Improving molecular detection of *Candida* DNA in whole blood: comparison of seven fungal DNA extraction protocols using real-time PCR

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The limitations of classical diagnostic methods for invasive *Candida* infections have led to the development of molecular techniques such as real-time PCR to improve diagnosis. However, the detection of low titres of *Candida* DNA in blood from patients with candidaemia requires the use of extraction methods that efficiently lyse yeast cells and recover small amounts of DNA suitable for amplification. In this study, a *Candida*-specific real-time PCR assay was used to detect *Candida albicans* DNA in inoculated whole blood specimens extracted using seven different extraction protocols. The yield and quality of total nucleic acids were estimated using UV absorbance, and specific recovery of *C. albicans* genomic DNA was estimated quantitatively in comparison with a reference (Qiagen kit/lyticase) method currently in use in our laboratory.

The extraction protocols were also compared with respect to sensitivity, cost and time required for completion. The TaqMan PCR assay used to amplify the DNA extracts achieved high levels of specificity, sensitivity and reproducibility. Of the seven extraction protocols evaluated, only the MasterPure yeast DNA extraction reagent kit gave significantly higher total nucleic acid yields than the reference method, although nucleic acid purity was highest using either the reference or YeaStar genomic DNA kit methods. More importantly, the YeaStar method enabled *C. albicans* DNA to be detected with highest sensitivity over the entire range of copy numbers evaluated, and appears to be an optimal method for extracting *Candida* DNA from whole blood.

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INTRODUCTION

Blood culture remains the accepted 'gold standard' method for diagnosis of candidiasis and can be highly sensitive. However, in some settings, culture can fail to detect *Candida* spp. in more than 50 % of patients with chronic disseminated candidiasis (Einsele *et al.*, 1997; Moreira-Oliveira *et al.*, 2005). Molecular methods, particularly real-time PCR, are increasingly used alongside conventional microbiological techniques for the diagnosis of systemic candidiasis (Klingspor & Jalal, 2006; Kasai *et al.*, 2006; Löffler *et al.*, 2000; Schabereiter-Gurtner *et al.*, 2007; Bretagne & Costa., 2005; White *et al.*, 2006; Maaroufi *et al.*, 2003; Moreira-Oliveira *et al.*, 2005). However, two major limitations of PCR are the difficulty associated with breaking yeast cell walls to release *Candida* spp. DNA suitable for amplification (Maaroufi *et al.*, 2004; Löffler

et al., 1997) and limited sensitivity when the assays are adapted for testing clinical specimens, such as blood samples, in which the amounts of fungal DNA may be very low (White *et al.*, 2003; Löffler *et al.*, 2000; Ahmad *et al.*, 2002; Challier *et al.*, 2004). As a consequence, these tests are not yet recognized as part of the consensus diagnostic criteria, in contrast to some antigenaemia detection kits (Ascioglu *et al.*, 2002).

The yeast cell is notoriously difficult to lyse due to a highly complex cell wall structure that provides rigidity (Müller *et al.*, 1998). Moreover, it has been suggested that the fungal load in blood from patients with candidaemia can be as low as 1 c.f.u. (ml blood)⁻¹ (White *et al.*, 2003; Löffler *et al.*, 2000); therefore, the sensitivity of the PCR assay is critical. As the DNA extraction protocols applied to clinical specimens have been shown to have a major impact on the overall assay sensitivity (Fredricks *et al.*, 2005; Bretagne & Costa., 2005), an acceptable fungal DNA extraction method must be able to recover small amounts of pure DNA in a rapid and efficient manner.

Abbreviations: BAGH, benzyl alcohol/guanidine hydrochloride; C_T, threshold cycle; CV, coefficient of variation; FAM, 6-carboxyfluorescein; Y-DER, yeast DNA extraction reagent.

Comparison of different DNA extraction methods for *Candida* has been performed using a variety of specimen types such as serum samples (Maaroufi *et al.*, 2004), inoculated bronchoalveolar lavage fluid (Fredricks *et al.*, 2005), contents of blood culture bottles (Millar *et al.*, 2000) and cultures (Löffler *et al.*, 1997; Lugert *et al.*, 2006; Karakousis *et al.*, 2006).

We elected to test DNA extraction from inoculated EDTA-treated blood specimens. To the best of our knowledge, four of the five commercially available kits tested have not previously been evaluated in the context of extraction of *Candida* spp. DNA from blood. As the kits were all designed for extraction of fungal DNA from culture and fungal suspensions, these methods were adapted for use on whole blood. Unlike previous studies that utilized very high numbers of yeast cells to spike the specimens, we inoculated the whole blood with low numbers of *Candida* cells, with the aim of simulating the low titres of *Candida* DNA in blood of patients with systemic fungal infections.

In the present study, one published DNA extraction method (Fredricks & Relman, 1998) and five kit-based methods for DNA isolation were compared with a Qiagen kit/lyticase 'reference' method currently used in our laboratory. The methods were assessed for their relative effectiveness in recovering low concentrations of *Candida albicans* DNA (measured using UV absorbance, and real-time PCR), the cost per sample and the speed of processing. The overall objective was to highlight the importance of using specific PCR-based methods when comparing extraction protocols, and the fact that DNA yield data do not correlate with assay sensitivity when detecting pathogens in clinical specimens.

METHODS

Yeast cell dilutions and inoculated specimen preparation. *C. albicans* ATCC 90028 was pre-cultured on Sabouraud dextrose agar (SDA; Oxoid) for 48 h at 30 °C. Fungal cell suspensions were prepared in sterile saline and adjusted to a 0.5 McFarland standard. Tenfold serial dilutions were plated onto SDA plates in duplicate, and the number of c.f.u. ml⁻¹ of *C. albicans* was determined by colony counts after a further 48 h of culture. Cell suspensions were aliquotted and stored in 20 % (v/v) glycerol at -80 °C before addition to blood specimens [40 µl (ml blood)⁻¹]. Defined numbers of *C. albicans* cells (1400, 140 or 18 c.f.u. ml⁻¹) were inoculated into aliquots of uninfected EDTA-treated human blood, collected in Vacuette 'K3E' K₃EDTA blood tubes (Greiner Bio-One). Inoculated blood specimens (1 ml) were pre-treated with a hypotonic red cell lysis buffer as described by Löffler *et al.* (1997). Erythrocyte-free cell pellets were then obtained by centrifugation (3000 g, 10 min), washed in 2 ml 20 mM Tris/HCl (pH 8.3) and centrifuged for a further 10 min before the supernatant was discarded (Flahaut *et al.*, 1998). Negative controls consisting of aliquots of 20 % (v/v) glycerol added to uninoculated blood were also subjected to extraction. The following extraction protocols were applied to the washed cell pellets.

DNA extraction methods

Method 1: Qiagen kit/lyticase. Nucleic acids were extracted as described by Löffler *et al.* (1997) using recombinant lyticase

(Sigma-Aldrich) and a QIAamp DNA mini kit (Qiagen) except that the treatment step with NaOH at 95 °C was omitted. Carrier RNA (10 µg µl⁻¹; Sigma-Aldrich) (Kishore *et al.*, 2006; Gallagher *et al.*, 1987) was added to Qiagen lysis buffer AL as recommended by Qiagen for low-copy-number targets. This method is the standard reference currently used in our laboratory to extract fungal DNA. Nucleic acids were eluted in 100 µl buffer AE (Qiagen).

Method 2: Qiagen kit/beads. Extraction was performed using a modification of method 1, where the lyticase step was replaced with bead beating using glass beads. After resuspending the erythrocyte-free pellet in Qiagen buffer ATL with proteinase K and incubating at 55 °C for 15 min, Qiagen buffer AL was added to each tube and a volume of sterile glass beads (0.1 mm diameter; BioSpec Products) approximately equal to 100 µl was added. Samples were processed for 30 s at maximum speed in a Mini-BeadBeater-8 (BioSpec Products), followed by centrifugation at full speed in a microfuge for 10 min. The supernatant fluid from each tube was transferred to sterile 1.5 ml microcentrifuge tubes and extraction was continued using a QIAamp DNA mini kit according to the manufacturer's instructions. The nucleic acid was eluted in 100 µl Qiagen buffer AE.

Method 3: MasterPure yeast DNA purification kit. For the MasterPure yeast DNA purification kit (Epicentre), samples were extracted according to the manufacturer's protocol and the resulting pellet was resuspended in 100 µl TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA].

Method 4: benzyl alcohol/guanidine hydrochloride (BAGH). A published method for BAGH organic extraction was used for the extraction of *Candida* spp. DNA from blood culture bottles as described by Fredricks & Relman (1998). The resulting pellet was resuspended in 100 µl TE buffer.

Method 5: Dr GenTle. Extraction using the Dr GenTle (gene trapping by liquid extraction; Takara Bio) method was performed according to the manufacturer's instructions except that an extra phenol clean-up step was performed after adding solution III, for the complete removal of proteins. The resulting pellet was washed using 70 % (v/v) ethanol, dried and resuspended in 100 µl TE buffer.

Method 6: yeast DNA extraction reagent (Y-DER). Extraction using the Y-DER (Pierce Biotechnology) was performed according to the manufacturer's instructions except that linear acrylamide (10 µg ml⁻¹; Ambion) was added to each tube just before 2-propanol precipitation. This was necessary due to the very small pellet sizes obtained using this method, and enabled visible nucleic acid pellets to be obtained. Pellets were resuspended in 100 µl TE buffer.

Method 7: YeaStar genomic DNA kit. This method using the YeaStar genomic DNA kit (Zymo Research) is based on enzymic lysis of yeast cells using zymolase, followed by spin-column purification. Extraction was performed according to the manufacturer's instructions using the chloroform-free protocol (protocol II) and the nucleic acid was eluted in 100 µl TE buffer.

All extraction methods were repeated four times, and each time, quadruplicates of the three inoculum concentrations were extracted and total nucleic acid yield was estimated by UV spectrometry (GeneQuant II DNA/RNA spectrophotometer; Amersham Pharmacia Biotech). Extract quality was assessed using the A₂₆₀/A₂₈₀ ratio, with low ratios indicating low quality due to protein contamination. Quantified extracts were stored at -20 °C. Specific and quantitative recovery of *C. albicans* genomic DNA from inoculated blood specimens was then estimated using *Candida* spp.-specific real-time PCR, and also with reference to the Qiagen kit/lyticase method.

Real-time PCR assay. Real-time PCR was carried out using a nested, pan-fungal primer set with a *Candida* spp.-specific probe targeting the 18S rRNA gene (White *et al.*, 2003). Primer sequences (Sigma-Genosys) were as follows: first-round (210 bp amplicon), CAN1A (forward), 5'-GAGGGCAAGTCTGGTG-3', CAN1B (reverse), 5'-CCTGCTTTGAACACTCTAA-3'; second round (140 bp amplicon): CAN1C (forward), 5'-CTCGTAGTTGAACCTTGG-3', CAN1D (reverse), 5'-GCCTGCTTTGAACACTCT-3'. The probe was modified for this study to use TaqMan chemistry (Metwally *et al.*, 2007) utilizing a non-fluorescent 3'-quencher [Black Hole Quencher 1 (BHQ1)]: CAN-P1, 6'-FAM-TTTTGATGCGTACTGGACCC-BHQ1. The probe was specific for four *Candida* species (*C. albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida dubliniensis*). First-round PCRs (5 µl extract in 25 µl total volume) used: 1× Taq polymerase buffer (Promega), 3 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega), 0.2 µM each first-round primer and 0.5 U Taq polymerase (Promega) µl⁻¹. The thermal cycling parameters were 94 °C for 3 min, followed by 30 cycles of 94 °C for 10 s, 58 °C for 10 s and 72 °C for 30 s, with final extension at 72 °C for 5 min. Second-round real-time PCR (1 µl first-round products in 20 µl total volume) used the same reagents, except that reactions contained 0.2 µM each second-round primer, 0.4 µM TaqMan probe (CAN-1P), 1 µM ROX passive reference dye (Invitrogen) and 2 µg BSA µl⁻¹. The thermal cycling parameters (ABI 7000 Sequence Detection System; Applied Biosystems) were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min, with data collection during each 60 °C annealing/extension step. Data were analysed using ABI7000 Sequence Detection Software (version 1.2.3). Each sample was tested in triplicate, and the final threshold cycle (C_T) was the mean of the three results. Negative controls containing no added template DNA were run with every batch of samples.

Analytical sensitivity. A first-round PCR amplicon (210 bp) from *C. albicans* ATCC 90028 was cloned into the pCR4-TOPO vector using the TOPO TA cloning system (Invitrogen). The resulting plasmid (pCAN1) was quantified and the insert sequenced by standard methods to confirm the identity of the amplicon. For plasmid copy-number calculations, the formula weight of the pCAN1 plasmid (3956 bp vector+210 bp insert=4166 bp; dsDNA) was estimated to be 2530586. Log dilutions of pCAN1 (from 10⁻¹ to 10⁻¹²) were made in 0.2× TE buffer containing yeast tRNA (100 ng µl⁻¹; Sigma-Aldrich; Stahlberg *et al.*, 2004), and dilutions were tested in triplicate using the real-time PCR assay to determine the detection end point. Given a starting plasmid concentration of 108 µg µl⁻¹ (2.56 × 10¹⁰ copies µl⁻¹), the calculated end point (i.e. the lowest dilution expected to contain at least one copy per 5 µl aliquot) was the 10⁻¹¹ log dilution, with 1.2 copies per reaction.

PCR assay reproducibility. The inter- and intra-assay reproducibility of the PCR assay was assessed by three experiments, each analysing ten replicates of the pCAN1 plasmid at two different starting concentrations (2.3 × 10⁴ and 23 copies µl⁻¹). The coefficient of variation (CV) calculated for C_T data was used as an indicator of relative precision and reproducibility. CV was determined by dividing the standard deviation (SD) by the arithmetic mean of the measured values: CV (%) = (SD/mean value) × 100.

Statistical analysis. A ΔC_T method was used for comparing the efficiency of the extraction methods used with reference to method 1 (ΔC_T = sample C_T - reference C_T). Lower C_T values (or more-negative ΔC_T values) indicated higher template copy number and increased assay sensitivity. Yield and ΔC_T values were compared using two-tailed Student's *t*-tests (TTEST function; Excel 2002; Microsoft) for different spike amounts within methods, and were compared with method 1.

RESULTS AND DISCUSSION

All of the extraction methods were used successfully; where kits were not specifically designed for the extraction of fungal DNA from whole blood, they were modified to enable this by using appropriate reagents. Overall efficiency was determined by DNA yield, specific real-time PCR testing, throughput time and labour intensity. No *Candida* DNA was detected in extraction controls consisting of glycerol added to uninoculated blood processed through each of the seven DNA extraction methods compared in this study, nor in nuclease-free water samples included in each PCR run.

Validation and quality-control data

The threshold for a positive signal by real-time PCR was set manually to be above all of the negative controls, and the C_T value at this point was recorded. As the study was a repeated measurement design, the reproducibility of the real-time PCR assay was crucial in order to be able to compare C_T values obtained by different extraction methods within and between runs. To assess intra- and inter-assay reproducibility, two different concentrations of *C. albicans* plasmid DNA were tested by real-time PCR, and the CV values within and between replicate sets were calculated and used as indicators of the precision of the assay. The assay exhibited a very good precision at both high and low template concentrations. At the higher template concentration (2.3 × 10⁴ copies µl⁻¹), the intra-assay CV was 0.19% and the mean ± SD C_T was 10.44 ± 0.02; the inter-assay CV was 1.9%, with a mean C_T of 10.15 ± 0.19. At the lower concentration (23 copies µl⁻¹), the intra-assay CV was 1.1% and the mean C_T was 20.49 ± 0.22; the inter-assay CV was 2.2% with a mean C_T of 20.17 ± 0.4.

The specificity of the probe used in the assay has been reported previously (Metwally *et al.*, 2007). The analytical sensitivity of the real-time PCR assay used in this study was determined using serial dilutions of *C. albicans* plasmid DNA with known copy numbers. The calculated end-point dilution (for PCR testing of a 5 µl extract aliquot) was 10⁻¹¹, and the experimentally determined end-point dilution was 10⁻¹⁰, which is equivalent to ~12 plasmid copies per reaction. DNA extracts (100 µl) were derived from 1 ml blood samples, and 5 µl extract aliquots were tested, so each aliquot was effectively derived from 200 µl blood. This limit of detection therefore equated to 240 copies (ml blood)⁻¹, which is theoretically equivalent to ~2.4 *C. albicans* cells ml⁻¹ (assuming ~100 18S rRNA gene copies per cell). This defined the maximum sensitivity that this assay could be expected to achieve in testing of clinical specimens. Whilst we adopted the approximate figure of 100 rRNA operon copies in the diploid *C. albicans* cell, based on the current physical map of the *C. albicans* genome, copy number estimates vary between ~80 and >200 (Scherer & Magee, 1990; Wickes *et al.*, 1991; Rustchenko *et al.*, 1993).

Yield and quality data

Within each method, no significant differences in yield or ratio were observed between the three inoculum concentrations used ($P=0.01$). This was unsurprising, as the quantities of *C. albicans* DNA added, even at the highest inoculum level, were negligible compared with the yield of human DNA from the whole blood. However, significant differences among methods were observed. As shown in Table 1, all but two of the methods tested gave a significantly lower yield of total nucleic acids than the reference Qiagen kit/lyticase method (method 1): the MasterPure yeast method (method 3) gave a significantly higher yield and the Qiagen kit/beads method (method 2) gave no significant difference in yield. The quality of the extracts (estimated using A_{260}/A_{280} ratio) from all but the YeaStar method (method 7) was also significantly lower than for the reference method.

ΔC_T data

Using the Qiagen kit/lyticase reference method, the mean $\pm$ SD C_T value for the 1400 c.f.u. ml^{-1} specimens was 12 ± 1.5 , for the 140 c.f.u. ml^{-1} specimens was 20 ± 0.7 and for the 18 c.f.u. ml^{-1} specimens was 26 ± 1.1 . Results using the other six extraction methods were compared with these data and presented as $-\Delta C_T$ values (Fig. 1), such that higher values indicated increased sensitivity (or reduced C_T value).

At the 1400 c.f.u. ml^{-1} inoculum level, four methods gave results that were significantly different ($P<0.05$) to the reference method; two were less sensitive (methods 3 and 4) and two were more sensitive (methods 6 and 7). At the 140 c.f.u. ml^{-1} inoculum level, method 5 was significantly less sensitive and method 7 was significantly more sensitive; no *C. albicans* DNA could be detected in the extract from method 4. At the 18 c.f.u. ml^{-1} inoculum level, method 3 was significantly less sensitive and methods 2, 6 and 7 were significantly more sensitive; PCR results for methods 4 and 5 were negative at this concentration.

In additional PCR experiments using inoculum sizes as low as 2 c.f.u. ml^{-1} , *C. albicans* DNA was successfully detected in extracts from methods 1 and 2, but none of the other methods (data not shown). Because the presence of individual *C. albicans* cells in any given volume of inoculum becomes highly stochastic at such high dilutions, much higher levels of replication than we used would be required to give reliable quantitative data. Nevertheless, detection at these dilutions confirmed that the PCR assay used here was capable of detecting *C. albicans* in blood at levels comparable to the analytical limits.

Only method 7 (YeaStar genomic DNA kit) performed significantly better in the PCR detection experiments at all of the tested *C. albicans* concentrations, despite the low DNA yields obtained with this kit (Table 1). According to the manufacturer's instructions, 30–80 % higher DNA yields may be obtained by using an alternative protocol (protocol I), which includes chloroform extraction, although we did not evaluate the modified protocol in this study. Overall, we found that the YeaStar method achieved highly effective yeast cell lysis and yielded high-purity DNA from the samples with minimal RNA contamination. Extracts from the other methods tested, especially method 3 (MasterPure) contained significant amounts of RNA, which was clearly visible in agarose gels stained with SYBR Gold (Invitrogen) (data not shown). The presence of RNA increases the apparent yield when measured by UV absorbance. It is also possible that large quantities of contaminating human rRNA can hybridize to some degree with the fungal 18S rRNA PCR primers used. Although not amplifiable, this may have affected the PCR by depleting the primers and could explain the very low PCR sensitivity that was observed for the MasterPure extracts. These results emphasize the importance of using a specific and sensitive method such as PCR to assess the yield of target DNA when validating extraction methods for clinical use.

Comparing mean C_T values for each method that detected *Candida* DNA at all of the tested inoculum sizes (Fig. 2) indicated that all of the methods except method 6 (Y-DER) behaved in a linear fashion as the inoculum size changed. It

Table 1. Total nucleic acid yield and purity by UV absorbance

Data are means of quadruplicate samples and three inoculum sizes (12 data points); P values indicate a significance difference from the reference method (Student's t -test).

Extraction method	Mean yield $\pm$ SD ($\mu\text{g ml}^{-1}$)	Yield P value	A_{260}/A_{280} ratio (mean $\pm$ SD)	Ratio P value
1. Qiagen kit/lyticase*	96 ± 21	Reference	2.0 ± 0.2	Reference
2. Qiagen kit/beads	89 ± 44	0.598	1.8 ± 0.1	0.035
3. MasterPure	215 ± 109	4.8×10^{-3}	1.7 ± 0.1	9.9×10^{-5}
4. BAGH	33 ± 42	1.9×10^{-3}	1.5 ± 0.3	3.3×10^{-4}
5. GenTle	36 ± 18	2.2×10^{-5}	1.5 ± 0.2	1.2×10^{-5}
6. Y-DER	23 ± 10	7.3×10^{-8}	1.5 ± 0.2	6.6×10^{-5}
7. YeaStar	11 ± 4	1.4×10^{-8}	1.9 ± 0.3	0.374

*Reference method.

Fig. 1. Box-and-whisker plots showing $-\Delta C_T$ values for different extraction methods at three *C. albicans* spike concentrations in blood relative to the Qiagen kit/lyticase reference method: (a) 1400 c.f.u. ml⁻¹; (b) 140 c.f.u. ml⁻¹; (c) 18 c.f.u. ml⁻¹. The methods are numbered as in the text and Table 1. Higher $-\Delta C_T$ values indicate increased sensitivity. Boxes show interquartile ranges, lines in boxes show median values and whiskers show ranges. *P* values are shown above the boxes for methods that gave significant differences from the mean reference method values (method 1). ND, Not detected by real-time PCR.

is notable that method 7, which gave the highest sensitivity, showed almost perfectly linear behaviour over the tested inoculum range.

Overall performance of the extraction methods

Whilst the sensitivity of a given DNA extraction method is important, other factors must be considered when selecting the most practical and appropriate methodology for the clinical laboratory. Several of these aspects are summarized in Table 2. Regarding the approximate cost per test, most of the methods were in a close range. However, method 4 offered the most economical method at just £0.10 per test,

Fig. 2. Variation of C_T values with inoculum size for extraction methods that allowed *C. albicans* to be detected at all three concentrations tested. The methods are numbered as in the text and Table 1. $\diamond$, Method 1; $\blacktriangle$, method 2; $\blacksquare$, method 3; $\circ$, method 6; $\bullet$, method 7. Error bars show ± 1 SD. Lower C_T values indicate higher sensitivity.

whilst methods 1, 2 and 6 were relatively more costly than any of the other methods evaluated.

The approximate time required to process a six-sample run was measured for each method. Timing began with resuspending the erythrocyte-free pellet in the appropriate reagent and concluded with recovery of the extracted DNA. The processing (hands-on) times required for manual manipulation of tubes, reagents and samples were tabulated separately from non-manipulation time (incubation, drying, etc.). Method 1 gave the highest processing time compared with the other methods, which showed little variation among each other, taking into consideration the time required for incubation and drying.

Because four of the seven methods described were actually designed for extraction of fungal DNA from pure colonies (methods 3, 5, 6 and 7), they were adapted for extraction of *C. albicans* DNA from inoculated whole blood. Problems necessitating these adaptations related to the Dr GenTle (method 5) and Y-DER (method 6) kits. With the former, an extra phenol clean-up step was mandatory to remove all proteins, presumably because the kit is not designed for the extraction of DNA from whole blood. With the latter kit, the resultant DNA pellet was very small or invisible; adding a co-precipitant to improve the recovery of DNA, such as linear acrylamide, helped increase the pellet size. All of the protocols were easy to perform and used standard equipment commonly available in most clinical laboratories.

Clinical diagnostic relevance of the data

Detection of fungal DNA in biological specimens by PCR using conserved or specific genome sequences is a promising technique and has been evaluated extensively as a diagnostic tool (Ellis, 2002). However, there are a

Table 2. Comparison of DNA extraction methods based on time, cost and detection limits

Processing time is the typical time required to extract DNA from six samples; total time includes hands-on time, incubation and drying periods; cost is per extract per test.

Extraction method	Processing time (h:min)		Cost	Detection limit (c.f.u. ml ⁻¹)	Method	Special equipment
	Hands-on	Total				
1. Qiagen/lyticase	0:40	2:00–2:20	2.60	18	Enzymic lysis, spin column	
2. Qiagen/beads	0:40	0:50	2.30	18	Mechanical lysis, spin column	Bead beater
3. MasterPure	0:20	0:50–1:30	0.57	18	Centrifugation and precipitation	
4. BAGH	0:20	1:00–1:20	0.10	1400	Centrifugation and precipitation	Refrigerated centrifuge
5. GenTle	0:20	1:20–1:30	1.70	140	Centrifugation and precipitation	Refrigerated centrifuge
6. Y-DER	0:30	1:20–1:30	2.10	18	Detergent lysis, centrifugation and precipitation	
7. YeaStar	0:15	1:00	1.20	18	Enzymic lysis, spin column	

number of limitations associated with PCR diagnosis in cases of invasive fungal infections. A major problem is the low number of positive whole blood samples in which fungal DNA is detected when consecutive samples are obtained from infected patients. In one study of patients with invasive aspergillosis, it was calculated that more than 26 samples per risk episode needed to be analysed in order to detect all disease-positive patients (Buchheidt *et al.*, 2004). This could indicate that the window of circulation of fungal DNA is limited either due to irregular release of DNA by the fungus or due to rapid clearance from the blood.

Several approaches have been adopted by previous investigators to increase the probability of detecting *Candida* DNA in blood from patients with invasive fungal infections; by targeting the multicopy 18S rRNA gene (Jaeger *et al.*, 2000; Löffler *et al.*, 1997; White *et al.*, 2003; Widjoatmodjo *et al.*, 1999), the sensitivity of the assay can be increased. Using nested PCR to diagnose systemic *Candida* infections can further enhance the sensitivity of many assays by one to three orders of magnitude (White *et al.*, 2003; Bougnoux *et al.*, 1999; Ahmad *et al.*, 2002). The use of PCR enhancers such as BSA in the PCR mix may help to overcome PCR inhibitors that might be present in the blood of patients (Maaroufi *et al.*, 2004); other workers recommend a 1 day incubation of blood samples at 34 °C before being evaluated to improve the sensitivity of detection of fungal DNA in the blood of patients with candidaemia (Arishima & Takezawa, 2006). The use of microconcentrators to concentrate fungal DNA after extraction has also been proposed (White *et al.*, 2003).

To date, there is no consensus concerning the best blood fraction to be tested for diagnosis of invasive candidiasis. Several PCR methods have been developed for use either on whole blood (Schabereiter-Gurtner *et al.*, 2007; Innings *et al.*, 2007; Pryce *et al.*, 2003; White *et al.*, 2006) or on serum samples (Bougnoux *et al.*, 1999; Maaroufi *et al.*, 2004; Ahmad *et al.*, 2002; Wahyuningsih *et al.*, 2000). For the present study, we reasoned that inoculating serum with

C. albicans cells would not simulate clinical serum samples from patients with candidaemia, as yeast cells are eliminated by centrifugation during serum processing without being lysed to release intracellular DNA (Bougnoux *et al.*, 1999), in contrast to the conditions in true candidaemia where host phagocytic cells play a pivotal role in increasing the release of *Candida* DNA into extracellular fluid during the process of phagocytosis-mediated damage to circulating *Candida* cells (Kasai *et al.*, 2006). For this reason, we elected to use whole blood inoculated with low copy numbers of *C. albicans* cells to simulate the low-level candidaemia present in blood from patients with invasive *Candida* infections.

There is a need for a rapid, standard method for DNA extraction from *Candida* to be developed and accepted in order to standardize molecular diagnostic assays and allow useful comparisons to be made among laboratories. The data generated in this study add to existing data and suggest that the YeaStar kit is probably superior to other available methods for the extraction of *C. albicans* DNA, employed here as a model yeast pathogen. Furthermore, the YeaStar procedure is technically simple requiring less than 1 h, with only 15 min of hands-on time; moreover, this kit is less expensive than most of the others described here. Overall, the data presented suggest that the YeaStar extraction method may be optimal for extraction of *Candida* DNA from whole blood.

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