Detection of *Candida albicans* DNA from blood samples using a novel electrochemical assay.

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Running title: Electrochemical detection of Candida DNA in blood.

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Summary

The genus *Candida* contains a number of yeast species which are opportunistic pathogens and are associated with life-threatening infections in immunocompromised individuals. Provision of appropriate therapy relies on the rapid identification of the infecting species and existing methods of identifying *Candida* species in clinical samples are time and resource intensive and are not always specific enough to differentiate between drug-susceptible and drug-resistant species. We have previously developed a system for the rapid detection of yeast pathogens in clinical samples using PCR followed by hybridisation with a suite of five species-specific, electrochemically labelled DNA probes. The limit of detection of the assay was shown to be 37fg (≈ one genome) per reaction using extracted genomic DNA. We carried out a study to test the limit of detection of one of the probes, CA PR3, using blood samples from a healthy donor that were spiked with genomic DNA or with *C. albicans* cells. Our results demonstrate a limit of detection of 37fg ml^{-1} (≈ 1 genome ml^{-1}) of blood using extracted DNA or 10c.f.u ml^{-1} of blood using *C. albicans* cells indicating that the assay is capable of detecting *C. albicans* nucleic acid at levels that are encountered in clinical samples.

Introduction

Some species of fungi are capable of causing life-threatening invasive infections most commonly in individuals with a weakened immune system due to underlying disease or immuno-suppressive therapy (Pfaller & Diekema, 2010) and species of the genus *Candida* are by far the most prevalent, being responsible for 70-80% of diagnosed fungal bloodstream infections in the United States (Fridkin and Jarvis, 1996; Trick *et al.*, 2002). *C. albicans* is usually the most commonly isolated species from patients with invasive candidiasis, accounting for approximately 45-65% of cases.

Diagnosis of invasive candidiasis can be difficult and culture remains the 'gold standard' for definitive diagnosis of invasive fungal infections. However, identification of fungi by culture and subsequent biochemical tests is time consuming (Morris *et al.*, 1996) and has been reported to lack sensitivity (Pemán & Zaragoza, 2009). A number of nuceic acid-based assays for detecting fungi have been developed (e.g. Hata *et al.*, 2008; Borman *et al,* 2008; Lau *et al.*, 2008) which are capable of species-level identification and some have been shown to be capable of direct detection of fungal DNA from blood (e.g. McMullan *et al.*, 2008; Zhao *et al.*, 2009).

A novel method for detection of nucleic acid was developed by Hillier *et al.* (2004a; 2004b) using oligonucleotide probes labelled with the electroactive compound ferrocene and detection was performed using differential pulse voltammetry (DPV) on solid electrodes. We previously extended this work and developed a pan-fungal probe as well as a suite of species-specific probes for the detection of the five most clinically relevant *Candida* species which were capable of detecting DNA equivalent to a single genome from target fungal species in a 50µl reaction (Muir *et al.*, 2009).

The aim of this work was to develop the assay further in order to optimise conditions to allow detection of clinically relevant amounts of fungal nucleic acid in blood samples. Fungal titres in blood can be extremely low and have been reported to be in the range of 5 – 100 colony forming units (c.f.u) ml^{-1} of blood (Loeffler *et al.*, 2000). Therefore a study was performed using the *C. albicans* specific probe to optimise reaction conditions for detection of low amounts of *C. albicans* DNA and cells in 1ml spiked blood samples.

Materials and methods.

Culture acquisition

The type strain of *C. albicans* (CBS 562) species was obtained from the Centraal Bureau voor Schimmelcultures (CBS) and used for all experiments. Cultures were grown on yeast-peptone-dextrose (YPD) agar (1% yeast extract (Oxoid), 2% Mycological peptone (Oxoid), 2% glucose (Sigma), 2% Bacto agar (Difco)) at 28°C for 48 hours. Liquid stocks of *C. albicans* were prepared using the following method: a growing colony was picked from YPD agar using a sterile loop, transferred to 5ml liquid YPD and incubated overnight at 28°C with shaking. 1ml samples of each culture were then aliquoted into 2ml microcentrifuge tubes. Finally, 1ml of 20% glycerol added as a cryoprotectant before transfer to -20°C storage.

Steps to limit contamination

In order to prevent contamination the setup of PCR reactions was performed in a different room to where the PCR amplification was performed and in a class 2 biological safety cabinet which was regularly disinfected with 70% ethanol and sterilized using ultra violet (UV) irradiation. The air flow remained switched off while the mix was prepared to avoid contamination of samples by airborne PCR amplicon. Blood samples were aliquoted at a different site, in a laminar flow hood that was regularly sterilised as above and additionally was regularly swabbed with the DNA degrading reagent DNA-ExitusPlus (Applichem). All stock reagents were aliquoted into working solutions to limit potential contamination and problems associated with freeze-thawing.

Whole blood acquisition and treatment

Blood from a healthy donor was collected by a trained individual into 6ml K_2EDTA-coated vacutainers (BD Biosciences) and inverted 8 – 10 times to ensure adequate mixing of the anti-coagulant with blood. 1ml aliquots were then pipetted into 2ml sterile cryovials and the samples stored at -80ºC until used.

Extraction of DNA from liquid cultures

The 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. The yeast cell count in a stock cell suspension was

estimated by spectrometry according to the method described in Amberg *et al.* (2005) and a solution of approximately $5x10^6$ yeast cells was prepared by appropriate dilution of the stock and centrifuged at 10,000 rpm for 10mins to pellet the cells. The supernatant was removed and the cell pellet resuspended in 500ul of a lyticase lysis buffer which comprised 10U ml^{-1} lyticase from *Arthrobacter luteus* (Sigma), 50mM Tris, pH 7.5, 10mM EDTA and 28mM β-mercaptoethanol; the lyticase digestion was performed at 37°C for 30mins. Following treatment by lyticase the sphaeroplasts were collected by centrifugation and the supernatant was removed. The disruption of the sphaeroplast cell membrane by proteinase and detergent treatments was performed according to manufacturer's instructions and, following the addition of absolute ethanol, the samples were left on ice for 30mins to increase precipitation of DNA and enhance final yield. The samples were then placed in silica-based spin columns and washed according to the manufacturer's instructions. DNA was then eluted twice from each column using 50µl of the elution buffer for each elution and a single 1.5ml tube was used to collect both eluates. The concentration of eluted DNA was estimated by spectrometry. All DNA was stored at -20^{o}C.

Extraction of DNA from blood samples spiked with *C. albicans* genomic DNA.

The required number of 1ml whole blood samples were removed from storage at -80°C and allowed to thaw. *C. albicans* DNA was removed from -20°C storage and allowed to thaw. A 5ml sterile bijou tube had the required amount of *C. albicans* genomic DNA added and 1ml of the blood sample was then added to the bijou. The solution was agitated gently to allow mixing of the DNA in the blood. Extraction of the *C. albicans* DNA from blood was performed using GenElute Bacterial Genomic DNA extraction kit (Sigma) with some modifications which are described below.

The spiked blood samples had 2ml of a proprietary lysis buffer added and were incubated for 5mins at room temperature with occasional gentle agitation to allow mixing and complete lysis of blood cells. Meanwhile the required number of extraction columns was prepared using Column Preparation Solution as per manufacturer's instructions. After the 5min incubation of blood samples 700µl of the lysate was applied to the spin column and centrifuged at 12,000g for 1min to allow DNA to bind to the column. The flow-through was discarded. The above step was repeated until all of the lysed blood sampled had been spun through the column.

After the final centrifugation the collection tube was discarded and replaced with a fresh one. Wash steps were then performed as per the manufacturer's instructions. Elution of DNA was initially performed by adding 100µl of elution buffer to the column, incubating at room temperature for 30s and centrifuging at 12,000g for 1min. 30µl of this eluate was used as template in PCR reactions. However, to improve the yield when very small amounts of *C. albicans* DNA were spiked into blood the elution buffer was prewarmed to 60°C and 34.25µl was applied to the spin column, incubated for 30s at room temperature and centrifuged at 12,000g for 1min. The eluate was then reapplied to the spin column, incubated and centrifuged for a second time. This entire eluate was then used as template in PCR amplifications.

Monitoring for DNA loss and inhibition of PCR

To monitor for potential loss of DNA during the extraction procedure, a comparison was made between assays performed with samples that were extracted from blood spiked with excess *C. albicans* DNA (37pg), and unspiked blood samples which were processed in parallel before having an excess amount (37pg) of *C. albicans* DNA added to the eluate. Monitoring for potential inhibition of PCR by excess non-target DNA and/or inhibitory compounds from blood that may have co-eluted with the DNA was performed by comparing the results of assays performed using DNA extracted from spiked blood samples (3.7pg *C. albicans* DNA) and DNA extracted from 1ml samples of sterile H_2O that were spiked with an equal amount of DNA.

Extraction of DNA from blood samples spiked with *C. albicans* cells.

To obtain cell suspensions of the appropriate number of cells to spike into blood samples, growing colonies of *C. albicans* were picked with a sterile loop and resuspended in 500µl sterile H_2O. This stock solution was diluted 10-fold and 50-fold and cell-density estimates of the three solutions were made using an improved Neubauer haemocytometer. Estimates of different preparations ranged between ~$2x10^7$ cells ml^{-1} to ~$6x10^7$ cells ml^{-1} and estimates were considered to be in good agreement with one another if the cell density estimates were within $0.5x10^7$ cells ml^{-1} of one another to allow for pipetting inaccuracies. Provided it satisfied this criterion, the 50-fold diluted cell suspension was further diluted to yield solutions of 1 cell $µl^{-1}$

and 0.1 cell μl^{-1}. Viable cell densities were confirmed retrospectively by performing plate counts.

The extraction procedure was very similar to the method outlined above but differed in the pre-treatment step. After thawing the blood samples, 10µl of the appropriate *C. albicans* cell suspension was added to a 5ml sterile bijou and then the 1ml blood sample was added and the solution gently mixed. 250µl of a lysis buffer containing 200U ml^{-1} lyticase was added and the reaction mix incubated at room temperature for 5min with gentle agitation. After this step the extraction continued with the addition of 1ml of the proprietary lysis buffer and was identical to the procedure outlined previously for genomic DNA extraction. Note that 34.25µl of prewarmed elution buffer was always used for elution of DNA extracted from *C. albicans* cells.

Primers and probes

The previously described primers (Muir *et al*, 2009) ITS3.3 (TGCCTGTTTGAGCGTCATTTC) and ITS4.2 (AGTCCTACCTGATTTGAGG) were used for all PCR reactions and the probe CA PR3 (ATCGCTTTGACAATGGCTTA), labelled with a proprietary ferrocene molecule linked to the 5′ terminal nucleotide by ATDBio (Southampton) was used to detect amplified target DNA.

PCR conditions

PCR amplification conditions used a total volume of 50µl containing the following reagents: PCR buffer (10mM Tris-HCl (pH 8.3), 50mM KCl), 100nM ITS3.3, 500nM ITS4.2, 0.15mM each dNTP, 3mM $MgCl_2$ and 2.5U Jumpstart *Taq* DNA polymerase (Sigma), extracted nucleic acid (30µl or 34.25µl, see above) and molecular biology grade water (Sigma) to the required volume. The cycling conditions used were: 95°C for 1min; 94°C for 30s plus 58°C for 30s plus 72°C for 1min for 40 cycles; 72°C for 3mins. A 10µl aliquot of the PCR products was added to 2µl of 6X loading dye and visualized on a 1% (w/v) agarose gel made up in Tris-Borate-EDTA (TBE) buffer (0.09M Tris base, 0.09M boric acid, 2mM EDTA pH 8.0) containing 0.5µg ml^{-1} ethidium bromide to confirm the presence of amplified target DNA. Samples were run at 7V cm^{-1}. 20µl of the remaining PCR product was used for the electrochemical assay.

Electrochemistry

A probe mix was prepared for each sample to be assayed containing the following reagents: 3µM of probe CA PR3 and 10U T7 exonuclease (New England Biolabs) made up to 5µl with molecular grade water (Sigma). This probe mix was added to 20µl of PCR product and incubated for 20mins at 37°C to allow hybridization of the probe to single stranded target DNA and subsequent digestion of the duplex by T7 exonuclease. Once incubation was complete 20µl of the probe reaction mix was pipetted onto screen printed carbon electrodes with a silver/silver-chloride reference electrode. The electrodes used for specificity testing were manufactured by Gwent Electronic Materials Ltd. and those used for sensitivity testing were manufactured by G. M. Nameplate Inc. The observed current due to oxidation of the released ferrocene-labeled nucleotide following DPV was measured using a potentiostat (Autolab PGSTAT30, EcoChemie) and dedicated software (GPES version 4.9, EcoChemie). DPV measurements were made using the following settings: modulation time 0.04s; interval time 0.1s; initial procedure -0.1V; end potential 0.5V; step potential 0.003V; modulation amplitude 0.05V.

Results

Detection of excess *C. albicans* DNA and study of inhibitory effects

Assays were carried out using excess *C. albicans* DNA (37pg) spiked into samples in different ways to examine the effects of the extraction procedure on the currents that were generated. Samples that were extracted from blood spiked with excess *C. albicans* DNA produced a mean current of 153.4 ± 6.6nA (n=3) and this compared with a mean current of 168.9 ± 17.1nA (n=3) which was produced by samples from unspiked blood which had been processed using the extraction protocol and the eluate spiked with 37pg fungal DNA. The PCR positive control samples - obtained by amplifying the same amount of DNA in a 50µl PCR - produced a mean current of 189.9 ± 20.7nA and an unspiked blood sample gave no current.

The higher current produced by the positive control was probably as a result of the complete availability of the 37pg template in this reaction, whereas in the other reactions the 37pg template was diluted in 100µl of elution buffer. The difference between the results obtained by spiked blood vs. spiked eluate demonstrated the effect of DNA loss due to the extraction procedure on the signals obtained in the

assay. The difference in mean currents (15.5nA) was not significant and demonstrated that DNA retention using the procedure was good. The zero current produced by the samples extracted from the unspiked blood confirmed that human DNA from leucocytes did not cross-react with the *C. albicans*-specific probe.

The assays comparing currents produced by samples extracted from spiked blood or H_2O demonstrated that some inhibition occurred in samples extracted from blood. The mean current produced by samples extracted from 1ml of H_2O spiked with 3.7pg of *C. albicans* DNA was 83.1 ± 14.9nA (n=3) compared with a mean of 55.5 ± 11.5nA (n=3) produced by assays using samples extracted from 1ml blood spiked with 3.7pg DNA. The difference in current (27.6nA) was statistically significant ($P<0.05$) and indicated that inhibition, either by sequestration of primers by excess human DNA or due to direct inhibition of *Taq* polymerase, occurred during PCR with samples extracted from whole blood.

Detection of clinically relevant levels of fungal DNA from spiked blood

Assays were performed to detect DNA from clinically relevant amounts of *C. albicans* spiked into blood samples. The mean currents produced by the samples spiked with 3.7pg and 370fg were 75.6 ± 8.9nA and 35.6 ± 6.4nA (≈100 and ≈10 genomes respectively, n=3) and these indicated successful detection but the mean current produced with 37fg DNA was 15.0 ± 17.2nA (≈ 1 genome, n=3) which demonstrated that detection at this level was unreliable: the mean current was low and the standard deviation high due to one of the samples failing to produce a signal (i.e. 0nA current, fig 1). With a minor adjustment to the elution stage of the extraction procedure (i.e. use of a 34.25µl eluate as template) more reliable identification of ≈1 genome of *C. albicans* DNA in a 1ml blood sample was achieved (mean of 26.3 ± 14.3nA, n=3).

It was noted that there was a highly significant difference ($P<0.001$) between the mean current produced by the positive control which contained 3.7pg of DNA in a 50µl PCR reaction compared to the mean current produced by samples which had been extracted from 1ml blood samples spiked with 3.7pg of DNA. It was also observed that the mean current produced by the positive control was approximately

twice that of the mean current produced by the same amount of DNA spiked into blood.

Detection of *C. albicans* cells from spiked blood samples.

Fungal cells represent the major source of fungal nucleic acid in a clinical sample so it was essential that the assay be capable of their detection. Results of assays attempting to detect 10 c.f.u of *C. albicans* cells present in a 50µl reaction and spiked into a 1ml blood sample showed that detection was successful in both sample types with mean currents of 64.5 ± 45.7nA (n=3) and 16.3 ± 12.6nA (n=6) respectively. Attempts were also made to detect suspensions containing a single c.f.u of *C. albicans* added to both PCR samples and 1ml blood samples but these were unsuccessful (data not shown), possibly due to the limitations inherent in the DNA extraction procedure. This may also have accounted for the difference ($P<0.0001$) between the mean current of 68.5 ± 3.6nA (n=3) produced by 37fg (≈ 1 genome) of *C. albicans* DNA in the PCR positive control and the mean current of 16.3 ± 12.6nA (n=6) produced by template from the spiked blood sample which contained 10 *C. albicans* c.f.u's.

Discussion

The limit of detection of *C. albicans* extracted DNA in blood was shown to be 37fg ml^{-1} (≈1 genome ml^{-1}) while the limit of detection of *C. albicans* cells in blood was shown to be 10 c.f.u ml^{-1}. The extraction method was shown to recover a good yield of the spiked *C. albicans* DNA from blood, and minor inhibitory effects due to co-elution of constituents from human blood were demonstrated. There were differences observed in the mean currents obtained from samples containing equivalent amounts of nucleic acid that were prepared in different ways; the currents produced by template extracted from blood spiked with extracted *C. albicans* genomic DNA were higher when compared to the currents produced by assays using equivalent amounts of *C. albicans* c.f.u spiked into blood, while the mean currents produced by genomic DNA and whole cells added directly to PCR were higher than the currents produced by material extracted from spiked blood. Furthermore, detection of a single *C. albicans* c.f.u in blood was not possible using the method.

These differences could have been due in part to the effects of DNA loss occurring during the extraction procedure which would likely be more critical when lower amounts of DNA were used, or by the potential for PCR primers to be sequestered due to interactions with non-target nucleic acid which would have been provided by the large excess of human genomic DNA and RNA that would have been present in the extracted DNA sample. It was also possible that fungal 5.8S and 26S rRNA molecules that co-eluted with DNA may have inhibited PCR by competing for primers because these molecules possess the ITS3.3/4.2 primer target sites (Li *et al.*, 1990; Metwally *et al.*, 2008).

The extraction procedure used in this initial study had not been fully optimised for detection of fungal DNA from blood samples. In particular, due to the volume of blood and lysis buffers used, several centrifugation steps were required to process each sample which increased the risk of contamination and loss of nucleic acid and this may have been of particular importance when attempting to detect DNA from a single *C. albicans* cell in a 1ml blood sample. Nonetheless, the fact that detection of fungal cells was possible using the method was a significant result and opportunities exist for optimisation of the DNA extraction procedure that may allow detection of a

single cell in blood. Improved methodologies will probably involve immobilisation of the fungal cells on a membrane in order to remove human material and reduce the volume of sample to be processed. Such modifications will be the subject of future research.

Whole blood was used in the assay both to reduce the turnaround time and because it is a more favourable sample type for the recovery of fungal nucleic acid. Plasma and serum samples, whilst containing fewer inhibitory substances such as human DNA and macromolecules, have the disadvantage of the removal of cell-associated fungal DNA, as well as the extra time required for preparation. Although reports by Bougnoux *et al.* (1999) and Kasai *et al.* (2006) suggested that cell-free fungal DNA can survive in blood for relatively long periods in a rabbit model, and studies have detected cell-free fungal DNA in serum samples (Wahyuningsih *et al.*, 2000; McMullan *et al.*, 2008), other studies using mouse models have shown that naked DNA is rapidly cleared from the blood, mainly due to uptake by the liver but also by the action of serum-associated nucleases (Kawabata *et al.*, 1995; Liu *et al.*, 2007). Therefore cell-free blood fractions may be an unsuitable sample type to use. Despite the difficulties inherent in using whole blood, our results demonstrate that detection of fungal DNA and cells is possible using the assay.

While spiked blood samples from a healthy patient are useful for preliminary testing of an assay's capability the use of clinical samples would provide much more definitive results. Samples taken from patients with an episode of invasive candidal infection may differ from healthy blood samples, particularly in terms of the leucocyte cell population and can also provide a better measure of the sensitivity of an assay – indicating the likelihood of detection of fungal DNA from a sample taken under clinical conditions. Further testing of the assay using clinical samples would be an essential undertaking for future work to validate this method of detection. Additionally, the remaining probes from fungal detection suite must also be tested to demonstrate a similar range of detection of target DNA in blood samples.

Nonetheless, the assay was shown to be sensitive enough to detect 37fg (≈1 genome) of *C. albicans* genomic DNA per millilitre of blood but was less sensitive when intact cells were used, achieving a limit of detection of only 10 c.f.u per millilitre

of blood. This detection limit is within the lower clinical range for candidaemia of 5 to 100 c.f.u ml^{-1} (Loeffler *et al.*, 2000) but a lower limit outside of the range would be preferable to avoid the possibility of false negative results and this may be achievable by minor adjustments to the DNA extraction procedure as suggested above.

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Figures.

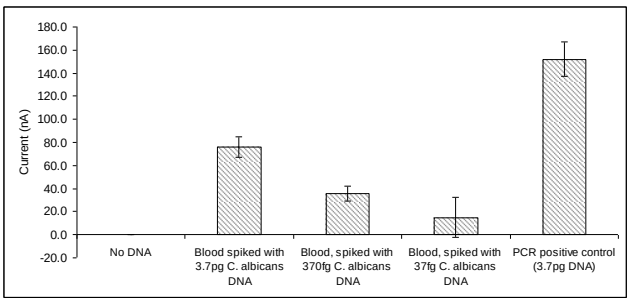

Figure 1. Mean currents produced in assays with DNA extracted from 1ml blood samples spiked with clinically relevant amounts of *C. albicans* DNA. Error bars represent SD (n=3).