Rapid electrochemical identification of pathogenic *Candida* species

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This study describes the development of a novel assay to detect fungal DNA and identify the most clinically relevant invasive human pathogenic fungi to the species level using oligonucleotide probes, labelled with electrochemically active groups, and solid-state electrodes. A panfungal probe designed against the 18S rRNA gene region, capable of detecting all fungal pathogens tested, and species-specific probes, designed against the ITS2 region for detection of the five *Candida* species most commonly encountered in the clinical setting (*Candida albicans*, *Candida glabrata*, *Candida parapsilosis* species complex, *Candida krusei* and *Candida tropicalis*), are described. When tested with PCR-amplified DNA from both type and clinical strains of the relevant species, the probes were able to positively identify the relevant fungi, indicated by production of a current significantly elevated above the background reading. No cross-reactivity was observed with any of the species-specific probes when compared with nine non-target *Candida* species or in the presence of human DNA equivalent to an equal number of ITS2 targets. The panfungal probe gave results that were similarly positive against 15 other fungal species and also did not cross-react with human DNA. The limit of detection of the assay was shown to be approximately 1 genome equivalent for all probes using extracted genomic DNA.

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INTRODUCTION

Increasingly, fungal species, particularly of the genera *Candida*, *Aspergillus* and *Cryptococcus*, are responsible for life-threatening invasive conditions in immunocompromised patients. A recent study in Denmark reported that 98% of fungaemia episodes were caused by *Candida* species (Arendrup *et al.*, 2008). Badiie *et al.* (2009) found in a southern Iranian hospital that two *Aspergillus* species were responsible for 27.3% of invasive fungal infections in a sample of patients who had undergone a kidney transplant or chemotherapy. Data from 134 study sites gathered by the ARTEMIS DISK Global Antifungal Surveillance Study showed that *Cryptococcus* species were the most common non-*Candida* yeast species implicated in invasive fungal infections (Pfaller *et al.*, 2009). In the UK between 2004 and 2006, the Mycology Research Laboratory identified 3033 yeast isolates and found that *Candida albicans* alone accounted for 39.3% of these, and together with five other species, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida lusitanae* and *Candida krusei*, collectively accounted for approximately 86% of isolates (Linton *et al.*, 2007). Up to 20 infrequently encountered *Candida* species such as *Candida rugosa* and

Candida guilliermondii have also been implicated in candidaemia (Kao *et al.*, 1999; Hajjeh *et al.*, 2004).

The mortality of candidaemia is high (Tortorano *et al.*, 2004; Horn *et al.*, 2007) due to the immunocompromised status of the infected patients, although co-morbidities exist in these patients making the causal link between *Candida* infection and mortality difficult to establish. Rapid detection of fungal species is essential, as a delay in initiation of therapy by even 24 h can lead to a significant increase in mortality (Garey *et al.*, 2006), whilst a varying spectrum of susceptibility to antifungal agents exhibited by clinically relevant fungi means that identification to the species level is essential for appropriate treatment.

Current methods of detection and identification of fungi lack sensitivity and are time-consuming. There are now several techniques based on molecular detection of fungal DNA following PCR amplification that have increased sensitivity and which are more rapid than culture, including the use of radiolabelled species-specific probes and Southern blotting of PCR products (van Deventer *et al.*, 1995), enzyme immunoassays (Fujita *et al.*, 1995; Shin *et al.*, 1997; Clancy *et al.*, 2008) and, more recently, numerous assays developed using fluorescent methods (Baskova *et al.*, 2007; Metwally *et al.*, 2007; Hata *et al.*, 2008; Lau *et al.*, 2008).

Abbreviation: DPV, differential pulse voltammetry.

Hillier *et al.* (2004) showed that electrochemical detection of target DNA was possible using ferrocene-labelled oligonucleotide probes and a suitable double-strand-specific exonuclease to digest the bound probe. This resulted in the release of a ferrocene-labelled mononucleotide, which could be oxidized at an electrode and the current produced measured using differential pulse voltammetry (DPV). In outline, following digestion, the released mononucleotide-ferrocene molecules are free to diffuse to an electrode surface where a potential is applied to the electrode. This causes oxidation of the ferrocene molecule and produces a measurable current which is proportional to the amount of mononucleotide-ferrocene present at the electrode surface, which in turn is proportional to the amount of target DNA that was initially present. Any undigested oligonucleotide that is present cannot diffuse freely to the electrode surface, or is inhibited in electron transfer, and so oxidation of the ferrocene label and production of current is minimal when the sample is free of DNA or contains non-target DNA. We have shown here that, with the addition of a PCR step and the design of species-specific probes, this procedure can detect DNA from a wide range of fungal pathogens and identify the five most clinically relevant species of *Candida* to the species level by using a single panfungal probe and five species-specific probes.

METHODS

Culture acquisition. The following type strains were obtained from the Centraal Bureau voor Schimmelcultures (CBS): *Candida albicans* CBS 562^T, *Candida parapsilosis* CBS 604^T, *Candida glabrata* CBS 138^T, *Candida tropicalis* CBS 94^T, *Candida krusei* CBS 573^T, *Candida lusitanae* CBS 4413^T, *Candida famata* CBS 767^T, *Candida dubliniensis* CBS 7987^T, *Candida kefyr* CBS 834^T and *Candida guilliermondii* CBS 566^T. Clinical strains were acquired from the culture collection at the University of Bath and from other donors. All species used had their identity confirmed by sequencing of the ITS2 region. All yeasts were cultured on yeast-peptone-dextrose (YPD) agar at 28 °C for 48 h and stored at 4 °C before being transferred to liquid YPD medium with 10 % glycerol and stored at -20 °C until DNA was extracted. Filamentous fungi were cultured on potato dextrose agar (PDA; Oxoid).

DNA extraction. A Qiagen DNeasy extraction kit was used for all extractions and the manufacturer's protocol was followed with some modifications that were appropriate for treatment of fungal material. In brief, approximately 5×10^6 yeast cells in liquid medium were collected by centrifugation at 8400 g for 10 min and the cell pellet was resuspended in 500 µl lyticase lysis buffer comprising lyticase from *Arthrobacter luteus* (10 U ml⁻¹; Sigma), 50 mM Tris/HCl (pH 7.5), 10 mM EDTA and 28 mM β-mercaptoethanol. For filamentous fungi, the PDA was flooded with 1 ml of a sterile solution of 0.9 % saline and agitated to transfer fungal material to the solution. The saline was then collected, centrifuged and resuspended in lyticase buffer as above. Lyticase digestion was performed at 37 °C for 30 min. Spheroplasts were collected by centrifugation at 8400 g for 10 min and resuspended in 180 µl buffer ATL. Proteinase K solution (20 µl) provided in the kit was added to the suspension and a 45 min incubation at 55 °C was carried out. After this treatment, 200 µl buffer AL and 200 µl absolute ethanol were added to the samples and mixed thoroughly. Following the addition of ethanol, the samples

were left on ice for 30 min to increase precipitation of DNA and enhance final yield. The samples were then placed in silica-based spin columns and wash steps with 500 µl buffer AW1 and AW2 were applied following the manufacturer's instructions. DNA was then eluted twice from each column using 50 µl elution buffer for each elution and a single 1.5 ml tube was used to collect both eluates. Estimates of DNA concentration were obtained by spectrophotometry, and dilutions of extracted genomic DNA were prepared with a solution of 10 mM Tris/HCl (pH 9.0)/0.5 mM EDTA for use in the limit of detection assays. All DNA was stored at -20 °C.

Primers and probes. Fungal universal primers ITS3 and ITS4 (White *et al.*, 1990) were obtained from Sigma. The primers amplified a region between the 3' end of the 5.8S rRNA gene and the 5' end of the 28S rRNA gene, which encompassed the ITS2 region. The novel primers ITS3.3 and ITS4.2 were designed manually in order to demonstrate the limit of detection of the assay and these also amplified the ITS2 region between the 5.8S and 28S rRNA genes. The sequences of probes that targeted this region for species-specific detection of *Candida* species were obtained from Shin *et al.* (1999). These species-specific probes were targeted to *Candida albicans* (CA PR), *Candida tropicalis* (CT PR), *Candida parapsilosis* species complex (CP PR), *Candida glabrata* (CG PR) and *Candida krusei* (CK PR), and were synthesized and labelled at the 5' terminal nucleotide with a proprietary ferrocene compound (ATDBio). CA PR and CG PR were found to produce unsatisfactory results and so improved probes, CA PR3 and CG PR3, were designed manually for the detection of *Candida albicans* and *Candida glabrata*, respectively. The panfungal probe (PanF PR) was designed manually by analysing a matrix of 18S rRNA gene sequences that had been obtained from GenBank and identifying an appropriate target site for a probe. This probe was then subjected to a BLAST search (Altschul *et al.*, 1997) against a database containing fungal 18S rRNA gene sequences, as well as human, plasmodium and trypanosome sequences, to ensure that it had high similarity to all of the fungal sequences but not to the non-target sequences. The same matrix was then used to identify potential primers to amplify the area surrounding the PanF PR target site. Following PCR trials, the forward primer Fungal18sF2 and the reverse primer Fungal18sR4 were chosen for use with the PanF PR probe. Table 1 lists the probe and primer sequences.

Assessing cross-reactivity of probes using bioinformatics. A matrix of fungal ITS2 sequences was constructed using MEGA version 4.0 software (Tamura *et al.*, 2007) and used as a database for a BLAST query with each of the *Candida* species-specific probes. Any non-target species that were found to have ≥60 % identity to any of the species-specific probes were used in assays to test for cross-reactivity.

PCR using *Candida* species DNA. All PCRs were performed in a Peltier PTC thermal cycler (MJ Research). Asymmetric PCR conditions using a 5:1 ratio of reverse:forward primers were used to generate an excess of single-stranded target DNA for the assay. For testing probe specificity and the ability to detect clinical isolates, these PCRs were set up in triplicate and, for all probes except CA PR3, were carried out in a volume of 30 µl containing the following reagents: PCR buffer [10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂; GE Healthcare], 100 nM forward primer, 500 nM reverse primer, 0.15 mM each dNTP, 1 U IllustraTaq DNA polymerase (GE Healthcare) and 2 µl extracted DNA corresponding to 10–100 ng. The remaining volume was made up to 30 µl with molecular-grade water (DNase-, RNase- and protease-free, 0.1 µm filtered; Sigma). PCRs to amplify the ITS2 region for sequencing were carried out as above but in a reaction volume of 50 µl and symmetrical PCR conditions were used (500 nM each primer). Sequencing reactions were carried out by MWG and BLAST searches were performed against the GenBank sequence database to confirm the identity of the fungal isolates. For amplification of *Candida albicans* DNA for the

Table 1. Novel primer and internal probe sequences used in the electrochemical detection assay

All probes had a diferrocene label attached by a carbon linker molecule to the 5' terminal nucleotide.

Primer/probe	Sequence (5'→3')	GenBank accession no.	Binding position (nt)	Species detected
Primers				
ITS3.3	TGCCTGTTTGAGCGTCATTTC	FJ662407	315–335	
ITS4.2	AGTCCTACCTGATTTGAGG	FJ662407	509–491	
Fungal18sF2	GCCATGCATGTCTAAGTATAA	X99399	45–65	
Fungal18sR4	CCTCGTTAAGGGATTTAAATTGT	X99399	535–513	
Probes				
CA PR3	ATCGCTTTGACAATGGCTTA	AB032172	373–392	<i>C. albicans</i>
CT PR	CAAAACGCTTATTTTGCTAGTGGCC	AY282528	224–248	<i>C. tropicalis</i>
CP PR	GGTACAAACTCCAAACTTCTTCCA	AJ635316	413–437	<i>C. parapsilosis</i>
CG PR3	TCAGTATGTGGGACACGAGCGCAAG	AB032177	632–656	<i>C. glabrata</i>
CK PR	AGTGGCCCGAGCGAACTAGACTTTT	EU266565	363–387	<i>C. krusei</i>
PanF PR	GGTGATTCATAATACTTTTCG	AY497766	99–120	All fungi

electrochemical assay, the buffer used comprised 10 mM Tris/HCl (pH 8.3)/50 mM KCl. MgCl₂ was added separately to give a final concentration of 3 mM. The lower pH and higher concentration of MgCl₂ were used because these provided better conditions for activity of the T7 exonuclease used in the assay (Kerr & Sadowski, 1972). The amplification conditions were: 94 °C for 1 min; 40 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s; and 72 °C for 3 min. The PCR conditions for the limit of detection assay were as above except that a 50 µl reaction volume was used. The PCR buffer was prepared in house and provided the reaction with 10 mM Tris/HCl (pH 8.3) and 50 mM KCl. MgCl₂ was added separately to give a concentration in the reaction of 3 mM and 2.5 U Jumpstar *Taq* DNA polymerase (Sigma) was used. Serial dilutions (1 µl) of genomic DNA over 2 orders of magnitude were added to give a range of template DNA from 3.7 pg to 37 fg, which was equivalent to a range of ~100 genomes to ~1 genome of a diploid *Candida* species based on estimates of fungal genome size reported by Kullman *et al.* (2005). For the assay with *Candida glabrata* DNA, 1.05 pg–10.5 fg genomic DNA was used, corresponding to <100 genomes to <1 genome (Kullman *et al.*, 2005). The amplification conditions were: 95 °C for 1 min; 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and 72 °C for 3 min. A 10 µl aliquot of the PCR products was visualized on an ethidium bromide-stained agarose gel to check for contamination and to confirm the presence of amplified DNA (data not shown). For all assays, 20 µl PCR product was used for the electrochemical assay.

Electrochemical assay. The probe mix for the assay contained the following reagents: 3 µM of the relevant probe and 10 U T7 exonuclease (New England Biolabs) made up to 5 µl with molecular-grade water (Sigma). The probe mix was added to the remaining 20 µl PCR product and incubated for 20 min at 37 °C. When incubation was complete, 20 µl of the probe reaction mix was pipetted onto screen-printed carbon electrodes with a silver/silver chloride reference electrode. The assays testing probe specificity and detection of clinical strains were performed using electrodes manufactured by Gwent Electronic Materials and those testing the limit of detection were performed using electrodes manufactured by G. M. Nameplate. The observed current due to oxidation of the released ferrocene-labelled nucleotide following DPV was measured using a potentiostat (Autolab PGSTAT30; EcoChemie) and dedicated software (GPES version 4.9; EcoChemie). DPV measurements allowed the measurement of low concentrations of electroactive species by effectively subtracting capacitive charging current which can other-

wise mask low concentration measurements. The following settings were used: modulation time 0.04 s, interval time 0.1 s, initial procedure –0.1 V, end potential 0.5 V, step potential 0.003 V, modulation amplitude 0.05 V.

RESULTS AND DISCUSSION

Results of PCR and sequencing

The primer pairs ITS3/ITS4 and ITS3.3/ITS4.2 were able to amplify the ITS2 region in all of the species tested, whilst the Fungal18sF2/Fungal18sR4 primer pair was able to successfully amplify a region in the 18S rRNA gene in all species tested. An amplicon was produced when both symmetrical and asymmetrical conditions were used and the size of each amplicon corresponded to the expected size based on sequence information (data not shown).

Testing probe specificity with *Candida* type strains

BLAST searches against the fungal ITS2 sequence matrix using the sequences of the species-specific probes revealed that five species contained regions with ≥60 % similarity to the CG PR probe binding site. Assays using the CG PR probe and these species displayed cross-reactivity (data not shown) and as a result the probe CG PR3 was designed, which had no significant similarity to any of the fungal species in the matrix. The CA PR3 probe displayed 70 % similarity to a region in the ITS2 sequence of *Candida dubliniensis*, but, when tested, the current produced was a similar value to the other non-target currents (Table 2). None of the other probes produced alignments with significant similarity to non-target species.

To test the specificity of the species-specific probes experimentally, an assay was performed with each of the probes using DNA extracted from 10 type strains of *Candida* species (Table 2) and a DNA-free sample. The first

Table 2. Mean currents $\pm$ SD obtained with the five species-specific *Candida* probes when tested against the target species and nine other non-target *Candida* species

The mean background current calculated for each probe was subtracted from all of the results for that probe. The mean currents produced by the probes against their target species are highlighted in bold.

Species	Mean current minus background current values obtained with species-specific probes (nA)				
	CA PR3	CP PR	CG PR3	CT PR	CK PR
<i>C. albicans</i>	112.2 $\pm$ 20.0	4.5 $\pm$ 9.2	2.5 $\pm$ 7.0	7.6 $\pm$ 8.0	-1.9 $\pm$ 6.9
<i>C. parapsilosis</i>	-3.2 $\pm$ 8.3	96.2 $\pm$ 19.6	-4.7 $\pm$ 5.7	6.6 $\pm$ 3.4	-1.4 $\pm$ 9.2
<i>C. glabrata</i>	3.1 $\pm$ 7.3	-1.7 $\pm$ 7.2	73.8 $\pm$ 16.8	9.6 $\pm$ 9.1	-0.4 $\pm$ 6.4
<i>C. tropicalis</i>	-1.6 $\pm$ 5.9	-5.7 $\pm$ 6.2	-7.7 $\pm$ 5.4	135.0 $\pm$ 7.0	-1.5 $\pm$ 8.2
<i>C. krusei</i>	-1.4 $\pm$ 2.2	2.2 $\pm$ 6.3	-8.7 $\pm$ 6.8	2.9 $\pm$ 8.3	112.1 $\pm$ 26.1
<i>C. lusitaniae</i>	-3.8 $\pm$ 1.2	1.2 $\pm$ 6.8	-2.5 $\pm$ 5.7	2.7 $\pm$ 5.2	0.7 $\pm$ 14.1
<i>C. dubliniensis</i>	1.1 $\pm$ 3.8	3.8 $\pm$ 6.7	-5.8 $\pm$ 7.7	5.6 $\pm$ 5.3	1.2 $\pm$ 7.4
<i>C. kefyr</i>	0.4 $\pm$ 7.8	-3.1 $\pm$ 6.9	-3.5 $\pm$ 6.2	3.9 $\pm$ 5.3	9.1 $\pm$ 6.7
<i>C. famata</i>	-5.5 $\pm$ 1.3	1.3 $\pm$ 13.1	4.6 $\pm$ 6.0	6.3 $\pm$ 4.9	-2.3 $\pm$ 7.8
<i>C. guilliermondii</i>	0.1 $\pm$ 4.2	3.9 $\pm$ 10.2	7.6 $\pm$ 11.3	4.2 $\pm$ 8.5	1.9 $\pm$ 7.3

Candida albicans-specific probe, CA PR, did not allow significant discrimination between target- and non-target-containing samples (data not shown), and small changes to the reagents used for PCR (higher MgCl_2 concentration and lower pH) as well as the design of a new probe, CA PR3, were implemented to improve the conditions of the assay and so obtain much higher currents with target DNA. After this redesign, all of the species-specific probes were found to be able to discriminate between their target species and nine non-target *Candida* species. Peak current heights were measured, examples of which are shown with the CT PR probe (Fig. 1). Fig. 2 shows examples of the mean background currents from DNA-free samples produced in assays with two of the *Candida* species-specific probes. When the probes were present in the assay without target DNA, background currents were produced and found to vary between probes, with the highest values being produced by CA PR3 and the lowest values by CK PR. This mean background current was subtracted from the mean values obtained from the target DNA-containing samples for each probe.

Testing DNA from clinical *Candida* isolates

To test the ability of the species-specific probes to detect clinical strains of *Candida*, PCRs were performed with DNA from four clinical isolates of the relevant species as well as from the type strain. DNA-free samples were also included to produce background values for each probe. All species-specific probes were able to detect DNA from clinical isolates (Fig. 3). The adjusted mean values (sample mean current minus background mean current) reported for the CK PR probe were relatively low compared with the values reported in Table 2 and with the values produced by the other probes in Fig. 3. This was caused by considerably lower currents than expected being produced by both the target-containing samples and by the DNA-free samples. However, after adjustment, the mean currents remained

high enough to indicate positive detection of the target DNA.

Testing the panfungal probe against a range of pathogenic fungi

A panfungal probe, PanF PR, was tested against DNA from a range of commonly occurring fungi in invasive infections together with three additional opportunistic fungal pathogens and one non-pathogenic fungal species (Fig. 4). The fungal DNA tested produced mean currents that ranged from 88.83 ± 15.44 nA for *Cryptococcus victoriae* to 356.03 ± 35.76 nA for *Cryptococcus gattii*.

Fig. 1. Electrochemical traces obtained by the probe CT PR tested against 10 *Candida* species and a DNA-free sample. The current produced in the assay is represented by the peak height. The large peak corresponded to the assay with *Candida tropicalis* DNA present, whilst the other peaks corresponded to the non-target species and the DNA-free sample.

Fig. 2. Example mean currents obtained with the target type species and a DNA-free sample with the *Candida albicans*- and *Candida krusei*-specific probes CA PR3 and CK PR. Error bars represent SD ($n=3$).

Testing the fungal probes against human DNA

Each of the probes was tested against fungal target DNA in the presence of sufficient human DNA to provide approximately the same number of rRNA gene copies as the fungal target. Discrimination was achieved with all probes and the mean currents produced by PanF PR and CK PR, which were the best- and worst-performing probes, respectively, are shown in Fig. 5. In a clinical sample, the ratio of human cells:fungal cells would be of the order of $10^9:1$, but to achieve this ratio experimentally, based on the amount of fungal DNA currently being used in the assay, would have required milligram quantities of human DNA to be added to the reaction, which was not feasible.

Limit of detection

Fig. 6 shows sample data using an assay with *Candida albicans* DNA and the corresponding specific probe, which demonstrated that detection of low amounts of genomic DNA was possible. Table 3 shows the mean currents produced from the assays with each of the probes tested against a range of template amounts of target genomic DNA equivalent to ~100, ~10 and ~1 genomes. The limit of detection of the PanF PR probe was tested using *Candida albicans* genomic DNA as this species had originally given the lowest mean current out of all of the pathogenic fungi in the test demonstrating the range of fungi detected by this probe (Fig. 4). All of the probes were capable of detecting down to approximately 1 genome equivalent of DNA. The assay conditions were modified substantially to achieve a limit of detection of 1 genome equivalent and the changes resulted in the DNA-free samples producing no visible peaks in the assay with concomitant zero current with all of the replicates. Furthermore, the changes resulted in higher currents in these assays with picogram amounts of DNA compared with nanogram amounts of DNA in the initial specificity work. The dose response was observed to be highly reproducible but non-linear, the reasons for which were not studied.

The results showed that the electrochemical assay was capable of detecting and identifying fungi to the species level using *Candida* species-specific probes and that DNA from a broad range of fungal species could be detected using the panfungal probe. The probes were shown to be specific for their target species when tested against a panel of nine other clinically relevant *Candida* species and were

Fig. 3. Mean currents obtained from assays using the type strain and four clinical isolates with all of the *Candida* species-specific probes. Error bars represent SD ($n=3$).

Fig. 4. Example mean currents produced when a range of fungal species were tested with the panfungal PanF PR probe. Error bars represent SD ($n=3$). The species *Cryptococcus victoriae* has not been reported as a pathogen of humans.

Fig. 5. Example mean currents obtained from assays with the *Candida krusei* species-specific probe CK PR and the panfungal probe PanF PR testing samples containing human DNA and mixed human and fungal DNA. The *Candida krusei* type strain was used with the CK PR probe and a clinical strain of *Candida lipolytica* was used with the PanF PR probe. Note the difference between the scales of the y-axis. Error bars represent SD ($n=3$).

Fig. 6. Example mean currents obtained in assays with ~100, ~10 and ~1 genome equivalents of *Candida albicans* and *Candida krusei* genomic DNA and the probes CA PR3 and CK PR, respectively. Note the difference between the scales of the y-axis. Error bars represent SD ($n=3$).

Table 3. Mean currents $\pm$ SD ($n=3$) obtained in assays with 100, 10 and 1 fungal genome equivalents with the species-specific probes and the PanF PR probe

Species and probe used	Mean current with ~100 genome equivalents (nA)	Mean current with ~10 genome equivalents (nA)	Mean current with ~1 genome equivalent (nA)
<i>C. albicans</i> – CA PR3	233.7 $\pm$ 6.0	169.7 $\pm$ 7.4	74.6 $\pm$ 0.7
<i>C. parapsilosis</i> – CP PR	228.3 $\pm$ 40.7	119.8 $\pm$ 21.3	51.6 $\pm$ 15.7
<i>C. glabrata</i> – CG PR3	130.7 $\pm$ 6.5	92.8 $\pm$ 15.8	38.5 $\pm$ 7.2
<i>C. tropicalis</i> – CT PR	86.3 $\pm$ 11.1	41.8 $\pm$ 9.3	15.3 $\pm$ 2.7
<i>C. krusei</i> – CK PR	35.4 $\pm$ 0.7	14.1 $\pm$ 2.9	4.8 $\pm$ 0.7
<i>C. albicans</i> – PanF PR	188.7 $\pm$ 58.0	121.7 $\pm$ 11.1	40.4 $\pm$ 12.8

able to detect clinical isolates of the relevant target species. The limit of detection for the species-specific probes was demonstrated to be ~1 genome equivalent.

The PanF PR probe was designed to be able to detect a wide range of fungal pathogens including emerging opportunistic fungi, such as *Rhizomucor*, *Absidia*, *Fusarium* and *Penicillium* species, as well as the more commonly occurring *Cryptococcus*, *Aspergillus* and *Candida* species. It successfully detected 14 species of opportunistic fungal pathogens, including several infrequently encountered species, and one non-pathogenic fungal species, *Cryptococcus victoriae*. Using DNA from *Candida albicans*, the limit of detection of the probe was shown to be ~1 genome equivalent.

The probe set was designed to detect DNA from any fungal pathogen, as well as to achieve species-specific detection of the most common species involved in invasive fungal infections including *Candida glabrata* and *Candida krusei*, which are associated with decreased susceptibility to fluconazole. Detection methods that permit species-specific detection of the most common fungal pathogens, including fluconazole-resistant species, are generally unsatisfactory as they cannot detect infecting species outside the test panel, and the majority of methods of detecting invasive fungal infections have concentrated on a few common fungi, usually *Candida* species (Shin *et al.*, 1999; Maaroufi *et al.*, 2003; Baskova *et al.*, 2007; Innings *et al.*, 2007; Metwally *et al.*, 2007). Only a few methods have attempted to achieve detection (but not identification) of a range of fungal species (e.g. Vollmer *et al.*, 2008) and very few have tried to design a probe capable of detecting all pathogenic fungi (e.g. Zhao *et al.*, 2009). The inclusion of a panfungal probe in this suite means that early detection of an invasive infection caused by any fungal pathogen could be achieved, enabling a patient to be monitored and treated more successfully. A potential drawback to the use of a highly sensitive panfungal probe is that it necessitates extremely careful handling of samples as it can detect a single cell and thus fungi present in the environment may be detected by the assay.

Multiplex capability is an attractive feature of detection assays as it reduces the number of tests that need to be performed to achieve detection with all of the probes.

Multiplex detection in this assay is currently limited by the number of different ferrocene compounds available, as well as by the need for internal positive controls. The development of unique ferrocene labels for the probes in the fungal suite would be desirable, enabling the probes to be used simultaneously in a single reaction.

In summary, the data show that the described methodology and probes used in this novel detection system have the required specificity and sensitivity to detect fungal DNA at clinically relevant levels and to identify individual species.

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