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Molecular detection and identification of *Candida* and *Aspergillus* spp. from clinical samples using real-time PCR

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ABSTRACT

This report describes the development of a real-time LightCycler assay for the detection and identification of *Candida* and *Aspergillus* spp., using the MagNa Pure LC Instrument for automated extraction of fungal DNA. The assay takes 5–6 h to perform. The oligonucleotide primers and probes used for species identification were derived from the DNA sequences of the 18S rRNA genes of various fungal pathogens. All samples were screened for *Aspergillus* and *Candida* to the genus level in the real-time PCR assay. If a sample was *Candida*-positive, typing to species level was performed using five species-specific probes. The assay detected and identified most of the clinically relevant *Aspergillus* and *Candida* spp. with a sensitivity of 2 CFU/mL blood. Amplification was 100% specific for all *Aspergillus* and *Candida* spp. tested. To assess clinical applicability, 1650 consecutive samples (1330 blood samples, 295 samples from other body fluids and 25 biopsy samples) from patients with suspected invasive fungal infections were analysed. In total, 114 (6.9%) samples were PCR-positive, 5.3% for *Candida* and 1.7% for *Aspergillus* spp. In patients with positive PCR results for *Candida* and *Aspergillus*, verification with conventional methods was possible in 83% and 50% of cases, respectively. In conclusion, the real-time PCR assay allows sensitive and specific detection and identification of fungal pathogens *in vitro* and *in vivo*.

Keywords *Aspergillus*, *Candida*, diagnosis, fungi, identification, real-time PCR

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INTRODUCTION

In Sweden, *Candida albicans* and other *Candida* spp., followed by *Aspergillus* spp., are the most common fungal pathogens causing invasive fungal infections (IFIs). Opportunistic IFIs are a major cause of morbidity and mortality in immunocompromised patients such as transplant recipients [1–4]. The clinical features of invasive candidiasis are non-specific, making early diagnosis of invasive candidiasis difficult [5,6]. There has been some progress in the diagnosis of invasive aspergillosis (IA) in recent years, mainly because of the use of high-resolution computerised tomography (CT) scanning and other imaging procedures, but established IA is difficult to treat, with a mortality rate of 80–90% [7].

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The incidence of nosocomial bloodstream infections caused by *Candida* spp. has increased during the last two decades. In a recent report from Sweden [8], the crude mortality rate of candidaemia was 31%. The highest mortality rates were observed in patients with haematological malignancies (41.2%), in those aged >70 years (41%), following surgery (38.5%), and in those infected with more than one *Candida* sp. (40%) or *Candida glabrata* (38%). However, blood cultures take time and have poor sensitivity [9–12]. As a consequence, the diagnosis of candidaemia and aspergillosis is generally established at a late stage, or even at autopsy, in a considerable number of cases [13].

Non-cultural techniques used previously have lacked sensitivity and specificity in immunocompromised patients [14,15]. New rapid methods that can detect IFI early in the course of disease, with high sensitivity and specificity, are thus required. More recently, PCR protocols for diagnosing fungal infections have been described [16–22]. Molecular diagnostic methods using uni-

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versal fungal PCR primers and species-specific probes have been developed and evaluated for the detection of fungal DNA in clinical specimens. Since non-*albicans Candida* spp. and *Aspergillus* spp. are increasing in importance, it is necessary for the fungal PCR assay to have high sensitivity for most pathogenic *Candida* and *Aspergillus* spp.

This report describes the development of an assay that uses a fully automated laboratory robot, the MagNA Pure LC Instrument, for extraction of *Candida* and *Aspergillus* DNA, in combination with the real-time PCR LightCycler System. The whole assay takes *c*. 6 h to perform. To assess its clinical applicability, a large number of blood samples, samples from other body fluids and biopsy samples from patients with suspected IFI were analysed by the assay, and the results were compared with those obtained by conventional methods.

MATERIALS AND METHODS

Fungal cultures

Standard fungal strains used in the study were *C. albicans* ATCC 10231, *C. glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* CCUG 5570, *Candida lusitaniae* UKNEQAS 6208, *Candida guillerimondi* SMI 8302, *Candida norvergensis* SMI 115-04, *Candida dubliniensis* (sequenced), *Candida kefyr* SMI 7-01, *Cryptococcus neoformans* ATCC 24067, *Aspergillus fumigatus* UKNEQAS 5526, *Aspergillus flavus* UKNEQAS 6657, *Aspergillus niger* ATCC 1640, *Aspergillus versicolor* UKNEQAS 6406, *Aspergillus nidulans* UKNEQAS 6020, *Aspergillus clavatus* UKNEQAS 7021, *Aspergillus glaucus* UKNEQAS 5647 and *Aspergillus terreus* UKNEQAS 6323. Clinical isolates were obtained from Karolinska University Hospital Huddinge, Stockholm, Sweden.

Sample preparation

Before DNA extraction, *Candida* cultures were grown on Sabouraud–glucose agar for 48 h at 30°C, and *Aspergillus* cultures were grown for 72 h at 30°C. Fungal suspensions in saline were adjusted (0.5 × MacFarland standard) to a concentration of 1×10^6 to 5×10^6 cells/mL. Ten-fold serial dilutions (10^6–10^1 cells) were prepared to test the sensitivity and specificity of the assay.

For determination of the detection limit in blood, EDTA-anti-coagulated whole-blood samples (5 mL) from healthy volunteers were spiked with serial dilutions of *Candida* and *Aspergillus* (10^6–10^1 cells/mL). DNA was extracted and analysed according to the protocol described below.

For specificity testing of the probes, cells from the following yeasts and moulds were tested: *Candida* spp., *Aspergillus* spp., *Malazessia* spp., *Crypt. neoformans*, *Saccharomyces cerevisiae*, *Trichosporon* spp., *Fusarium* spp., *Zygomycetes* spp., *Scedosporium* spp. and *Paecilomyces* spp. (Table 1).

Preparation of total genomic DNA from clinical samples

Manual steps: blood. Blood specimens (and fluids containing red blood cells) were incubated initially with a hypotonic red cell lysis buffer (RCLB) as described previously [19]. Following lysis of the erythrocytes, the sample was centrifuged at 2500 *g* for 10 min. The pellets were transferred to 1.5-mL Eppendorf tubes

Table 1. *Candida* and *Aspergillus* spp. and additional fungal species tested by real-time PCR

	Positive by hybridisation with DNA probe specific for						
	Common *Candida*	*C. albicans/ C. dubliniensis*	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	*Aspergillus* spp.
Candida albicans	+	+	–	+	–	–	–
Candida glabrata	+	–	+	–	–	–	–
Candida parapsilosis	+	+	–	+	–	–	–
Candida tropicalis	+	–	–	–	+	–	–
Candida krusei	+	–	–	–	–	+	–
Candida lusitaniae	+	–	–	–	–	–	–
Candida dubliniensis	+	+	–	+	–	–	–
Candida kefyr	+	–	–	–	–	–	–
Candida norvergensis	+	–	–	–	–	–	–
Candida guillerimondi	+	–	–	–	–	–	–
Saccharomyces cerevisiae	+	–	–	–	–	–	–
Trichosporon spp.	–	–	–	–	–	–	–
Malassezia spp.	–	–	–	–	–	–	–
Cyptococcus neoformans	–	–	–	–	–	–	–
Aspergillus fumigatus	–	–	–	–	–	–	+
Aspergillus flavus	–	–	–	–	–	–	+
Aspergillus niger	–	–	–	–	–	–	+
Aspergillus versicolor	–	–	–	–	–	–	+
Aspergillus terreus	–	–	–	–	–	–	+
Aspergillus nidulans	–	–	–	–	–	–	+
Aspergillus glaucus	–	–	–	–	–	–	+
Aspergillus clavatus	–	–	–	–	–	–	+
Zygomycetes spp.	–	–	–	–	–	–	–
Fusarium spp.	–	–	–	–	–	–	–
Scedosporium spp.	–	–	–	–	–	–	–
Paecilomyces spp.	–	–	–	–	–	–	–
Penicillium spp.	–	–	–	–	–	–	–

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containing glass beads (1.180 μm; Sigma, Deissenhofen, Germany), and vortexed thoroughly for 3 min; 200 μL of the supernantant was then pipetted into the wells of the MagNA Pure LC sample cartridge (Roche, Mannheim, Germany).

Manual steps: biopsies. Tissue samples were mechanically homogenised before being suspended in 1 × RCLB (only biopsies containing red blood cells). The mixture was then vortexed briefly, incubated at room temperature on a rotating platform for 10 min, and centrifuged at 13 200 *g*. The supernatant was discarded and 1 mL of white cell lysis buffer [19] was added. The tube was vortexed and incubated overnight in a water bath at 55°C, and then centrifuged for 15 min at 13 200 *g*. The pellet was transferred to a 1.5-mL Eppendorf tube containing glass beads, and processed as described above.

MagNA Pure DNA isolation. DNA was extracted and purified by the MagNA Pure DNA robot (Roche), using the MagNA Pure LC DNA I isolation kit for all preparations. The preparation and settings of the instrument were according to the manufacturer's instructions.

Primer and hybridisation probes for LightCycler-based amplification of *Candida* and *Aspergillus* DNA

The oligonucleotide primer pair (5′-ATTTGGAGGGCAAGTCTGGT and 5′-GATCCCTAGTCGGCATAGTT), as well as the probes described below, were designed following a comparison of the sequences of 18S rRNA genes in the GeneBank database. The primers target a consensus sequence for a variety of fungal pathogens. Probes bind within the variable areas of the gene.

The 'common *Candida*' probe was designed to detect most pathogenic *Candida* spp., and comprised a 'donor' sequence (5′-CGAAAGTTAGGGGATCGAAGATG) labelled at the 5′-end with the LightCycler Red705 fluorophore, and an 'acceptor' sequence (5′-CCAAGGACGTTTTCATTAATCAAGA) labelled at the 3′-end with fluorescein. Similarly, the *Candida* species-specific probes were labelled at the 5′-end of the first (donor) sequence with the LightCycler Red640 fluorophore, and at the 3′-end of the second (acceptor) sequence with fluorescein. Probes used were as follows: for *C. albicans*, 5′-CGAAAGTTAGGGGATCGAAGATG and 5′-AGCCTTTCCTTCTGGGTAGCCAT; for *C. tropicalis*, 5′-GTCCATCTTTCTGATGCGTACTGG and 5′-TAGTTGAACCTTGGGCTTGGTTGGC; for *C. glabrata*, 5′-TCCTTGTGGCTTGGCGGCGAACCAG and 5′-GGCCTTTCCTTCTGGCTAACCCCA; for *C. parapsilosis*, 5′-GACCCAGCCGAGCCTTTCCTTCTG and 5′-CCGGTCCATCTTTTTTGATGCGTAC; and for *C. krusei*, 5′-CCTCGGGCGAACCAGGACGATT-3′ and 5′-GGCCGGGTCTTTCCTTCTGGCT.

The *Aspergillus* probe was designed to detect *A. fumigatus*, *A. flavus*, *A. niger*, *A. versicolor*, *A. terreus*, *A. nidulans*, *A. glaucus* and *A. clavatus*. The donor sequence (5′-TGAGGTTCCCCAGAAGGAAAGGTCCAGC) was labelled at the 5′-end with the LightCycler Red640 fluorophore, and the acceptor sequence (5′-GTTCCCCCCACAGCCAGTGAAGGC) was labelled at the 3′-end with fluorescein.

All primers and probes were synthesised by Tibmolbiol (Berlin, Germany). The primers and hybridisation probes for *Candida* spp. were developed as part of the present study; the *Aspergillus* probe was that described by Loeffler *et al.* [23].

PCR amplification and detection

The LightCycler system (Roche) was used for amplification of *Candida* and *Aspergillus* DNA. LightCycler hot-start PCR was performed in glass capillaries with a LightCycler Fast Start DNA Master Hybridisation Probes kit (Roche) as specified by the manufacturer. The PCR master mix (10 μL) contained 1 × Fast Start reaction mixture with Fast Start *Taq* DNA polymerase, reaction buffer, dNTPs, 1.6 μL of 25 mM $MgCl_2$, 1 μL of each primer (3 μM), and 1 μL (2 μM) of each hybridisation probe.

PCR was performed in a final volume of 20 μL (10 μL of master mix + 10 μL of DNA extract) with 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 10 s at 58°C and 20 s at 72°C, with a temperature transition rate (TTR) of 20°C/s. The PCR was followed by a melting temperature analysis cycle comprising 95°C for 10 s (TTR of 20°C/s), 50°C for 60 s (TTR of 20°C/s) and 75°C for 0 s (TTR of 0.1°C/s) to check the specificity of the PCR product.

Each PCR included a negative control consisting of RCLB without template DNA to monitor possible contamination. Furthermore, DNA extracts from clinical samples were analysed in parallel with an extraction control and a PCR control containing fungal DNA.

Clinical samples

All samples were screened for *Aspergillus* and *Candida* to the genus level in the real-time PCR assay. If a sample was positive for the *Candida* genus, the sample was tested further with the *Candida* species-specific probes described above. The samples analysed comprised blood (3 mL of EDTA blood from children, 5 mL of EDTA blood from adults) and other body fluids (in sterile tubes), as well as small biopsy samples (in sterile saline). The samples were either analysed directly on arrival at the laboratory, or were kept refrigerated overnight or stored frozen during weekends before analysis.

Patients

Between 1 June 2002 and 31 October 2003, the real-time PCR assay was used to analyse 1650 patient samples sent to the Mycology Laboratory at Karolinska University Hospital Huddinge from patients at risk for fungal infection, from patients with suspicion of fungal infection, or from patients for whom confirmation of fungal infection before, during and after treatment was required. Classification of opportunistic invasive fungal infections in immunocompromised patients with cancer or haematopoietic stem-cell transplants was according to the EORTC/MSG criteria [24].

RESULTS

Combination of MagNA Pure DNA extraction and LightCycler PCR

The in-vitro sensitivity obtained with serially diluted genomic DNA from *Candida* and *Aspergillus* spp. was 2 CFU/mL blood. The hybridisation probes for the genus *Candida* detected DNA from the ten *Candida* spp. listed in Table 1. Identification

to the species level was possible with the probes for *C. albicans/C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. The probes for *C. glabrata*, *C. tropicalis* and *C. krusei* did not cross-react with other *Candida* spp., but the *C. albicans/C. dubliniensis* probe cross-reacted with *C. parapsilosis*, and the *C. parapsilosis* probe cross-reacted with *C. albicans/C. dubliniensis*. However, it was possible to differentiate between *C. albicans/C. dubliniensis* and *C. parapsilosis* following melting curve analysis. Cross-reaction was observed between the 'common *Candida* probe' and *Sacch. cerevisiae*, but the species-specific *Candida* probes did not cross-react with *Sacch. cerevisiae.* Fungal pathogens that were negative with the common *Candida* probe, the species-specific *Candida* probes and the *Aspergillus* probe were *Trichosporon* spp., *Malassezia* spp., *Crypt. neoformans*, *Zygomycetes* spp., *Fusarium* spp., *Scedosporium* spp., *Paecilomyces* spp. and *Penicillium* spp. (Table 1).

All negative controls, consisting of RCLB or DNA extracted from blood from healthy volunteers, gave negative results in LightCycler runs. A wide range of template DNA, extracted from various clinical isolates of non-fungal pathogens and human cells, remained negative with the primer pairs, as also described previously [20].

Candida and *Aspergillus* PCR results with patient samples

In total, 1650 samples from 379 patients were investigated (Table 2). Of these, 114 (6.9%) from 74 patients (1–74) were PCR-positive, comprising 86 (5.2%) from 50 patients positive for *Candida* spp., and 28 (1.8%) from 24 patients positive for *Aspergillus* spp. (samples from two patients were positive for both *Candida* spp. and *Aspergillus* spp.). Table 2 summarises the results obtained with different types of specimens (described in more detail below). Table 3 compares the results obtained by PCR for the identification of *Candida* to the species level with the results obtained by conventional culture.

Characteristics of PCR-positive patients

The 74 PCR-positive patients comprised 38 females and 36 males, with a median age of

Table 2. PCR results showing *Candida*-positive samples (n = 86) and *Aspergillus*-positive samples (n = 28)

Samples	No of samples	PCR-positive Cand/Asp	Culture-positive Cand/Asp	Culture-negative Cand/Asp	Not done Cand/Asp	Not known Cand/Asp	CT-verified Cand/Asp	BAL-positive Cand/Asp	Other positive tests Cand/Asp
Blood	1330	19/19	8/0	5/10	6/7	0/2		0/2[b]	0/Not done
BAL	89	9/7	7/3	2/4					0/2[c]
CSF	24	4/0	1/0[a]	1/0	2/0		1/0		
Biopsy	25	6/0	4/0		2/0		2/0		
Bile	43	31/0	23/0	1/0	7/0				
Pleura	32	0/1	0/1						
Abscess	50	6/0	5/0	1/0					
Urine	10	3/0	2/0	1/0					
Sputum	7	1/1	1/1						
Tracheal secretion	6	1/0	1/0						
Drainage	19	6/0	5/0	1/0					
Various	15	0/0							
Total	1650	86/28	57/5	12/14	17/7	0/2	3/0	0/2	0/2

[a]Microscopy-positive.
[b]Two cultures positive for *Aspergillus* spp. in bronchoalveolar lavage (BAL) fluid.
[c]One microscopy- and one antibody-positive sample for *Aspergillus* spp.
Candida verified, 60 (83.4%) of 72; *Aspergillus* verified, nine (50%) of 18.
Cand, *Candida*; Asp, *Aspergillus*; CT, computerised tomography; CSF, cerebrospinal fluid.

Table 3. *Candida* spp. identified by PCR, compared with results of conventional culture

PCR results	(n = 86)	Culture results	Positive = 57	Negative = 11	Not done = 18
Candida albicans	31	*C. albicans*	27		4
Candida tropicalis	4	*C. tropicalis*	4		
C. albicans/tropicalis	5	*C. albicans*	2	2	1
C. albicans/Candida parapsilosis	1	*C. albicans*	1		
Candida glabrata	12	*C. glabrata*	9		3
C. glabrata/Candida krusei	4	*C. glabrata/C. krusei*	3		
		C. glabrata	1		
C. parapsilosis	2	*C. parapsilosis*	1	1	
C. krusei	3	*C. krusei*	3		
Candida spp.	24	*C. albicans*	4	8	10
		C. albicans/C. tropicalis	2		

Identification to the species level was possible by PCR in 62 (72%) of 86 samples.

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35 years (range 6 months to 84 years). Significant underlying conditions were allogeneic stem cell transplantation (SCT) (n = 27), liver transplantation (n = 17), kidney transplantation (n = 2), heart transplantation (n = 1), abdominal surgery and treatment in an intensive care unit (n = 5), abdominal surgery (n = 1), vascular surgery (n = 1), treatment in an intensive care unit (n = 3; comprising two patients with myeloma, and one tetrapares with pneumonia), haematological malignancies (n = 8) and extracorporeal membrane oxygen treatment (n = 3); there was also one infant with hydrocephalus and shunt (n = 1), one neonate, and four other patients.

Blood samples

Candida. Nineteen (1.4%) of 1330 blood samples were PCR-positive for *Candida* spp. The 19 positive samples came from 18 patients, and eight (61%) of 13 samples from seven patients (two haematology, three abdominal surgery, one liver transplant and one allogeneic SCT) were verified by blood culture (Table 2; four *C. albicans*, two *C. parapsilosis* and three *C. krusei*). Blood cultures from five patients were negative (one haematology and four allogeneic SCT), and blood cultures were not performed for six patients (all allogeneic SCT). In two patient samples, although the melting curves were positive, the PCRs were negative initially (perhaps because of inhibition caused by excess total DNA, with 2748 ng/sample and 975 ng/sample, respectively), but were positive following dilution of the DNA extract. For ten patients (eight allogenic SCT, one haematology and one abdominal surgery), PCR was positive for *Candida* to the genus level, but was negative with all the species-specific probes. For the patient with abdominal surgery, identification to the species level could not be performed because of insufficient DNA.

Aspergillus. Nineteen (1.4%) of 1330 blood samples were PCR-positive for *Aspergillus*, all from 17 immunocompromised patients (Tables 2 and 4). All blood cultures taken were negative for *Aspergillus*, but the diagnosis of pulmonary aspergillosis could be verified by bronchoalveolar lavage (BAL) cultures growing *A. fumigatus* for two patients (61 and 66). Following X-ray, patient 6 was diagnosed with suspected necrotising aspergillosis, while patient 8, who had a negative X-ray at the time of the positive *Aspergillus* PCR test, later developed an aircrest sign on a CT scan of the chest that was suggestive of pulmonary aspergillosis. No X-ray or CT scan was performed for six patients (4, 60, 63, 64, 67 and 70), and no data were available for six patients (35, 52, 53, 68, 69 and 71). Patient 54 had a negative X-ray at the time of a positive *Aspergillus* PCR test (Table 4).

Cerebrospinal fluid samples

Four (17%) of 24 cerebrospinal fluid samples from four patients were PCR-positive for *Candida*. None were PCR-positive for *Aspergillus*. These four patients comprised: an infant with hydrocephalus and shunt who had meningitis and was PCR-positive for *C. albicans*, but culture-negative (although cultures from cerebrospinal fluid taken during an earlier episode had grown *C. albicans*); two allogeneic SCT recipients from whom cultures were not taken (one had meningitis and the other had a fungal lesion of the brain on CT scan); and a patient with neurological symptoms (yeast cells were observed with direct microscopy, but culture was negative; it was believed that

Table 4. *Aspergillus* PCR-positive blood samples from 17 patients

	Antifungal treatment instituted:			
	Before positive PCR sample was taken (patients 35, 61, 63, 66, 67, 71)	After positive PCR sample was taken (patients 4, 60, 69)	No treatment (patients 6, 8, 52 54, 64, 70)	No data (patients 53, 68)
SCT (n = 12)	4	3	5[a] (patient 52 died)	
ALL (n = 1)				1
Cancer (n = 1)				1
Kidney transplant (n = 1)	1			
Liver transplant (n = 1)	1			
COPD + surgery (n = 1)			1 (patient 6 died)	

[a]Two patients (54 and 64) later developed suspected pulmonary aspergillosis; one patient (8) developed probable aspergillosis.
SCT, stem cell transplantation; ALL, acute lymphoblastic leukaemia; COPD, chronic obstructive pulmonary disease.

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contamination occurred at sampling, as the patient received no antifungal treatment, but repeated PCR, direct microscopy and culture remained negative).

Biopsies

Six biopsy samples from three patients were analysed. None was positive for *Aspergillus* by either PCR or culture (if performed). A patient with acute myeloid leukaemia developed chronic disseminated candidiasis of the liver, verified by CT scan. Two liver biopsy samples, taken 3 weeks apart, were PCR-positive for *C. albicans*. A second patient had two biopsy samples taken from the vitreous fluid of the eye that were positive by PCR for *C. albicans*, with the positive results confirmed by cultures and direct microscopy. Finally, an allogeneic SCT recipient had two colon biopsy samples taken that were PCR-positive for *Candida*, with the results confirmed by enrichment cultures that grew *C. albicans*.

BAL samples

Candida. Nine (10%) of 89 BAL samples from seven patients (one allogeneic SCT patient, two intensive care unit patients, two patients who had received extra-corporeal membrane oxygen, and two leukaemic patients) were PCR-positive for *Candida* (four *C. albicans*, four *C. tropicalis*, one *Candida* sp.), with verification by culture for seven BAL samples (Table 2).

Aspergillus. Seven (7.9%) of 89 BAL samples, taken from seven patients (two allogeneic SCT patients, two leukaemic patients, one patient who had received extra-corporeal membrane oxygen, and two other patients) were PCR-positive for *Aspergillus*, with culture verification in five patients (Table 2). For one patient, PCR was positive with BAL fluid from the left lung, where the patient had an infiltrate on X-ray, but was negative on the right side without an infiltrate. For a second patient, PCR was negative with BAL samples, but *A. fumigatus* was grown on culture. The two samples of BAL fluid came from different parts of the lung.

Pleura, sputum and tracheal secretion samples

Two patient samples (one pleural fluid and one sputum) were PCR-positive for *Aspergillus*, and cultures grew *A. fumigatus*. One tracheal secretion was PCR-positive for *C. glabrata* and *C. krusei*, with *C. glabrata* and, some days later, *C. krusei* being grown following culture.

Bile samples

Thirty-one (72%) of 43 bile samples were PCR-positive for *Candida* to the genus level in 15 liver transplant recipients. None was positive for *Aspergillus* by either PCR or culture. Of the 31 samples from 12 patients that were PCR-positive for *Candida*, identification to the species level was achieved in 26 cases. The PCR results were verified by culture in 23 of 24 samples, cultures were not performed in seven cases, and culture was negative for one PCR-positive patient. One patient was PCR-positive for both *C. glabrata* and *C. krusei*, but cultures grew only *C. glabrata* on that occasion, although *C. krusei* was also isolated subsequently (Table 2).

Ascites and abscess fluid samples

Of 50 samples of abscess fluid or ascites, all were PCR-negative for *Aspergillus* and cultures were negative for *Aspergillus*. Six (from five patients) of the 50 samples were PCR-positive for *Candida* and were also culture-positive in five cases. Cultures were negative for all the *Candida* PCR-negative samples ($n = 44$). One ascites sample was PCR-positive for *C. krusei*, two abscess fluid samples were positive for *C. albicans*, and two were positive for *C. glabrata*; cultures grew *C. krusei*, *C. albicans* and *C. glabrata*, respectively. One pancreatic abscess sample was PCR-positive for *C. albicans/C. tropicalis*, but cultures were negative.

Drainage samples

The 19 drainage samples were all PCR- and culture-negative for *Aspergillus*, but six samples (from three patients) were PCR-positive for *Candida*. One patient yielded two samples that were PCR-positive for *C. albicans*, with cultures growing yeast (species identification was not performed). A second patient had negative cultures, while the third yielded three samples that were PCR-positive for *C. albicans* and confirmed by culture (Table 2).

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Urine samples

Three of ten samples, from three patients, were PCR-positive for *C. albicans*. Urine cultures from two patients grew *C. albicans*, while a bladder puncture sample from the third (neonate) patient was culture-negative.

DISCUSSION

Real-time PCR has been used successfully to diagnose infections that are difficult to detect by conventional culture [23,25–30]. Various molecular approaches have been used for the diagnosis of invasive aspergillosis and candidiasis, e.g., PCR amplification followed by restriction enzyme analysis using species-specific probes, and nested PCR amplification [17–20]. However, some of these methods detect only *Aspergillus* spp. or *C. albicans* [17], or only a few of the non-*albicans Candida* spp. known to cause candidaemia [19]. A PCR assay amplifying an 18S rRNA gene has also been described [20].

The real-time PCR assay described in the present study is capable of analysing blood and other body fluids, as well as small biopsy samples, from patients with suspected IFI. The high sensitivity of detection (2 CFU/mL of blood) was achieved by optimising the DNA extraction method with a manual preparation step before performing the DNA extraction with the MagNA Pure robot, and by amplifying a gene found in multiple (> 100) copies.

The DNA extraction method used for fungi is crucial, since DNA extracted from different body fluids contains not only human DNA, but possibly also fungal, bacterial, viral and parasite DNA. The total amount of DNA may vary greatly from sample to sample. Inhibition may occur when using the LightCycler probe system if the total amount of DNA is > 500 ng/sample, and inhibition was observed with two PCR-negative blood samples when the melting curve analysis showed a PCR product with the same melting point as the *Candida* control. Therefore, the DNA concentration should be measured routinely for all samples by spectrophotometry, with dilution of samples before PCR analysis when necessary.

In 60 (83.5%) of 72 PCR-positive samples, the *Candida* PCR results were verified by culture (78%) or other tests (5.5%). Samples that were culture-negative, but *Candida* PCR-positive, were mostly blood samples from allogeneic SCT patients, for whom blood cultures for *Candida* are known to have a low sensitivity (< 50%) [9–12]. PCR was more sensitive than culture for some patients with *Candida* infection verified by CT scan (hepatosplenic and central nervous system lesions).

Only nine (10%) of 89 BAL samples were PCR-positive for *Candida*. All seven patients involved had severe underlying diseases, and for two patients, the PCR and cultures were positive on two occasions each for *C. tropicalis*. Contamination with yeasts when performing BAL with the technique used today seems to be unusual. The patients with *Candida* PCR-positive bile samples were all liver transplant recipients. More than one *Candida* sp. could be detected in the same sample by PCR, with the results confirmed by culture in 96% of cases, and reliable identification of the causative *Candida* pathogen to species level by the assay. Identification of *Candida* spp. is essential for appropriate clinical decision-making concerning the significance of a particular isolate and the choice and dosage of antifungal therapy. The specificity of the assay appeared to be high, and detection of more than one fungal pathogen could be achieved. Cross-reaction between the *Candida* genus probe and *Sacch. cerevisiae* occurs, but *Sacch. cerevisiae* was not isolated in any culture from patients in the present study.

Aspergillus was detected by PCR in 28 (1.7%) of 1650 samples. *Aspergillus* was detected only from blood, BAL, pleura and sputum. In all other samples, PCR and cultures (when taken) were negative for *Aspergillus*. For BAL, pleura and sputum samples, the PCR result was verified in seven (78%) of nine cases. It is well-known that blood cultures are often negative in patients with proven aspergillosis, and it was therefore not possible to compare the PCR-positive results for *Aspergillus* with blood cultures. Interestingly, positive PCR results for *Aspergillus* with blood were found only with blood from 17 severely immunocompromised patients at high risk for IA (Table 4). Of these patients, six were receiving antifungal prophylaxis and three had received early treatment. Five patients did not receive any antifungal treatment; of these, two died, two developed suspected pulmonary aspergillosis, and one developed probable aspergillosis. No data were available for two patients. The final patient (70), a child who was an allogeneic SCT

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recipient and had engraftment at the same time as the PCR was positive for *Aspergillus*, did not develop clinical symptoms of IA despite an absence of antifungal treatment (Table 4).

Construction work in the vicinity is a known risk-factor for IA in immunosuppressed individuals because of the possible exposure to *Aspergillus* spores [31]. During the 17-month study period, 11 allogeneic SCT patients were hospitalised at Karolinska University Hospital Huddinge. During the first 5 months, only one patient was PCR-positive for *Aspergillus* in blood. During the following 6 months, extensive construction work took place in the vicinity of the SCT ward, and blood samples for seven patients were PCR-positive for *Aspergillus*. During the subsequent 6-month period, with no construction near the ward, three patients were PCR-positive.

DNA may have been detected at an early stage of infection in some patients, i.e., before clinical signs of disease. 'Infection' and 'disease' might not be synonymous when DNA detection is used for screening. The pathogenesis of IA is poorly understood. Spores are inhaled and may form hyphae when deposited in the alveoli of the lungs. Fungal DNA (spores and/or hyphae) may be released into the bloodstream at this stage, with clinical signs (e.g., patients 6, 61 and 66), but also without real evidence of disease (e.g., patient 8), which does not equate to an absence of disease; indeed, patient 8 later developed aircrest signs on CT scan that were suggestive of pulmonary aspergillosis (Table 4). The spread of *Aspergillus* through the bloodstream to internal organs may take place long before the patient develops clinical symptoms.

The goal of diagnostic research in invasive mycoses should be to detect the presence of the fungus as early as possible. With a rapid and highly sensitive assay, such as a real-time PCR, it may be possible to identify infected patients at an early stage. If so, real-time PCR may be useful also for excluding patients at risk; thus, the negative predictive value might be of great importance.

In conclusion, the real-time PCR assay described in this study provides high sensitivity and specificity for the detection of fungal DNA in blood, various body fluids and biopsy samples within 6 h, and identifies most *Candida* spp. Further clinical studies are needed to elucidate the true potential of such new techniques for different patient groups. There is a need for prospective studies to evaluate the potential benefits of early therapy, based on real-time PCR, in patients at risk for invasive *Candida* and *Aspergillus* infection.

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