Rapid diagnosis of candidaemia by real-time PCR detection of *Candida* DNA in blood samples

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This study prospectively evaluated an 18S rRNA gene-targeted real-time PCR approach in comparison with standard blood culture (BC) diagnostics for rapid diagnosis of candidaemia in a large study population of 384 patients, including 902 whole blood samples from 468 infectious episodes (IEs) of 329 adults and 55 children with haematological malignancies and various forms of immunodeficiency, and intensive care unit patients. Seven out of eight BC-proven cases (87.5 %) of candidaemia and seven out of twelve BC-positive samples (58.3 %) were positive by the *Candida*-specific PCR. A positive PCR result was also obtained for 28/460 BC-negative samples from IEs, including 8 patients with culture-confirmed *Candida* infection at primary sterile body sites. Of the PCR-positive, culture-negative patients, more than 50 % received systemic antifungal therapy. In 432/460 BC-negative IEs, the *Candida* specific-PCR was negative, resulting in a negative predictive value of 99.8 %. In conclusion, the *Candida* specific-PCR approach facilitates rapid detection of *Candida* DNA in blood samples of patients at risk of candidaemia within a few hours. Although standard BC diagnostics appear to remain indispensable for the detection of all cases of candidaemia, this PCR assay allowed the detection of candidaemia at a mean of 3 days earlier than BC diagnostics. Thus, it enables earlier antifungal therapy for patients with suspected candidaemia and may prevent further complications.

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INTRODUCTION

Candidaemia accounts for up to 10 % of bloodstream infections and is associated with an exceptionally high mortality rate, reaching up to 50 % in intensive care unit (ICU) patients (Engel *et al.*, 2007; Laupland *et al.*, 2004; Wisplinghoff *et al.*, 2004). Rapid initiation of antifungal therapy is crucial for the survival of patients (Kumar *et al.*, 2006). At present, blood cultures (BCs) incubated in automated BC systems remain the 'gold standard' for diagnosing candidaemia. However, it usually takes 3–5 days of incubation until growth of *Candida* spp. is detected.

Detection of fungal DNA in blood samples, without prior cultivation, is a promising approach for the rapid detection of *Candida* spp. in patients with suspected candidaemia. Fungal-specific PCR assays, mostly targeting regions of the 5.8S, 18S or 28S rRNA genes, or internal transcribed spacer regions, have been evaluated for use with serum or blood samples of various patient groups, such as neonates, patients with haematological malignancies or ICU patients (Ahmad *et al.*, 2002; Alam *et al.*, 2007; Dunyach *et al.*, 2008; Innings *et al.*,

2007; Klingspor & Jalal, 2006; Maaroufi *et al.*, 2003; Moreira-Oliveira *et al.*, 2005; Tirodker *et al.*, 2003). Recently, a new pre-analytical tool for PCR analysis of blood samples, MolYsis (Molzym), has been brought to market. The principle of MolYsis involves lysis of human blood cells prior to degradation of bacterial and fungal cell walls, allowing digestion of human DNA by DNase and thus enrichment of pathogen DNA (Horz *et al.*, 2008). In addition, PCR inhibitors can be removed efficiently by the MolYsis procedure. In a recent study, the use of MolYsis pre-incubation showed promising results regarding the rapid detection of *Candida* species in BC samples (Gebert *et al.*, 2008).

In the present study, we prospectively evaluated a real-time PCR approach, in conjunction with sample preparation using MolYsis, for rapid diagnosis of candidaemia in a large study population of 384 patients at risk of candidaemia, including children and adults with haematological malignancies and various forms of severe immunodeficiency, and ICU patients.

METHODS

Study population. The study was performed at three hospitals belonging to the University of Ulm (Ulm, Germany) in the

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Abbreviations: BC, blood culture; C_h, threshold cycle; ICU, intensive care unit; IE, infectious episode.

Departments of Medicine, Paediatrics and Surgical Intensive Care from 7 December 2007 to 31 August 2008. The criteria for inclusion of patients in the study were as follows: ICU patients with systemic inflammatory response syndrome or sepsis (Bone *et al.*, 1992), haematology/oncology patients with fever $>38.5^{\circ}\text{C}$ in neutropenia ($<1 \times 10^9$ neutrophils l^{-1}) or patients with other forms of hereditary or acquired immunodeficiency and fever $>38.5^{\circ}\text{C}$. Within one infectious episode (IE), subsequent samples were included only when obtained within 7 days of sampling of the initial sample. A patient was eligible for the study a second time if the new IE occurred more than 4 weeks after the initial inclusion of the patient into the study and if a BC-confirmed infection with an identical species had been ruled out. The study was approved by the ethics committee of the University of Ulm (reference no. 175/07) and informed consent was obtained from all patients or parents of patients included in the study. For ICU patients, post-hoc informed consent was obtained.

Blood samples. Blood samples for PCR were collected in 5 ml (adults), 2.5 ml or 1.4 ml citrate tubes (children) (Sarstedt). Prior to the start of the study, the use of EDTA and citrate anti-coagulated blood was compared. In three independent experiments, we found a slightly higher sensitivity of the PCR assays in citrate anti-coagulated blood in comparison with EDTA anti-coagulated blood. The blood collection team was advised to obtain a citrate blood sample for PCR and a 20 ml blood sample for inoculation of a pair of aerobic and anaerobic BACTEC Plus/F BC bottles (BACTEC PED bottles in children) from the same venepuncture or catheter blood sample, respectively. The blood sample for PCR analysis was fixed to the parallel BC to allow definite allocation. Blood samples and BCs were transported to the laboratory within 16 h. BCs were incubated in an automated BACTEC 9240 system for 7 days and cultured *Candida* isolates were identified by API ID32C (bioMérieux). Blood samples for PCR analysis were stored at -2 – -8°C until DNA isolation.

DNA isolation. Isolation of DNA was performed twice daily from Monday to Friday, using a MolYsis Complete5 kit (Molzym). The principle of the MolYsis Complete kit involves lysis of human blood cells prior to degradation of bacterial and fungal cell walls, allowing enrichment of pathogen DNA (Gebert *et al.*, 2008; Horz *et al.*, 2008). The kit was used as indicated by the manufacturer with the following adaptations: the volumes of the CM and DB1 buffers were adapted depending on the blood sample volume: 250 μl each buffer was added to 1 ml blood samples, 800 μl each to 2 ml blood samples, and 2 ml each to 5 ml blood samples. Before incubation of the samples with BugLysis solution (37°C , 30 min; contained in the kit), 1.4 μl β -mercaptoethanol was added. The isolated DNA was eluted in 100 μl EB buffer and stored at -20°C until use. With each kit, a PCR-grade water control for DNA isolation was used. DNA isolation was carried out under a HEPA-filtered PCR working bench, which was decontaminated daily by UV radiation and strictly separated from PCR working places.

PCR analysis. For detection of *Candida* species, a *Candida*-specific 18S rRNA gene-targeted PCR was run on a LightCycler 1.0 instrument. The amplification mixture consisted of 0.25 μM primer FungL543 and 0.75 μM primer FungL1046R (Einsele *et al.*, 1997), 0.15 μM probe Can-LC (5'-CCAAGGACGTTTTCATTAATCAAGA-3'), 0.15 μM probe Can-FL (5'-LCRed640-CGAAAGTTAGGGGATCGAAGATG-3') (Klingspor & Jalal, 2006), 4 μl LightCycler FastStart DNA Master^{PLUS} HybProbe-Mix and 5 μl template DNA, in a total volume of 20 μl . The primer FungL1046R was used at a higher concentration than the forward primer in order to decrease an observed 'hook' effect seen with equimolar concentrations. Samples were amplified with the following program: initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation for 10 s at 95°C , annealing for 10 s at 58°C and elongation for 20 s at 72°C , followed by melting-curve analysis. In every PCR run, a negative

control (PCR-grade water) and a positive control (*Candida albicans* DNA at 1 pg μl^{-1}) were included. DNA extracted from three healthy volunteers was investigated before initiation of the study and was negative in the PCR assay (data not shown).

The sensitivity and specificity of the PCR probes have been determined elsewhere (Klingspor & Jalal, 2006). We also investigated DNA samples (10 pg μl^{-1}) of each of the following fungal isolates with the PCR assay used in this study and all isolates resulted in correct positive and negative results: *C. albicans* (ATCC 14053), *Candida ciferrii* [control strain from the World Health Organization Collaborating Center for Quality Assurance and Standardization in Laboratory Medicine (INSTAND)], *Candida dubliniensis* (control strain 01-2004; INSTAND), *Candida famata* (DSM 3428), *Candida glabrata* (ATCC 90030), *Candida kefyr* (DSM 11954), *Candida krusei* (ATCC 90878), *Candida lusitanae* (clinical isolate), *Candida parapsilosis* (ATCC 22019), *Candida rugosa* (DSM 70761), *Candida tropicalis* (clinical isolate), *Candida valida* (control strain from INSTAND), *Absidia corymbifera* (clinical isolate), *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger* (all clinical isolates), *Cryptococcus neoformans* (ATCC 62066), *Saccharomyces cerevisiae* (ATCC 9763), *Scedosporium apiospermum* (clinical isolate), and *Trichosporon inkin* (clinical isolate). A BLAST analysis revealed 100% identity of the combined probe sequence with the environmental fungi *Macrorhabdus ornithogaster* (GenBank accession no. AF350243), *Pichia anomala* (EF550479), *Saccharomyces cerevisiae* (EF153844), *Saccharomycopsis microspora* (EU057528) and *Zygosaccharomyces lentus* (Y16814). The limit of detection was repeatedly determined as 10 fg *C. glabrata* DNA μl^{-1} , i.e. 50 fg per PCR, in sterile water. In addition, the sensitivity of the assay in spiked citrate blood of healthy volunteers was investigated in three repeated experiments. A detection limit of 1 c.f.u. per PCR or 3 c.f.u. (ml blood) $^{-1}$ was determined in all three experiments.

RESULTS AND DISCUSSION

Study population

A total of 902 blood samples from 384 patients (329 adults and 55 children <18 years) were included in the study. The samples were obtained in 468 IEs, including 379 from adults and 89 from children. A single blood sample was obtained in 197 IEs, two in 126 IEs, three in 134 IEs and four or more in 11 IEs.

PCR results of BC-positive samples

Candida species were cultured in 12 of the 902 BCs, comprising 7 isolates of *C. albicans*, 1 *C. glabrata* isolate and 4 isolates of *C. tropicalis*. *Candida* DNA was detected by PCR in 7 of the 12 corresponding blood samples (Table 1). Separate analysis of adult ICU patients and patients with severe immunosuppression (haematology/oncology patients and patients with hereditary immunodeficiency, severe immunosuppressive therapy or AIDS) revealed a higher sensitivity of the PCR assay in samples from ICU patients (Table 2). It can be speculated that this was caused by a higher load of fungal DNA in ICU patients, indicated by lower threshold cycle (C_t) values of PCR-positive samples (Table 3) or by an inhibitory effect of chemotherapeutic therapy of haematological patients on the fungal DNA in blood. Nevertheless, due to the small

Table 1. BC and PCR results

Sample	BC result for <i>Candida</i> spp.	PCR positive	PCR negative	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Blood/BC (<i>n</i> =902)	Positive	7	5	12	58.3	96.2	17.0	99.4
	Negative	34	856	890				
IEs (<i>n</i> =468)	Positive	7	1	8	87.5	94.0	20.0	99.8
	Negative	28	432	460				

PPV, Positive predictive value; NPV, negative predictive value.

Table 2. BC and PCR results in samples from ICU patients and patients with immunosuppression

Blood/BC sample	BC result for <i>Candida</i> spp.	PCR positive	PCR negative	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
ICU patients (<i>n</i> =420)	Positive	5	0	5	100	97.1	29.4	100
	Negative	12	403	415				
Patients with immunosuppression (<i>n</i> =487)	Positive	2	5	7	28.6	95.4	8.3	98.9
	Negative	22	458	480				

PPV, Positive predictive value; NPV, negative predictive value.

Table 3. Characteristics of BC- and PCR-positive patients

Patient no.	Age (years)	Ward	Underlying disease	BC result	No. positive BC/no. sampled	BC time to positivity (h)*	No. PCR-positive samples/no. sampled	C _t value of PCR-positive samples	No. PCR- and BC-positive samples	Sampling days†	Antifungal therapy‡
137	72	Hema	Adenocarcinoma from an unknown primary tumour	<i>C. albicans</i>	1/1	57	1/1	29	1	Day 1	Day 4, FLU 200
176	59	Hema	Malignant thymoma	<i>C. tropicalis</i>	2/4	18/18	1/4	36	0	Day 1, 4, 7, 8	Day 9, FLU 400
199	65	Hema	Metastatic breast cancer, polythrombotic disease	<i>C. glabrata</i>	1/2	83	1/2	34	0	Day 1	Day 3, VORI 800
355	56	ICU	Gastric cancer	<i>C. albicans</i>	1/3	72	3/3	29/32/36	1	Day 1, 2	Day 4, CASPO
359	46	ICU	Small bowel ischaemia, aortic thrombosis	<i>C. albicans</i>	3/3	40–48	3/3	32/34/36	3	Day 1	Day 3, VORI 800
422	66	ICU	Near-drowning, mechanical ileus	<i>C. albicans</i>	1/3	124	2/3	32/32	1	Day 1, 3	Day 3, FLU 400
845	42	Hema	Acute myeloid leukaemia	<i>C. tropicalis</i>	2/2	25/38	1/2	37	1	Day 1	Day 2, CASPO
411	48	Hema	Acute myeloid leukaemia	<i>C. albicans</i>	1/1	74	0/1	–	0	Day 1	Day 6, CASPO

Hema, Haematology/oncology.

*Incubation time until positive signal was obtained using the BACTEC system.

†Days at which blood samples were taken for PCR and BC (day 1=first sample).

‡Day of initiation (day 1=day of sampling of first sample) and antifungal treatment used: FLU 200, 200 mg fluconazole day⁻¹; FLU 400, 400 mg fluconazole day⁻¹; VORI 800, 800 mg voriconazole day⁻¹; CASPO, caspofungin.

Table 4. Characteristics of BC-negative, PCR-positive patients

Patient no.	Age (years)	Ward	Underlying disease	No. PCR positive samples/no. sampled	C _t value of PCR positive samples	Antifungal therapy or prophylaxis*	Positive <i>Candida</i> spp. culture in other specimens†
43	48	ICU	Vertebral osteomyelitis	1/3	30	None	+ + + <i>C. albicans</i> , bronchial secretions (day -1 and 0)
76	49	ICU	AIDS, <i>Pneumocystis jiroveci</i> pneumonia	2/2	32/32	None	+ + + non- <i>albicans Candida</i> , CVC (day +1)
98	65	ICU	Community-acquired pneumonia	1/3	35	200 mg fluconazole per day	+ <i>C. albicans</i> in tracheal aspirate (day 0); + + <i>C. albicans</i> in bronchial secretion (day -3 and +2)
183	47	ICU	Oesophagus perforation	1/3	35	None	+ <i>C. albicans</i> in tracheal aspirate (day +1); + non- <i>albicans Candida</i> in pleural effusion (day +2)
207	62	ICU	Aortic aneurysm	1/3	37	None	+ <i>C. albicans</i> in bronchial secretion and tracheal aspirate (day -3)
221	60	Hema	Multiple myeloma	1/3	33	3 g amphotericin OS per day	+ + <i>C. albicans</i> in stool (day +3)
304	38	ICU	Thoracic spine spondylodesis	1/3	34	None	+ + <i>C. albicans</i> in bronchial secretion and tracheal aspirate (day +2)
327	46	ICU	Stomach perforation	1/3	37	None	+ + + <i>C. albicans</i> in intra-abdominal swab (day -1 and +2)
341	64	ICU	Coronary artery disease	1/3	33	None	+ <i>C. non-albicans</i> in sputum (day -1)
834‡	65	Hema	Metastatic breast cancer, poly-thrombotic disease	1/1	34	600 mg posaconazole per day	<i>C. glabrata</i> in BC (day -21); + + non- <i>albicans Candida</i> in sputum (day 0)
8	45	Hema	Acute myeloic leukaemia	1/5	36	3 g amphotericin OS per day	No
29	7	Hema	Acute lymphatic leukaemia	1/2	34	None	No
44	23	Hema	Acute lymphatic leukaemia	1/2	34	600 mg posaconazole per day	No
65	62	Hema	Myelodysplastic syndrome	1/2	35	800 mg posaconazole per day	No
66	43	Hema	Non-Hodgkin lymphoma	1/2	37	600 mg posaconazole per day	No
100	51	Hema	Acute myeloic leukaemia	1/3	37	3 g amphotericin OS per day	No
162	72	Hema	Multiple myeloma	1/1	36	100 mg fluconazole per day	No
165	59	Hema	Osteomyelofibrosis	1/1	37	600 mg posaconazole per day	No
170	75	Hema	Non-Hodgkin lymphoma	1/3	35	None	No
181	39	Hema	Mantle cell lymphoma	1/1	35	100 mg fluconazole per day	No
220	40	ICU	Sarcoidosis	1/3	36	None	No
235	79	ICU	Coronary artery disease, preterminal renal insufficiency	1/2	36	None	No
293	71	Hema	Acute myeloic leukaemia	1/2	36	400 mg voriconazole per day	No
302	55	Hema	Chronic lymphatic leukaemia	1/1	34	600 mg posaconazole per day	No
316	5	Hema	Burkitt lymphoma	1/2	37	None	No
356	0.5	Hema	Nephroblastoma	1/3	37	None	No
836	70	Hema	Acute myeloic leukaemia	1/1	34	600 mg voriconazole per day	No
837	28	Hema	Acute myeloic leukaemia	1/3	37	600 mg posaconazole per day	No

CVC, Central vascular catheter; Hema, haematology/oncology; OS, oral suspension.

*Actual therapy or prophylaxis at day of blood sampling for PCR. In all patients the antifungals were already given for a minimum of 2 days preceding blood sampling.

†Day of positive culture result with regard to the sampling day of the PCR-positive blood sample is given in parentheses; day 0=day of blood sampling.

‡Second IE of patient no. 199 (Table 2), 40 days after first inclusion into the study.

number of positive samples, the data are still preliminary. In all BC-positive, but PCR-negative samples, PCR inhibition was excluded by spiking of the DNA sample with *C. albicans* DNA (1 pg μl^{-1}).

The 12 positive BCs were sampled from 8 patients (Table 3). Defining a PCR-positive patient as a patient with a positive PCR result in at least one blood sample of all samples obtained within the same IE, seven of the eight BC-positive patients were positive in the *Candida* specific-PCR (Tables 1 and 3), corresponding to an IE-related sensitivity of 87.5 %. This sensitivity is in accordance with published studies reporting sensitivities of 72.1 to 100 % (Ahmad *et al.*, 2002; Alam *et al.*, 2007; Dunyach *et al.*, 2008; Innings *et al.*, 2007; Klingspor & Jalal, 2006; Maaroufi *et al.*, 2003; Moreira-Oliveira *et al.*, 2005; Tirodker *et al.*, 2003). In contrast to former studies, however, we investigated a large, heterogeneous study population at risk of candidaemia, consisting of both patients with immunodeficiency and patients treated in the ICU, in order to evaluate the PCR approach regarding its hospital-wide applicability in a tertiary-care university hospital.

In patients with more than one blood sample investigated, the samples were taken within 1–7 days (Table 3). Remarkably, in patient 176, the PCR was already positive in the first sample (day 1), but the candidaemia was first detected by BC in the day 7 sample. In the other patients, BC and PCR were positive in samples obtained on the same day. After receiving the positive BC result, all patients were treated with systemic antifungals. Whilst the BCs were positive after a mean of 60 h of incubation (Table 3), a positive PCR result could be obtained within 4 h of arrival of the sample in the laboratory and may thus enable significantly earlier initiation of antifungal therapy.

PCR results of BC-negative samples

A positive *Candida* specific-PCR result was obtained in 34/890 BC-negative samples and in 28/460 IEs, respectively (Tables 1 and 4). PCR was positive in 2/2 samples in a patient with culture-confirmed central venous catheter infection with *C. albicans* (Table 4). In another seven patients, *Candida* spp. was cultured in specimens from a primary sterile body site ± 3 days from the day of blood sampling (Table 4). Therefore, in these patients, invasive *Candida* infection associated with candidaemia is very likely. Two further patients were colonized with *Candida* spp. in sputum or stool (Table 4). Of the remaining 18 culture-negative but PCR-positive patients, 10 (55.6 %) were receiving systemic antifungal therapy and 2 (11.1 %) were treated with amphotericin oral suspension on the day of blood sampling (Table 4). Unfortunately, *Candida* colonization of the patients was not systematically recorded in this study, but due to their underlying disease and/or actual medical treatment, all patients were at increased risk of systemic *Candida* infection. Positive *Candida* specific-PCR results in BC-negative patients with suspected invasive

Candida infection have also been reported sporadically in other studies (Ahmad *et al.*, 2002; Alam *et al.*, 2007; Dunyach *et al.*, 2008; Klingspor & Jalal, 2006; Maaroufi *et al.*, 2003). Although growth of *Candida* spp. in BC is considered the 'gold standard' for diagnosis of candidaemia, the sensitivity of BC is limited (Bretagne & Costa, 2006) and may be further decreased by prophylactic administration of antifungal agents. We assume that the positive PCR in BC-negative patients most likely reflects the presence of at least temporary candidaemia. However, a false-positive PCR result cannot be fully excluded.

In conclusion, the *Candida* specific-PCR approach presented here enables rapid detection of *Candida* DNA in blood samples of patients at risk of candidaemia. Although standard BC diagnostics appear to remain indispensable for the detection of all cases of candidaemia, this PCR assay allowed detection of candidaemia at a mean of 3 days earlier than BC diagnostics and detected *Candida* DNA in the blood samples of patients with culture-documented but BC-negative *Candida* infection. Although identification of the *Candida* species is important for appropriate treatment and a reduction of the risk of development of resistance, it is critical to get a rapid result of candidaemia to achieve an optimal outcome for the patient. The total assay time of 4 h, including isolation of DNA and PCR analysis, even allows reporting of the result on the same day that the sample is taken. Thus, it enables earlier antifungal therapy of the patients and may prevent further complications of candidaemia such as dissemination to other organs. In addition, in BC-negative patients, PCR may also be used as an adjunctive tool for exclusion of candidaemia in patients at high risk of systemic *Candida* infection, especially those receiving antifungal agents at a prophylactic dosage.

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