A. flavus</i>	CTCTAAATGGGTGGTAAATTTTCATCTAAAGCTAAATACTGGCCGAGACCGATAGCGCACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U28460	<i>A. fumigatus</i>	CTCTAAATGGGTGGTAAATTTTCATCTAAAGCTAAATACTGGCCGAGACCGATAGCGCACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U29859	<i>A. nidulans</i> *	CTCTAAATGGGTGGTAAATTTTCATCTAAAGCTAAATACCAGCCGAGACCGATAGCGCACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U28817	<i>A. niger</i>	CTCTAAATGGGTGGTAAATTTTCATCTAAAGCTAAATACTGGCCGAGACCGATAGCGCACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U28845	<i>A. terreus</i>	CTCTAAATGGGTGGTAAATTTTCATCTAAAGCTAAATACTGGCCGAGACCGATAGCGCACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
AF454195	<i>A. versicolor</i>	CTCTAAATGGGTGGTAAATTTTCATCTAAAGCTAAATACCAGCCGAGACCGATAGCGCACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
Z48339	<i>C. albicans</i>	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U57685	<i>C. dubliniensis</i>	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U44808	<i>C. glabrata</i>	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATACAGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
AF374616	<i>C. guilliermondii</i> *	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
AF335978	<i>C. kefir</i> *	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U76347	<i>C. krusei</i> *	CTCCAACTGGGTGGTAAATTTTCATCTAAAGCTAAATACTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
AF335977	<i>C. lipolytica</i> *	CTCAAACTGGGTGGTAAATTTTCATCTAAAGCTAAATACTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
U44817	<i>C. lusitaniae</i> *	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
AF374609	<i>C. parapsilosis</i>	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
AF267497	<i>C. tropicalis</i>	CTCTAACTGGGTGGTAAATTTTCATCTAAAGCTAAATATTGGCCGAGACCGATAGCGAACAAGTAGAGTGAATCGAAAGATGAAAGCACTTTGAAAAAGAG
M11167	Human 28S rib. gene	CCCAAGCGGTGGTAAATCCCATCTAAGGCTAAATACCAGCCGAGACCGATAGTCAACAAGTACCGTAAGCGAAAGTTGAAAGCACTTTGAAGAGAG
U13369	Human ribosomal DNA	CCCAAGCGGTGGTAAATCCCATCTAAGGCTAAATACCAGCCGAGACCGATAGTCAACAAGTACCGTAAGCGAAAGTTGAAAGCACTTTGAAGAGAG
Oligonucleotide sequences for the Pan-AC RQ-PCR assay		<div style="display: flex; justify-content: space-between;"> <div style="text-align: center;"> <p>185210</p> <p style="background-color: #e0e0e0; padding: 2px;">TCGCTGCTAAATTTTCATCTAAGCTA</p> <p>5'3'</p> <p>Pan-AC forward primer</p> </div> <div style="text-align: center;"> <p>217241</p> <p style="background-color: #e0e0e0; padding: 2px;">CCGCTCTGCTATCGCTCTCTT</p> <p>3'5'</p> <p>Pan-AC probe (reverse)</p> </div> <div style="text-align: center;"> <p>245270</p> <p style="background-color: #e0e0e0; padding: 2px;">CACTGCTTTCTACTTTTCTGAAAC</p> <p>3'5'</p> <p>Pan-AC reverse primer</p> </div> </div>

Wobble positions used: Y = C or T; R = G or A; S = G or C; K = G or T; W = A or T

Fig. 1. Alignment of a highly conserved region within the 28S rDNA gene of six *Aspergillus* species, ten *Candida* species and the corresponding region from the human genome. The primer pair and the probe (grey boxes) for the Pan-AC RQ-PCR assay were placed within this region. The indicated nucleotide positions refer to the sequence of *C. albicans* (NCBI accession no. Z48339). The asterisks indicate species that are listed under another name in the NCBI database: *A. nidulans*=NCBI *Emericella nidulans*; *C. guilliermondii*=NCBI *Pichia guilliermondii*; *C. kefir*=NCBI *Kluyveromyces marxianus*; *C. krusei*=NCBI *Issatschenkia orientalis*; *C. lipolytica*=NCBI *Yarrowia lipolytica*; *C. lusitaniae*=NCBI *Clavispora lusitaniae*.

Table 2. Primers and probe in the Pan-AC RQ-PCR assay

Sample	Oligonucleotide sequence (5'-3')	Target	NCBI accession no.	Nucleotide position*	Concn (nM)
Fw primer	TGGGTGGTAAATTTCATCTAAAGCTA	<i>C. albicans</i>	Z48339	185–210	400
Rev primer	CAAGTKCTTTTCATCTTTCSWTCAC	<i>C. albicans</i>	Z48339	245–270	400
Probe (rev)	ACTGTGKCGCTATCGGTCTCYSGCC	<i>C. albicans</i>	Z48339	217–241	200

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

reaction. A single primer pair and a universal probe were designed within a highly conserved region of the 28S large ribosomal subunit (Fig. 1). The selected primer and probe sequences display a degenerated code (Table 2), which was a prerequisite for sensitive detection of fungal species differing from others at single nucleotide positions.

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 *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. kefyi*, *Candida lipolytica*, *C. lusitaniae*, *C. dubliniensis*, *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. versicolor* and *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 *Aspergillus* and *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 (*C. albicans*) and moulds (*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 – 100 pg) were prepared. After PCR amplification, the cycle threshold (C_T) values of individual dilutions 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 *A. fumigatus* and C_T 37.9 for *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 *Candida* and *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 3. Influence of formamide concentration on the C_T and the fluorescence intensity of the reporter (ΔR_n)

The effect of formamide was tested for different fungal DNA template concentrations of both *Candida* and *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 *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.

Formamide concn (%)	C_T value	ΔR_n
0	33.60	2.41
0.5	34.17	1.72
1	34.12	1.55
1.5	35.46	1.10
2	36.61	0.95
2.5	40.86	0.50

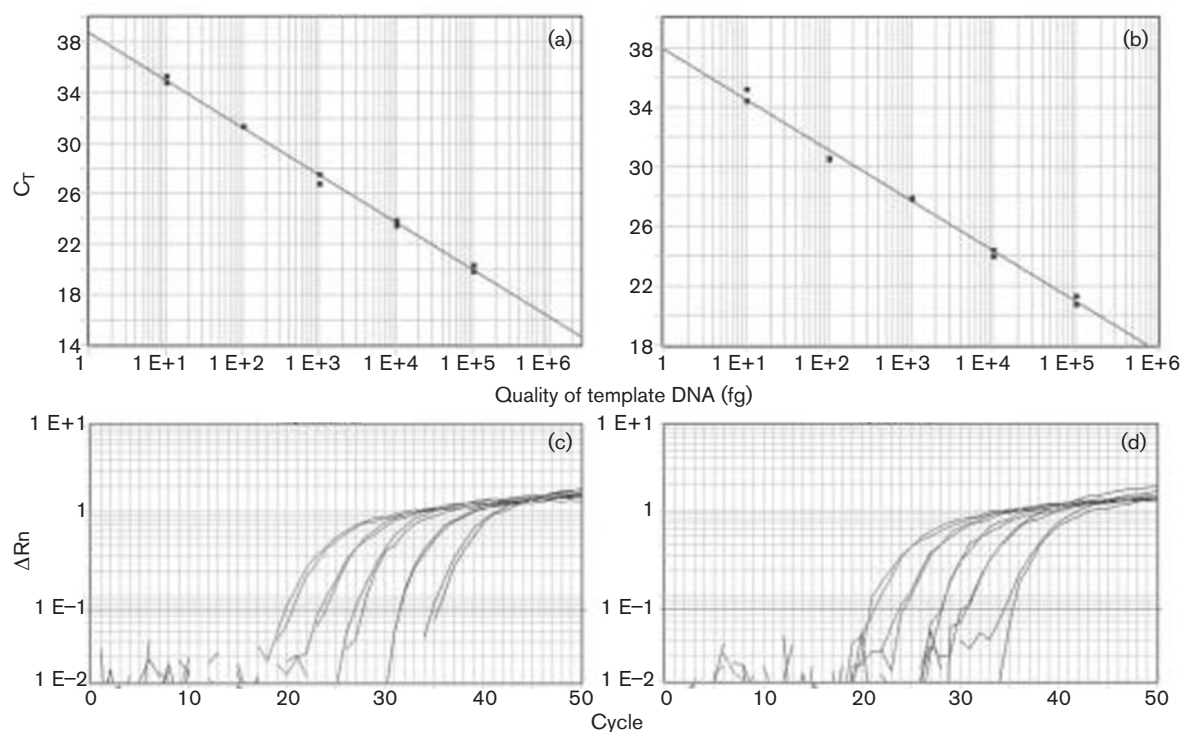


Fig. 2. Standard curves (a, b) and amplification plots (c, d) based on serial dilutions of fungal gDNA ranging from 10 fg to 100 pg, derived from representatives of moulds (*A. fumigatus*) (a, c) and yeasts (*C. albicans*) (b, d). The assays underlying the generation of standard curves were performed in duplicate.

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 haemato-oncological 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. lusitanae* 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. solanii*. 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

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

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.

ative therefore to control all materials used, both self-made and commercially available, for fungal contaminants, and to include multiple negative controls in each assay. The fungus detection assays must be performed under adequate experimental conditions, which include the preparation of reagents and processing of clinical samples under a laminar flow biohazard hood. With appropriate precautions and controls, the RQ-PCR assay presented can serve as a reliable diagnostic tool for the detection and quantitative monitoring of pathogenic fungi in clinical specimens.

In view of its broad specificity, the Pan-AC assay could serve as a screening technique for the presence of IFI. In patients testing positive, antifungal agents, such as voriconazole or caspofungins, could be used as first-line treatment, because these substances can be expected to cover the entire range of fungi detected by this assay. The use of agents with a narrower spectrum of antifungal activity, such as fluconazole or amphotericin B, would require the identification of the fungal species present, which can be performed by a number of molecular techniques, including, for example, the analysis of the variable *ITS2* region or species-specific hybridization (Chen *et al.*, 2000; Elie *et al.*, 1998; Sandhu *et al.*, 1995; Turenne *et al.*, 1999).

In comparison to most earlier RQ-PCR approaches to the detection of invasive aspergillosis or candidiasis (Bu *et al.*, 2005; Buchheidt *et al.*, 2004; Kami *et al.*, 2001; Kasai *et al.*, 2006; Klingspor & Jalal, 2006; Spiess *et al.*, 2003), the Pan-AC assay covers a considerably larger spectrum of

pathogenic *Aspergillus* and *Candida* species in a single reaction, using a universal detection probe and a single primer pair. Our data indicate that the Pan-AC assay can be readily implemented in routine clinical diagnosis and monitoring of the majority of IFIs.

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.

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