An improved rapid quantitative detection and identification method for a wide range of fungi

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To develop a rapid and quantitative diagnostic technique for the detection and identification of a wide range of fungi, an improved molecular method based on real-time PCR and the analysis of its products that targets the internal transcribed spacer (ITS) 2 region was established. The real-time PCR could quantitatively and specifically detect the ITS2 region from all 24 tested pathogenic fungal species at between 10^1 and 10^7 copies per test without amplification of bacterial or human DNA. The sequences of the primer-binding sites are conserved in the registered sequences of 34 other pathogenic fungal species, suggesting that the PCR would also detect these species. The hyperpolymorphic nature of the ITS2 region between fungal species in terms of length and nucleotide sequence provided valuable information for the determination of species. By labelling the 5' end of the reverse primer with NED fluorescent dye, the fragment lengths of the real-time PCR products and their 3'-terminal fragments, derived using restriction enzyme *Sac*FI digestion, were easily evaluated by capillary electrophoresis. Using this analysis, the number and species of fungi present in samples could be estimated. Moreover, sequence analysis of the real-time PCR products could accurately determine species in samples containing a single species. This diagnostic technique can estimate a wide range of fungi from various clinical samples within 1 day and accurately identify them in 2 days. Quantitative results for fungal titre in samples can also provide useful information for understanding the progression of disease and the efficacy of antifungal chemotherapy.

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INTRODUCTION

In recent years, the incidence of invasive opportunistic fungal infections has been increasing due to increases in the number of immunocompromised patients (Warnock, 2007). The survival of these patients is closely correlated with early antifungal chemotherapy (Morrell *et al.*, 2005). However, conventional diagnostic techniques for fungal infections, such as blood culture and biochemical tests, lack sufficient sensitivity and specificity, and require several days or even weeks to obtain results (Espinel-Ingroff *et al.*, 1998; Goodrich *et al.*, 1991; Wald *et al.*, 1997). Therefore, many cases of fungal infection have been treated through

the empirical use of antifungal compounds, although relatively lower susceptibilities of some fungi to certain compounds have been observed, such as *Trichosporon* to amphotericin B, *Candida glabrata*, *Candida krusei* and *Aspergillus* to fluconazole, and *Candida parapsilosis* and *Cryptococcus neoformans* to micafungin.

To meet the need for more rapid and accurate species identification, methods based on PCR, such as random amplified polymorphic DNA (RAPD)-PCR, restriction fragment length polymorphism (RFLP) analysis, real-time PCR with species-specific probes, and sequence analysis of amplicons, have been developed (Esteve-Zarzoso *et al.*, 1999; Kami *et al.*, 2001; Löffler *et al.*, 2000; Millon *et al.*, 2002; Pryce *et al.*, 2003). In particular, the use of real-time PCR technology has provided not only rapid and accurate results, but also clinical markers for understanding the progression of diseases and the efficacy of antifungal therapy via the fungal titre in samples (Kami *et al.*, 2001).

Abbreviations: ITS, internal transcribed spacer; T-RFLP, terminal restriction fragment length polymorphism.

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In these studies, most of the improved PCR protocols employ universal primers that are designed for conserved regions, such as the 18S, 5.8S and 28S rRNA genes, since an ever-increasing number of species of fungi have been associated with opportunistic infection (Chen *et al.*, 2001; De Baere *et al.*, 2002; Esteve-Zarzoso *et al.*, 1999; Jordanides *et al.*, 2005; Kurtzman & Robnett, 1997; Löffler *et al.*, 2000; Perfect & Schell, 1996; Turenne *et al.*, 1999; Velegraki *et al.*, 1999). However, these methods have not been optimized to cover a wide range of fungal species or to differentiate species. Therefore, in this study we report an improved rapid diagnostic technique targeting a wide range of fungi that can determine the titre of the fungus, clarify whether the infection is caused by a single species or a mixed infection, and identify pathogens from clinical samples, even if those samples contain human DNA.

METHODS

Strains. The reference strains listed in Table 1 were obtained from the German Collection of Micro-organisms and Cell Cultures Biological Resource Centre (Braunschweig, Germany) and NITE Biological Resource Centre (Kisarazu, Japan). Yeasts were grown on a malt agar medium for 48 h at 25 °C, and moulds were grown on a potato dextrose agar medium for up to 7 days at 30–35 °C in accordance with the supplier's instructions.

Nucleotide sequences of the internal transcribed spacer (ITS) 2 region from the reference strains were determined by a PCR-directed sequencing method. The target region was amplified with PCR using the primers 18S-F (forward primer; 5'-GAACCTGCGGAAGGATC-ATTA-3') and 28S-R (reverse primer; 5'-TACTTGTTCGCTAT-CGGTCT-3'), which were located outside the universal fungal primers ITS86M-F and ITS4M-R (Fig. 1b). The sequencing reaction was carried out using the same primers, and sequences were analysed on an ABI PRISM 3100 genetic analyser (Applied Biosystems).

Table 1. Predicted and evaluated fragment length of PCR products from the 24 reference strains

No.	Organism	Ref. strain* (accession no.)	GenBank†			Sequence‡		GENESCAN§	
			Accession no.	Length of PCR product (bp)	Length restricted by <i>ScrFI</i> (bp)	Length of PCR product (bp)	Length restricted by <i>ScrFI</i> (bp)	Length of PCR product (bp)	Length restricted by <i>ScrFI</i> (bp)
1	<i>Candida albicans</i>	DSM1386 (AB305093)	AY939789	297	297	297	297	297	297
2	<i>Candida glabrata</i>	DSM6425 (AB305094)	AY198398	376	324	378	326	377	323
3	<i>Candida krusei</i>	DSM6128 (AB305095)	AY939808	307	255	307	255	305	253
4	<i>Candida parapsilosis</i>	DSM5784 (AB305096)	AY217022	270	270	270	270	270	270
5	<i>Candida famata</i>	DSM70590 (AB305097)	AY125962	340	340	340	340	339	340
6	<i>Candida guilliermondii</i>	DSM11947 (AB305098)	AF218967	338	89	338	89	338	87
7	<i>Candida tropicalis</i>	DSM11953 (AB305099)	AY939810	287	192	287	192	287	190
8	<i>Candida kefyr</i>	NBRC0008 (AB480229)	AF218997	384	339	386	340	383	339
9	<i>Aspergillus flavus</i>	DSM 818 (AB305100)	AY939782	313	68	312	68	309	64
10	<i>Aspergillus fumigatus</i>	DSM 790 (AB305101)	DQ459328	312	105	312	105	307	102
11	<i>Aspergillus niger</i>	DSM 737 (AB305102)	DQ985232	313	68	313	68	308	64
12	<i>Aspergillus terreus</i>	NBRC4100 (AB480225)	AY939788	313	67	315	68	314	66
13	<i>Trichosporon cutaneum</i>	DSM70675 (AB305103)	AF444325	312	178	310	176	309	174
14	<i>Trichosporon mucoides</i>	DSM12017 (AB305104)	AF455482	308	174	310	176	309	174
15	<i>Cryptococcus neoformans</i>	DSM11959 (AB305105)	L14068	334	135	333	134	332	132
16	<i>Rhodotorula mucilaginosa</i>	DSM18184 (AB305106)	AF444635	363	113	363	113	363	110
17	<i>Fusarium solani</i>	DSM1164 (AB305107)	DQ486879	312	211	316	215	309	211
18	<i>Mucor circinelloides</i>	DSM1175 (AB305108)	AY243943	331	331	330	330	329	329
19	<i>Rhizopus oryzae</i>	DSM 853 (AB305109)	AY803931	335	107	335	107	336	104
20	<i>Clavispora lusitaniae</i>	NBRC1019 (AB480227)	(–)¶	(–)	(–)	211	125	211	124
21	<i>Scedosporium apiospermum</i>	NBRC31146 (AB480226)	AJ888399	338	111	339	111	332	103
22	<i>Absidia corymbifera</i>	DSM1144 (AB305110)	AY944897	425	425	424	424	424	424
23	<i>Trichophyton rubrum</i>	DSM4167 (AB305111)	AY213689	330	102	327	96	326	93
24	<i>Rhizomucor pusillus</i>	DSM1331 (AB305112)	DQ119005	349	349	349	349	349	349

*Reference strains and accession numbers (in parentheses) for the sequences determined in this study.

†Predicted fragment length from the nucleotide sequence registered in GenBank.

‡Predicted fragment length from our sequencing results of the 24 reference strains.

§Fragment length of PCR products from the 24 reference strains as determined by GENESCAN analysis.

||Terminal fragment length of PCR products digested with the restriction enzyme *ScrFI*.

¶Data not registered in GenBank.

Fig. 1. Design of universal fungal primers. Location and direction of the newly designed primers (a) and comparison of the sequences of these primers with those of previously reported primers (b). Direction and position of primers ITS86M-F and ITS4M-R are shown by arrows and the NED-labelled 5' end of ITS4M-R is shown by an open circle. The sequences of the primers designed in this study and those reported by Turenne *et al.* (1999) are enclosed with solid and dashed lines, respectively. Nucleotides identical to those observed in humans are marked with asterisks.

Extraction of fungal DNA. DNA was extracted from 500 μ l of whole EDTA-anticoagulated blood or fungal suspension in water by a previously described method with some modifications (Löffler *et al.*, 1996). Briefly, after the addition of 3 ml erythrocyte lysis buffer [10 mM NaCl, 10 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$], blood samples were incubated on ice for 10 min, and then leukocytes and microbes were pelleted by centrifugation at 3000 r.p.m. (800 g) for 10 min with a low-speed centrifuge. The pellet was washed once with erythrocyte lysis buffer and resuspended in 450 μ l lyticase buffer [50 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.2% 2-mercaptoethanol]. After sonication at full power for 3 min with an ELESTEIN035 ultrasonic homogenizer (Elekon, Tokyo, Japan), 50 μ l enzyme solution [100 U ml^{-1} of recombinant lyticase (Sigma-Aldrich) and 10 mg ml^{-1} of lysozyme chloride in lyticase buffer] was added to the samples. After incubation at 37 °C for 30 min, the resultant spheroplasts were broken using an ultrasonic homogenizer for 5 min and DNA was spun down at 18000 r.p.m. (30000 g) for 10 min with a microcentrifuge. DNA was purified from the pellet with a QIAmp DNA Mini kit (Qiagen) according to the manufacturer's instructions. The DNA recovered from 50 μ l elution buffer was analysed immediately or stored at -20 °C until testing.

Construction of DNA standards. The ITS2 region was amplified from *Candida glabrata* by PCR using the universal fungal primers ITS86M-F and ITS4M-R (Fig. 1b). The PCR products were then cloned into pGEM-T Easy Vector (Promega) using a TA cloning method. After confirming the nucleotide sequence of the insert, plasmid DNA (pGEM-CG-ITS2) was purified by a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions.

Real-time PCR assay. PCR was performed with 20 μ l of PCR mixture containing 0.2 μ M of each primer, ITS86M-F and ITS4M-R, 10 μ l SYBR Premix EX Taq (Takara Bio) and 2 μ l of sample DNA in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). For this PCR, we used tailed ITS86M-F, which consists of ITS86M-F with seven additional nucleotides at the 5' end, as the forward primer and ITS4M-R, labelled with the fluorescent dye NED (Applied Biosystems) at the 5' end for T-RFLP analysis (described below), as the reverse primer (Applied Biosystems). Initial denaturation was at 95 °C for 10 s, followed by 35 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 66 °C for 31 s. For each run, the standard curve was determined using 10-fold diluted purified plasmid pGEM-CG-ITS2 at 10^1 – 10^7 copies μ l⁻¹. The copy number of the genomic DNA in 1 μ l of sample was then determined from the standard curve with no account taken of the amount of human DNA present in the samples. The specificity of the PCR was analysed by melting curve analysis.

To determine the specificity of the primers ITS86M-F and ITS4M-R, PCR was carried out using DNA prepared from *Candida glabrata*, *Staphylococcus aureus*, *Escherichia coli*, and a human fibroblast cell line, hTART-BJ1 (BD Biosciences Clontech). Conditions for the real-time PCR for fungi using ITS86M-F and ITS4M-R are described above. A fragment of approximately 400 bp from the human suppressor of cytokine signalling-3 (SOCS-3) gene was amplified using primers SOCS3-Nhe (5'-TAGCTAGCGGCGCACGGAGCC-AGCGTGG-3') and SOCS3-400Kpn (5'-TATGGTACCCAGGC-AGTCCCGGGGGCCCC-3'). Initial denaturation was at 95 °C for 2 min, followed by 40 cycles of denaturation (30 s at 95 °C), annealing (1 min at 58 °C) and extension (30 s at 72 °C). Using the universal primers for the bacterial 16S rRNA gene, 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 803r (5'-CATCGTTTACGGCGTGGAC-3'), a fragment of approximately 800 bp from the bacterial gene was amplified under thermal cycling conditions as follows: initial denaturation (95 °C for 2 min) and 40 cycles of denaturation (30 s at 95 °C), annealing (1 min at 56 °C) and extension (1 min at 72 °C).

Fragment length analysis of PCR products. The DNA fragment lengths were determined to estimate the number and approximate the species of fungi present in the sample. One microlitre of the real-time PCR products, labelled with NED by the reverse primer, was treated with or without the restriction enzyme in 10 μ l of reaction mixture. Digestion was performed by incubating a 1 μ l aliquot of the PCR products with the restriction enzyme *ScrFI* in a final reaction volume of 10 μ l at 37 °C for 1 h. After digestion, 1 μ l of the reaction mixture was mixed with 10 μ l capillary electrophoresis mixture, consisting of 9.5 μ l Hi-Di formamide and 0.5 μ l GENESCAN 400HD (ROX) or 500 (LIZ) Size Standard (Applied Biosystems), and analysed with an ABI PRISM 3100 genetic analyser using GENESCAN analysis software version 3.7 (Applied Biosystems).

RESULTS AND DISCUSSION

Design of universal fungal primers for real-time PCR

Four regions, the 18S rRNA gene, ITS1 region, ITS2 region and 28S rRNA gene (D1/D2 region), have been previously reported as potential targets for the development of a universal fungal PCR primer (Chen *et al.*, 2000; Kurtzman & Robnett, 1997; Lott *et al.*, 1993; Makimura *et al.*, 1994; Turenne *et al.*, 1999). We aligned these regions from the human DNA sequence and various fungal DNA sequences

derived from our original sequencing results and the GenBank database, and searched the regions with a consensus sequence for most fungal species but not the human sequence. From these regions, we chose two candidate sites, in the 5.8S and 28S rRNA genes, for the construction of a primer set for real-time PCR whereby the PCR products would be kept below 400 bp through a limitation of the real-time PCR and at least one primer would be specific for fungi, so as to avoid amplification of any human DNA present in the clinical samples (Fig. 1a). The primer set designed for the regions had been reported as ITS86 and ITS4 (Turenne *et al.*, 1999); however, we modified these primers to create ITS86M-F and ITS4M-R (Fig. 1b) with the following advantages; (i) the lengths of ITS86M-F and ITS4M-R were 2- and 8-mer longer than the original primers, respectively, leading to an increase in T_m and specificity, (ii) ITS86M-F was synthesized as a tailed primer consisting of a specific sequence for the fungal 5.8S rRNA gene and seven additional nucleotides at the 5' end to stimulate *Taq* polymerase for the generation of single-base 3' adenine overhangs and to produce PCR products of uniform length, and (iii) ITS4M-R was labelled with the fluorescent marker NED at the 5' end for the detection of PCR products by capillary electrophoresis. These primers amplified a single PCR product from *Candida glabrata* but none from human or bacterial DNA, and were able to successfully amplify the ITS2 region from each of the 24 fungal reference strains.

Sample preparation

To increase the usefulness of our assay for practical diagnostic purposes, a reduction in time taken from sample collection to final diagnosis was also required. DNA extraction is generally a time-consuming and troublesome step, requiring more than half a working day (Löffler *et al.*, 1996; Lugert *et al.*, 2006). Therefore, we improved this step through the use of an ultrasonic homogenizer instead of proteinase treatment for disrupting fungi and established a rapid and quantitative DNA extraction method (see Methods).

Sensitivity of real-time PCR

The detection limit and quantification capacity of the real-time PCR assay for fungi with the primer set ITS86M-F/ITS4M-R was evaluated using the *Candida glabrata* target region cloned into the pGEM-T plasmid as a template. The 100% detection limit of PCR was 10 copies per microlitre of the template (Fig. 2a). Melting curve analysis indicated that the reaction products were specific and a quantitative result was obtained from 10^1 – 10^7 copies μL^{-1} (Fig. 2b, c). This PCR efficacy would be influenced by the amount of human DNA present in samples. It is impossible to adjust the amount of human DNA present in samples isolated from a variety of clinical specimens – blood, pus, cerebrospinal fluid and so on – giving rise to possible inaccuracies; however, the results of the continuous

Fig. 2. Results of real-time PCR with control plasmid pGEM-CG-ITS2. Profile of PCR products (a), melting curve analysis (b) and standard curve (c). The plasmid, serially diluted from 10^1 to 10^7 copies per assay, was amplified and the amount of PCR product was monitored at the end of each cycle with SYBR Green. The slope of the standard curve was -1.2536 cycles per log decade and the correlation coefficient was 0.9954 .

monitoring of one patient and the existence of significant differences in log-order between corresponding specimens from different patients would nevertheless provide us with valuable information.

Fragment length analysis of PCR products

Data from the GenBank database and our sequencing results from the 24 fungal reference strains showed that

Fig. 3. Capillary electrophoresis (GENESCAN) analysis of (a) PCR products from 24 reference strains and (b) T-RFLP for the 24 reference strains. In the T-RFLP analysis, PCR products were digested with the restriction enzyme *ScrFI*. Numbers from 1 to 24 indicate fungal species corresponding to those in Table 1.

PCR product length is highly variable among fungal species, ranging from 211 bp for *Clavispora lusitaniae* to 424 bp for *Absidia corymbifera* (Table 1), and that the sequences of the ITS2 region are hyper-variable between species, as described previously (Chen *et al.*, 2001; Rakeman *et al.*, 2005). Therefore, the length of amplicons or fragments after restriction enzyme digestion can provide valuable information regarding the number and identification of species in samples. For this purpose, we used a NED-labelled primer (ITS4M-R) as the reverse primer for real-time PCR and analysed the 3' terminal fragment length of PCR products from the 24 reference strains with and without restriction enzyme digestion (T-RFLP).

Of the 24 fungal species, nine (numbers 20, 4, 7, 1, 24, 16, 2, 8 and 22) could be differentiated from the other 15 without restriction enzyme digestion (Fig. 3a). Within the genus *Candida* (designated 1–8 in Fig. 3a; refer to Table 1), the ITS2 fragments ranged from 270 to 386 bp in length. However, the product size from (i) *Candida famata* (5) and *Candida guilliermondii* (6), (ii) *Cryptococcus neoformans* (15), *Rhizopus oryzae* (19), *Mucor circinelloides* (18) and *Scedosporium apiospermum* (21), and (iii) *Aspergillus* spp. (9–12), *Trichosporon* spp. (13, 14), *C. krusei* (3) and *Fusarium solani* (17), were of similar lengths, ranging from 338 to 339 bp, 329 to 336 bp and 305 to 312 bp, respectively.

To differentiate between these species, we selected *ScrFI* as the most suitable restriction enzyme and established a

T-RFLP analysis system. Using this method, the ITS2-fragment lengths from each species ranged from 64 bp for *Aspergillus flavus* (9) and *Aspergillus niger* (11) to 424 bp for *Absidia corymbifera* (22; Fig. 3b). Fourteen species from the 24 test fungal species were clearly differentiated (Table 1, Fig. 3). The lengths of PCR products and the 3' terminal fragments obtained by *ScrFI* digestion for 34 other pathogenic fungal species predicted from sequence data in GenBank are summarized in Table 2.

We observed slight differences between the fragment lengths predicted from the sequence data and those evaluated by GENESCAN for *Aspergillus* and *Trichosporon* (Table 1). This discrepancy can be explained by the fact that electrophoretic migration is partially sequence dependent (Chen *et al.*, 2001). Fragment length polymorphisms involving a few base pairs were also observed between strains registered in GenBank. These indicated that small interspecies differences in fragment length, which were observed in two groups of around 310 bp and 340 bp, could not be used to differentiate fungi at the species level. We employed T-RFLP analysis in the assay using the restriction enzyme *ScrFI* and obtained adequate results for species identification. However, as we have not analysed the influence of polymorphisms in the nucleotide sequence in a large number of strains from one species, the effectiveness of T-RFLP analysis for the differentiation of strains has not yet been confirmed.

Table 2. Predicted fragment length of PCR products from other less common species known to cause invasive fungal infection

Fragment lengths amplified by PCR using primers ITS86M-F and ITS4M-R were predicted from the registered sequences in GenBank. Asterisks signify no restriction enzyme site in the PCR product.

Species	Accession no.	Length of PCR product (bp)	Length restricted by <i>ScrFI</i> (bp)
<i>Candida lipolytica</i>	AF218983	196	196*
<i>Candida lusitanae</i>	AF218970	211	125
<i>Candida norvegensis</i>	AY939799	280	235
<i>Candida rugosa</i>	AF218971	229	229*
<i>Candida zeylanoides</i>	AY382337	326	326*
<i>Aspergillus nidulans</i>	AY373888	305	103
<i>Aspergillus restrictus</i>	AY373864	309	68
<i>Aspergillus versicolor</i>	AY373883	304	100
<i>Trichosporon asahii</i>	AF444466	310	310*
<i>Trichosporon asteroides</i>	AF444416	310	310*
<i>Trichosporon inkin</i>	AF444420	310	310*
<i>Trichosporon ovoides</i>	AF444439	309	309*
<i>Malassezia furfur</i>	AY743635	509	238
<i>Saccharomyces cerevisiae</i>	AF219005	371	326
<i>Geotrichum candidum</i>	DQ907937	201	201*
<i>Pneumocystis jirovecii</i>	L27658	321	321*
<i>Histoplasma capsulatum</i>	AF038354	318	68
<i>Paracoccidioides brasiliensis</i>	AY631237	350	125
<i>Alternaria alternata</i>	AY714479	296	296*
<i>Acremonium strictum</i>	DQ297552	307	203
<i>Cunninghamella bertholletiae</i>	AF254931	357	226
<i>Scedosporium prolificans</i>	AY228120	321	105
<i>Phialophora verrucosa</i>	AF397136	318	82
<i>Fonsecaea pedrosoi</i>	AB087202	331	103
<i>Cladosporium carrionii</i>	AF397181	316	82
<i>Exophiala dermatitidis</i>	AY663828	349	123
<i>Exophiala jeanselmei</i>	DQ836795	320	91
<i>Paecilomyces lilacinus</i>	DQ641505	301	209
<i>Paecilomyces variotii</i>	AY373941	307	186
<i>Fusarium oxysporum</i>	DQ452455	289	289*
<i>Penicillium marneffei</i>	DQ399414	291	177
<i>Blastomyces dermatitidis</i>	AF038358	328	68
<i>Sporothrix schenckii</i>	AF484468	328	176
<i>Pseudallescheria boydii</i>	AY939802	338	108

Identification of species by nucleotide sequence analysis of PCR products

It was previously reported that 372 of 373 tested fungal strains from 86 species (99.7 %) were correctly identified by sequence analysis of the ITS2 region. Only *Cryptococcus albidus* could not be differentiated from *Cryptococcus adeliensis* because of 100 % sequence identity between the ITS2 regions of these two species (Leaw *et al.*, 2006). We confirmed that the 24 strains tested in this study were correctly identified by sequence analysis of real-time PCR products (data not shown).

Analysis of a clinical case

To evaluate the efficacy of our method, serial whole-blood samples from a patient diagnosed with candidaemia due to

central-venous catheter infection were analysed retrospectively (Fig. 4). The patient developed antibiotic-resistant fever and *Candida albicans* was isolated and identified from his blood sample on day 2. Thus, antifungal chemotherapy with micafungin sodium (100 mg day⁻¹) was started and *Candida albicans* was no longer detectable in blood sampled on day 8 or 15. These three blood samples, which had been stored in a freezer, were examined using our real-time PCR system. Twenty-four copies of fungal DNA per microlitre were detected in the blood sample taken on day 2, but no fungal DNA was detected in the blood sampled on day 8 or 15. The real-time PCR products from the sample taken on day 2 showed a single fluorescent peak corresponding to a product of 296 bp both with and without *ScrFI* digestion. These results

Fig. 4. Clinical course of a patient diagnosed with candidaemia due to central-venous catheter (CVC) infection. On day 2, *Candida albicans* was isolated from the blood culture. Retrospective analysis detected 24 copies μl^{-1} of fungal DNA in the blood. The CVC infection had cleared by day 3. After antifungal chemotherapy, no fungal DNA was detected in blood sampled on day 8 or 15. ◆, Patient's temperature pattern; ●, patient's (1,3)- β -D-glucan pattern. The grey bar indicates the copy number of the fungal DNA. MCFG, micafungin; F-FLCZ, fosfluconazole (dose 400 mg per day).

indicated that the patient was infected with one species and that it might be *Candida albicans*. By analysis of the nucleotide sequence of the amplicon, the pathogen was identified as *Candida albicans*, which corresponded to the results obtained from the blood culture.

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