Rapid differentiation between fluconazole-sensitive and -resistant species of *Candida* directly from positive blood-culture bottles by real-time PCR

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In view of both the delay in obtaining identification by conventional methods following blood-culture positivity in patients with candidaemia and the close relationship between species and fluconazole (FLC) susceptibility, early speciation of positive blood cultures has the potential to influence therapeutic decisions. The aim was to develop a rapid test to differentiate FLC-resistant from FLC-sensitive *Candida* species. Three TaqMan-based real-time PCR assays were developed to identify up to six *Candida* species directly from BacT/Alert blood-culture bottles that showed yeast cells on Gram staining at the time of initial positivity. Target sequences in the rRNA gene complex were amplified, using a consensus two-step PCR protocol, to identify *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida dubliniensis*, *Candida glabrata* and *Candida krusei*; these are the most commonly encountered *Candida* species in blood cultures. The first four of these (the characteristically FLC-sensitive group) were identified in a single reaction tube using one fluorescent TaqMan probe targeting 18S rRNA sequences conserved in the four species. The FLC-resistant species *C. krusei* and *C. glabrata* were detected in two further reactions, each with species-specific probes. This method was validated with clinical specimens (blood cultures) positive for yeast (n=33 sets) and the results were 100 % concordant with those of phenotypic identification carried out concomitantly. The reported assay significantly reduces the time required to identify the presence of *C. glabrata* and *C. krusei* in comparison with a conventional phenotypic method, from ~72 to <3 h, and consequently allows optimization of the antifungal regimen at an earlier stage.

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INTRODUCTION

Disseminated fungal infection is a significant cause of mortality in hospitalized patients (Trick *et al.*, 2002; Wey *et al.*, 1988). *Candida* species account for between 70 and 80 % of fungal bloodstream infections, and collectively represent the fourth most common group of pathogens responsible for nosocomial bloodstream infection, with a mortality rate of approximately 50 % (Beck-Sague & Jarvis, 1993; Fridkin & Jarvis, 1996; Trick *et al.*, 2002). Of the *Candida* species that cause invasive infections, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata* typically account for between 80 and 90 % of isolates encountered in the clinical laboratory, although this may vary for a given clinical unit over time (Aliyu *et al.*, 2006; Pfaller, 1996; Pfaller *et al.*, 2001). Fluconazole (FLC), which has a relatively favourable adverse-effect profile and comparatively modest drug-acquisition cost, has been reported to be as effective as amphotericin B for the treatment of candidaemia in non-neutropenic patients (Rex *et al.*, 1994). Therefore, it is frequently prescribed as the first-line empirical antifungal agent in such patient populations.

However, *C. glabrata* and *C. krusei* possess the capacity to be resistant to FLC, and MIC values for *C. krusei* and *C. glabrata* are typically much higher than those for *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis* (Pfaller *et al.*, 1999). Such high MICs are not consistently

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Abbreviations: FLC, fluconazole; ITS, internal transcribed spacer; PNA-FISH, peptide nucleic acid fluorescent *in situ* hybridization.

within the achievable therapeutic range when standard FLC dosing regimens are employed. Therefore, it is logical that the availability of information at an early stage regarding the species recovered in patients with candidaemia may help physicians to select the most appropriate antifungal agent in a timely manner; this has the potential to positively impact patient outcomes while maintaining the cost-effectiveness of antifungal therapy (Garey *et al.*, 2006).

Final identification of yeasts in positive blood cultures normally requires at least 72 h, during which time patients receive empirical treatment. A similar time is required to complete antifungal susceptibility testing. Therefore, a test to rapidly and accurately identify *Candida* isolates, categorized into species groups strongly predictive of FLC susceptibility, directly from positive blood-culture bottles would be of significant clinical and therapeutic value.

Efforts have been directed toward molecular testing of *Candida* isolates from solid media or blood-culture bottles. These include amplification of a target gene(s) followed by a post-amplification analysis to identify the amplicons based on either electrophoretic migration or hybridization with specific nucleotide probes (Einsele *et al.*, 1997; Elie *et al.*, 1998; Fujita *et al.*, 1995; Park *et al.*, 2000; Fadda, 2000, Playford *et al.*, 2006; Luo & Mitchell, 2002; Shin *et al.*, 1997; Das *et al.*, 2006; Morace *et al.*, 1997). These methods have shown promise in the diagnosis of fungal infections but have problems that prevent their use on a routine basis; for example, the use of nested PCR or PCR in conjunction with restriction enzyme analysis may add needless complexity to these assays.

Other investigators have demonstrated the accuracy of peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH) for rapid detection of *C. albicans* directly from blood-culture bottles (Forrest *et al.*, 2006; Alexander *et al.*, 2006; Wilson *et al.*, 2005; Rigby *et al.*, 2002; Oliveira *et al.*, 2001). This is a rapid and relatively straightforward technique; however, this approach does not facilitate the detection of other species. Therefore, it cannot be assumed that a negative result with this test implies the presence of an FLC-resistant species, since it is unable to distinguish between, for example, *C. parapsilosis* and *C. krusei*.

We report a rapid and sensitive real-time PCR method to distinguish the typically FLC-sensitive species of *Candida* (*C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*) from usually resistant species (*C. glabrata* and *C. krusei*) directly from positive blood-culture bottles which can be completed in less than 3 h after the initial detection by Gram staining.

METHODS

***Candida* isolates.** American Type Culture Collection (ATCC) strains and laboratory strains identified by standard phenotypic methods were used to generate *Candida* species DNA templates. The strains *C. albicans* ATCC 90028, *C. tropicalis* ATCC 20336, *C. glabrata* ATCC 66032, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and laboratory strains of *C. dubliniensis*, *Candida guillermondii*, *Candida kefyr*, *Candida sphaerica*, *Candida famata* and *Candida lusitaniae* were obtained from the mycology laboratory, Royal Victoria Hospital, Belfast.

Clinical specimens. BacT/Alert blood-culture bottles (Organon Teknika) were normally inoculated with 5–10 ml blood from adult patients; in the case of children and neonates, Pedi-BacT bottles were typically inoculated with 3–5 ml and 1–2 ml, respectively, in the course of routine clinical care. Bottles were inserted into the BacT/Alert instrument (Organon Teknika) and incubated at 37 °C. Once bottles became positive, aliquots were removed for Gram staining and subsequent culturing.

A total of 33 consecutive *Candida*-positive blood-culture sets (20 aerobic, 15 anaerobic and six paediatric bottles) were collected from the Microbiology Laboratory, Royal Victoria Hospital, Belfast, from November 2005 to July 2006. In addition, 10 specimens from blood-culture bottles (five aerobic, four anaerobic and one paediatric) that were negative for yeast were included as negative control samples.

Routine phenotypic identification consisted of isolation of *Candida* species from positive blood-culture bottles by plating 50 µl aliquots onto one Malt agar plate incubated at 30 °C, one Malt agar plate at 37 °C and CHROMagar plates (Hardy Diagnostics) at 37 °C, and subsequent identification by microscopic (germ-tube formation in horse serum) and biochemical (API *Candida* and/or API 32C, bioMérieux) tests.

Extraction of *Candida* DNA

(i) From fungal suspensions. Fungal isolates were subcultured onto Sabouraud medium (Difco) at 30 °C; yeast isolates were cultured for 48 h and mould isolates for 72 h. Thereafter, fungal saline suspensions were adjusted to equivalence with the 0.5 McFarland standard. The fungal suspensions were centrifuged at 5000 ***g*** for 10 min and then the pellet was incubated with 500 µl lyticase buffer [10 U ml^{-1} recombinant lyticase (Sigma), 50 mM Tris, pH 7.5, 10 mM EDTA, 28 mM β-mercaptoethanol] at 30 °C for 30 min. The resultant spheroblasts were pelleted by centrifugation for 5 min at 5000 ***g*** and resuspended in buffer and proteinase K. DNA extraction was continued according to the tissue protocol in the QIAamp DNA Mini kit (Qiagen). Extracted DNA was stored at −20 °C.

(ii) From blood-culture bottles. Aliquots from all blood-culture (n=43) bottles were subjected to DNA extraction by two established protocols.

QIAamp extraction (method A). The total DNA from 0.2 ml of yeast-positive BacT/Alert blood-culture medium was purified according to the manufacturer's instructions by using the recombinant lyticase enzyme and QIAamp blood kit (Qiagen) and eluted in 0.1 ml elution buffer.

Benzyl alcohol/guanidine hydrochloride organic extraction (method B). A published method was adopted (Fredricks & Relman, 1998). The extract was stored at −20 °C until it was used for PCR amplification.

Real-time PCR assay

(i) Oligonucleotide design. Primers used to amplify all typically FLC-sensitive *Candida* species have been published previously (White *et al.*, 2003; Shin *et al.*, 1999), whereas those for *C. krusei* and *C. glabrata* were designed in-house using the Lasergene software program, version 5 (DNAstar) (Table 1). Publically available GenBank sequences for the rRNA genes of different *Candida* species were aligned and inspected for regions of conserved and variable

Table 1. Primers and probes used in this study

Primer	Sequence (5′→3′)*	Product size	Target gene(s)
FLC sensitive			
Can 1c	F-CTCGTAGTTGAACCTTGG	140 bp	18S
Can 1d	R-GCCTGCTTTGAACACTCT		
Can p1	6FAM-TTTTGATGCGTACTGGACCC-BHQ1		
C. glabrata			
Gla1c	F-CCTGTTTGAGCGTCATTTCC	229 bp	ITS1, 5.8S, ITS2
Gla1d	R-AGCACGCACAAAACACTCACTTAT		
Gla p1	VIC-TAGGTTTTACCAACTCGGTGTTGAT-BHQ1		
C. krusei			
Kru 1c	F-CCTGTTTGAGCGTCATTTCC	219 bp	ITS1, 5.8S, ITS2
Kru 1d	R-CCTGCTTTGAACACTCTAA		
Kru p1	6FAM-AGCTGGCCGAGCGAACTAGACTTTT-TAMRA		

*F, forward primer; R, reverse primer.

sequences by the CLUSTAL_W tool using the Lasergene software program. Regions specific for the chosen fungal species were selected for primer targeting of *C. glabrata* and *C. krusei*. The assay for the FLC-susceptible group utilized universal fungal primers can 1c (forward primer) and can 1d (reverse primer) and an FLC-sensitive species-specific probe to amplify a conserved portion of the 18S rRNA sequences, whereas the sequences of probes for *C. glabrata* and *C. krusei* assays were specific to the variable sequences of the internal transcribed spacer (ITS)2 region. The probe for detection of FLC-sensitive *Candida* species has been published elsewhere (White *et al.*, 2003); however, it was modified by TaqMan chemistry, utilizing a non-fluorescent quencher (BHQ1) at it 3′ end. Primers and probes were purchased from Sigma-Genosys.

(ii) PCR amplification. Real-time PCR was performed with 2 μl template DNA in a total reaction volume of 10 μl containing 1 × PCR buffer (Promega), 3.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 0.25 U μl^{-1} *Taq* DNA polymerase (Promega), 0.2 μM of primers for the rRNA genes, 0.2 μM TaqMan probe, 1 μM 5-carboxy-X-rhodamine (ROX) passive reference dye (Invitrogen) and 2 μg BSA μl^{-1} (Sigma). The ABI 7000 Sequence Detection System (Applied Biosystems) was switched on 30 min prior to use. Each sample was run in duplicate in standard PCR strips but with optical PCR lids using the following PCR cycling conditions: after an initial denaturation step of 3 min at 95 °C, a two-step PCR procedure was used consisting of 30 s at 95 °C and 1 min at 60 °C for 40 cycles.

A negative control consisting of the reaction mixture and water (in place of template DNA) was added in each run. In addition to negative controls, 1 ml of medium withdrawn from a sterile blood-culture bottle was spiked with the yeast under test (<100 c.f.u. ml^{-1}) and the sample was included as a positive extraction control.

Data were obtained during the annealing period. Fluorescence was measured once every cycle immediately after the 60 °C incubation (extension step). Fluorescence curves were analysed with the ABI 7000 Sequence Detection System software and results were expressed by determination of the threshold of detection, C_T, which marked the cycle at which the fluorescence of the sample became significantly different from the baseline signal. A sample was regarded as positive when the ABI 7000 software determined a C_T in the quantification analysis screen.

(iii) Synthetic controls. To establish the analytical sensitivity of the assay, we created a plasmid containing *Candida* DNA amplicons by using the TOPO TA cloning procedures with a pCR4-TOPO vector (Invitrogen). Insertion of the correct amplicons was confirmed by nucleotide sequencing using the Thermo Sequenase fluorescently labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). The purified recombinant plasmid was quantified using the Amersham Gene Quant II DNA/RNA spectrophotometer (Amersham Pharmacia Biotech). The pre-quantified plasmid standards were diluted in nuclease-free water and stored at −20 °C.

Assay specificity. The analytical specificity of the three developed assays was determined by testing a panel of archived micro-organisms, identified by standard methods, including bacterial species (*Enterobacter cloacae*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis* and *Moraxella catarrhalis*), different *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. kefyr*, *C. guillermondii* and *C. sphaerica*), non-*Candida* yeast strains of *Cryptococcus laurentii*, *Cryptococcus neoformans*, *Geotrichum* species, *Rhodotorula rubra*, *Saccharomyces cerevisiae* and *Trichosporon asahii*, as well as strains of filamentous fungi such as *Penicillium viridicatum*, *Alternaria alternata*, *Aspergillus niger*, *Fusarium* species, *Rhizopus*, *Aspergillus fumigatus* and *Cladosporium* species. The identity of all fungal isolates was confirmed phenotypically by the mycology laboratory, Royal Hospitals Trust, Belfast.

Analytical sensitivity. Tenfold dilutions (typically in the range 10^{-1}–10^{-12}) were made by serial dilution of *Candida* plasmid DNA with nuclease-free water. The sensitivity of the assay was determined by carrying out real-time PCR on serial tenfold dilutions of plasmid DNA and recording the detection end point copy number.

Quality control. Each reaction was carried out in duplicate. Blood-culture specimens that were inoculated with yeast cells were used as positive controls for each sample run. Carryover contamination was reduced by using aerosol-resistant pipette tips and separate laboratory areas for DNA sample preparation and PCR amplification, along with other standard contamination precautions.

Nucleotide sequence accession numbers. The database accession numbers of the existing GenBank depositions investigated were as follows: *C. albicans*, M60302; *C. parapsilosis*, AY055855; *C. tropicalis*, M55527; *C. krusei*, M60305; *C. glabrata*, M60311; *C. dubliniensis*, X99399; *A. fumigatus*, M60300; *Aspergillus terreus*, X78540; *A. niger*, X78538; *Aspergillus flavus*, X78537; and *Aspergillus nidulans*, X78539.

RESULTS

Extraction of *Candida* DNA

Sodium polyanetholesulfonate (SPS) is a common component in commercially available blood-culture media and is a potent PCR inhibitor. We found that the organic extraction method, with benzyl alcohol and guanidine hydrochloride, was able to overcome its effect and the effects of other inhibitory substances that were present in both aerobic and anaerobic bottles, such as haemoglobin and lactoferrin. We were able to obtain PCR products from all blood-culture bottles extracted by this method. On the other hand, when we used the enzymic lysis method (followed by the Qiagen extraction protocol), the inhibitory effect of SPS could be overcome only by diluting the extracts.

Assay specificity

The probe for the FLC-sensitive group hybridized appropriately with *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*, but did not hybridize with DNA from other species of *Candida*, such as *C. famata*, *C. kefyr*, *C. guillermondii*, *C. sphaerica*, *C. lusitaniae*, *C. krusei* and *C. glabrata*, or with the bacteria and other fungi that were tested. The *C. krusei* probe hybridized only with *C. krusei* and the *C. glabrata* probe only with *C. glabrata*.

Assay sensitivity

The sensitivity of the real-time PCR assay was determined using serial dilutions of *Candida* species plasmid DNA with known copy numbers. The detection end point was 10^{-9} for the three assays. As there are more than 100 copies of the ribosomal genes per yeast cell, the sensitivities of the three assays equated to no more than one *Candida* cell per reaction. The end point copy numbers for each of the three assays were:

FLC-sensitive *Candida* assay, 160 copies ml^{-1}

C. glabrata assay, 600 copies ml^{-1}

C. krusei assay, 280 copies ml^{-1}

Assay verification with clinical specimens

Routine subculture and phenotypic identification of 33 yeast-positive blood-culture bottles revealed 26 as *C. albicans*, three as *C. parapsilosis*, one as *C. dubliniensis* and three as *C. glabrata*; there were no isolates of *C. krusei* or *C. tropicalis* in any of the clinical specimens obtained.

Among the 33 positive blood-culture bottles containing yeasts, two were mixed with coagulase-negative staphylococci. Coexisting bacteria in these specimens did not produce any products or did not interfere with yeast identification. There were no episodes of mixed candidaemias among the blood-culture bottles analysed.

There was 100 % agreement between the results obtained by the real-time PCR method and those of the conventional techniques in routine use (Table 2). All blood cultures (n=10) that had shown no growth after 7 days incubation in the BacT/Alert instrument were negative by real-time PCR.

DISCUSSION

Widespread use of FLC in the prophylaxis and treatment of candidosis has contributed to the emergence of *Candida* infections caused by FLC-resistant species. Given the fulminant and frequently fatal outcome of acute disseminated candidosis, the rapid discrimination of azole-sensitive from -resistant species has a critical role in the timely implementation of targeted antifungal drug therapy.

Extant, culture-based, phenotypic methods to identify *Candida* species from positive blood-culture bottles require at least 24 h following initial positivity by Gram staining to obtain positive growth on solid culture medium. If germ-tube formation can be seen on microscopic examination, a presumptive identification of *C. albicans* may be made; however, a further 48 h is usually needed to speciate non-*albicans* isolates by sugar assimilation strip or fermentation tests (Dooley *et al.*, 1994); this amounts to a total of 72 h following blood-culture positivity to reach a final identification. Although CHROMagar *Candida* (Hardy Diagnostics) can be helpful in providing early presumptive speciation of some common *Candida* species, based on a chromogenic indicator, such identification is not definitive; furthermore, some species produce subtle, indeterminate colour changes. Even the 4 h RapID test (Innovative Diagnostics) still requires a time of at least 1 day after initial blood-culture positivity to obtain pure isolated colonies from which to inoculate test wells for analysis (Espinel-Ingroff *et al.*, 1998).

At the molecular level, fungal rRNA sequence variation offers an alternative to phenotypic identification for detection and identification of yeasts. The multicopy ribosomal gene complex is a useful target for PCR assays because of the enhanced sensitivity attributable to its multiple copies, the high sequence conservation of its 18S, 5.8S and 28S regions (for panfungal primers), and the high variability of its intervening ITS regions (for species-specific probes) with high interspecies and low intraspecies heterogeneity. As a result, molecular biology-based diagnostic methods that use rRNA sequences have been used to overcome the problems of sensitivity, specificity and delay encountered with conventional methods (Moreira-Oliveira *et al.*, 2005; Ellepola & Morrison, 2005; Klingspor & Jalal, 2006; Maaroufi *et al.*, 2003).

Although other investigators have employed molecular techniques for yeast identification from positive blood-culture bottles, these techniques have been associated with several problems, ranging from inability to discriminate *C. glabrata* (a potentially FLC-resistant species) to time-consuming, labour-intensive and costly methods, some of which require equipment which is not readily available in a typical molecular-diagnostic laboratory (Chang *et al.*, 2001; Selvarangan *et al.*, 2003). We believe that we have

Table 2. Comparison between results of phenotypic identification and those of the real-time PCR assay

Abbreviations: A, aerobic; AN, anaerobic; P, paediatric; CNS, coagulase-negative staphylococcus; CPS, coagulase-positive staphylococcus.

Blood culture set no.	Type of bottle tested	Phenotypic identification	Assay results	Agreement/disagreement between phenotypic identification and PCR
1	A, AN	*C. albicans*	Positive by assay 1 only*	Agreement
2	P	*C. albicans*	Positive by assay 1 only*	Agreement
3	A, AN	*C. parapsilosis*	Positive by assay 1 only*	Agreement
4	A	*C. albicans*	Positive by assay 1 only*	Agreement
5	A	*C. parapsilosis*	Positive by assay 1 only*	Agreement
6	AN	*C. albicans*	Positive by assay 1 only*	Agreement
7	P	*C. albicans*	Positive by assay 1 only*	Agreement
8	A, AN	*C. albicans*	Positive by assay 1 only*	Agreement
9	A	*C. dubliniensis*	Positive by assay 1 only*	Agreement
10	AN	*C. albicans*	Positive by assay 1 only*	Agreement
11	A	*C. albicans*	Positive by assay 1 only*	Agreement
12	A	*C. albicans*	Positive by assay 1 only*	Agreement
13	AN	*C. albicans*	Positive by assay 1 only*	Agreement
14	A, AN	*C. albicans*	Positive by assay 1 only*	Agreement
15	AN	*C. glabrata*	Positive by *C. glabrata* assay only	Agreement
16	A	*C. albicans*	Positive by assay 1 only*	Agreement
17	A, AN	*C. albicans*	Positive by assay 1 only*	Agreement
18	A	*C. albicans*	Positive by assay 1 only*	Agreement
19	AN	*C. albicans*	Positive by assay 1 only*	Agreement
20	P	*C. albicans*	Positive by assay 1 only*	Agreement
21	A, AN	*C. albicans*	Positive by assay 1 only*	Agreement
22	P	*C. albicans*	Positive by assay 1 only*	Agreement
23	A	*C. parapsilosis*	Positive by assay 1 only*	Agreement
24	A, AN	*C. albicans*	Positive by assay 1 only*	Agreement
25	P	*C. albicans*	Positive by assay 1 only*	Agreement
26	A	*C. albicans*	Positive by assay 1 only*	Agreement
27	P	*C. albicans*	Positive by assay 1 only*	Agreement
28	AN	*C. albicans*	Positive by assay 1 only*	Agreement
29	A	*C. glabrata*	Positive by *C. glabrata* assay only	Agreement
30	AN	*C. albicans*	Positive by assay 1 only*	Agreement
31	A, AN	*C. glabrata*	Positive by *C. glabrata* assay only	Agreement
32	A	*C. albicans*	Positive by assay 1 only*	Agreement
33	A	*C. albicans*	Positive by assay 1 only*	Agreement
34	A	CNS	Negative by all assays	Agreement
35	AN	*Streptococcus mitis*	Negative by all assays	Agreement
36	A	*E. coli*	Negative by all assays	Agreement
37	P	CNS	Negative by all assays	Agreement
38	A	CNS	Negative by all assays	Agreement
39	AN	CPS	Negative by all assays	Agreement
40	AN	*Bacillus* species	Negative by all assays	Agreement
41	AN	*E. coli*	Negative by all assays	Agreement
42	A	CNS	Negative by all assays	Agreement
43	A	CNS	Negative by all assays	Agreement

*Assay for FLC-sensitive *Candida* species.

overcome many such barriers in developing the current method. For example, the *Candida* species are grouped such that those predictive of FLC susceptibility may be identified in a single assay. As this assay will be positive in the majority of circumstances, it can serve as a rapid test to guide the choice of antifungal drug even before the other two described assays are carried out.

The PNA-FISH technique also targets rRNA, although its evaluation has not extended beyond a *C. albicans*-specific probe in the published literature (Forrest *et al.*, 2006; Alexander *et al.*, 2006; Wilson *et al.*, 2005; Rigby *et al.*, 2002; Oliveira *et al.*, 2001). While this rapid test has proven reliability in a multicentre trial, it cannot distinguish between the non-*albicans* species; since this group

represents a growing proportion of candidaemias, such deficiencies may not be acceptable. As a result, one could be inclined to assume that specimens testing negative represent FLC-resistant species, which will be inappropriate in a large number of instances, depending on the epidemiology of the institution. This has the potential to threaten both the cost-effectiveness of this test and prudent antifungal stewardship. Furthermore, this technique is incapable of discriminating mixed candidaemias (containing more than one *Candida* species). One may, therefore, wrongly conclude that *C. albicans* is the only species present in a mixture of *C. albicans* and *C. krusei* or *C. glabrata*; this could lead to inadequate initial therapy and pose a risk to patient outcomes.

TaqMan probes were used in the real-time PCR assay we describe to discriminate the typically FLC-sensitive *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*) from the more frequently FLC-resistant species (*C. glabrata* and *C. krusei*); this allowed both a reduction in the number of sample-manipulation steps that needed to be performed and the detection of four *Candida* species in the same reaction tube. We favour the organic extraction method to the enzymic lysis method, as described, since it was better able to overcome the effects of inhibitors present in blood-culture bottles. The organic extraction method has been used successfully by Fredricks & Relman (1998) to purify bacterial DNA from inoculated blood-culture bottles.

Our study has some limitations. First, the number of positive blood cultures was limited by the incidence of candidaemia in our institution during the study; nonetheless, the available results provide robust pilot data. Second, most of the isolates that were collected were *C. albicans* and no *C. krusei* was isolated during the study period. Although this is not entirely surprising, given our knowledge of the pattern of species causing candidaemia (McMullan *et al.*, 2002), it is disappointing that the *C. krusei* assay could not be verified using clinical specimens. However, this does not substantially threaten the potential utility of the assay. Third, since this was a single-institution study, only one blood-culture system was in use; hence, the applicability of this technique, particularly the extraction step, cannot be assured for other systems.

Furthermore, no blood-culture bottles included in the study contained more than one yeast species. It would be interesting to evaluate our hypothesis that the assays would be capable of simultaneously amplifying different species so that in mixed blood cultures, more than one *Candida* species would be detectable. Finally, we were unable to demonstrate any specific clinical benefit or the cost-effectiveness of this assay, because of the size and scope of the dataset. Although the cost-effectiveness of the PNA-FISH technique has been suggested elsewhere (Forrest *et al.*, 2006; Alexander *et al.*, 2006), generalization of this suggestion is not universally appropriate, since cost-effectiveness is critically dependent upon the empirical antifungal-prescribing preferences of a given institution.

Currently, our system relies on identifying the six *Candida* species in three reaction tubes in a single run. Because we are using the same cycle parameters for the three assays, there is potential for multiplexing the three probes in a single reaction tube. *C. glabrata* and *C. krusei* probes could be labelled with other fluorescent dyes that have similar excitation wavelengths but different emission wavelengths from those used in the FLC-sensitive group probe, so identification and discrimination of the six species could be performed in one tube. Therefore, further modifications to these assays have the potential to facilitate even greater ease of use.

Nonetheless, with the clinical specimens to which they were applied, our assays demonstrated 100 % concordance with phenotypic assays, and significantly reduced the time required to accurately identify the presence of typically FLC-resistant species, from ~72 to <3 h. Furthermore, the equipment required to perform these assays is likely to be readily available in a routine molecular-diagnostic laboratory, and consequently there is a real potential that these assays could be applied to this clinical diagnostic problem.

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