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Application of a Non-amplification based Technology to Detect Invasive Fungal Pathogens

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Abstract

Current diagnostic techniques for fungal diseases could be improved with respect to sensitivity, specificity and timeliness. To address this clinical need, we adapted a non-amplification based nucleic acid detection technology to identify fungal pathogens. We demonstrate a high-specificity, detection sensitivity, reproducibility and multiplex capacity for detecting fungal strains.

Keywords

Invasive fungal disease; Diagnosis; *Aspergillus*; *Candida*; nCounter analysis system

The incidence of invasive fungal diseases (IFDs) is increasing. Microbiological culture lacks sensitivity in patients with proven IFD. Polymerase chain reaction (PCR) IFD assays are limited by moderate specificity, methodological heterogeneity, and lack of an available FDA-approved test (Hsu, et al., 2011). Table 1 highlights parameters of current technologies for fungal diagnosis. We evaluated the nCounter Analysis system (NanoString, Seattle WA) to quantify and differentiate 10 clinically relevant fungal species. This assay is an

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amplification free, fluorescence-based technology that captures and counts nucleic acid molecules (Geiss, et al., 2008).

We designed ITS/5.8S probes for *Candida glabrata*, *C. albicans*, *C. tropicalis*, *C. krusei*, *C. metapsilosis*, HCV, and 2009 H1N1 influenza from GenBank sequences. We designed *C. parapsilosis* and *Aspergillus* species probes from sequences in the Broad Institute Repository (Wapinski, et al., 2007) and *Aspergillus* Genome Database (Arnaud, et al., 2012), respectively. Probes for HCV genotype 2a and 2b, and H1N1 were included as negative controls. Probe sequences (~50 base pairs per probe) incorporated *Bfa*I restriction enzyme sites to facilitate consistent DNA fragmentation.

Candida glabrata and *C. albicans* were obtained from American Type Culture Collection (ATCC 90030 and 90028, respectively). Other strains were derived from clinical isolates and were provided by Dr. David A. Stevens' laboratory. *A. fumigatus* strain 10AF DNA was extracted from 50 µl of conidial suspension ($10^{8.5}$ conidia/ml). Other strains were grown on Sabouraud dextrose agar and 1 cm² of culture was added to 200 µl phosphate buffered saline. Beads (garnet matrix with ¼ inch ceramic sphere, MP Biomedicals, Solon, OH) were added and samples shaken (5000 rpm) for 3 minutes using a Mini bead beater (Cole Parmer, Vernon Hills, IL). DNA was extracted with QiAmp DNA Mini Kit (Qiagen Inc, Valencia, CA) per manufacturer's instructions. We verified target DNA by PCR, using ITS-1, ITS-4 primer sequences (5'TCCGTAGGTGAACCTGCGG and 5'TCCTCCGCTTATTGATATGC, respectively) and confirmed amplicons by gel electrophoresis. DNA was quantified by spectrophotometer (Nanodrop, Wilmington, DE) and digested with *Bfa*I as follows: 7 µl of target DNA, 1 µl of *Bfa*I (5000 units/ml) and 1 µl of restriction 10X buffer (1X buffer: 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT) for 2 hours at 37°C. DNA was denatured at 95°C for 5 minutes, then incubated at 4°C for 2 minutes. Ten µl of digested DNA was added to reporter and capture probe solution as per manufacturer's instructions (Geiss, et al., 2008) and allowed to hybridize at 65°C for 16 hours. The solution was processed on the nCounter prep station (2 hours) and read at high resolution (600 fields of view) on the analyzer station (4 hours) (Geiss, et al., 2008). Singlet assays were performed in 2 runs in duplicate and multiplex assays were performed in duplicate. We evaluated limit of detection (LOD) for *C. glabrata*, *C. albicans*, and *A. fumigatus* using a 10-fold dilution series starting at 10^6 genome equivalents/µl in five replicate runs. For multiplex assays, fungal DNAs were added in equal volumes, maintaining a final species DNA concentration of > 20 ng/µl. Multiplex studies mixed DNA from 10 or 8 (excluding *C. krusei* and *A. fumigatus*) fungal strains.

We compared mean positive signal with mean non-specific signal determined from the background fluorescent signal intrinsic to each probe in the absence of sample DNA. Positivity was determined by a difference between positive and non-specific mean signals at $p < 0.05$ level, using a two-tailed Student's t-test. Mean intra-assay coefficient of variation (CV%) was determined for all single target samples. A correlation coefficient (R^2) of linear fit was used to determine LOD reproducibility.

In singlet experiments, nCounter identified all fungal species (Table 2). Mean fluorescent gene count (FGC) for ITS/5.8S probes ranged from 152384 for *C. glabrata* to 3056 for *C.*

krusei. For single copy genes, mean FGC ranged from 2584 for the *C. parapsilosis* probe-2 to 33 for the *A. nidulans* probe-1. Because probes were designed for single or multi-copy genes, cross-probe FGC comparisons are not meaningful. Thus, a *C. glabrata* signal of 152384, using the multi-copy ITS sequence is not necessarily more robust than an *A. fumigatus* signal of 1802 using a single gene probe.

Eighty percent (12/15) of the probes demonstrated an intra-assay CV% <20% (mean CV% 10.7% for all probes, Table 2). *C. krusei* displayed substantial cross-reactivity with *C. parapsilosis* probes (mean FGC 3056 vs. non-specific *C. parapsilosis* probe-1, -2 of 2121 and 2660, respectively). The *C. parapsilosis* probe-2 also demonstrated cross reactivity with *C. metapsilosis*, albeit to a lesser degree (mean FGC 3837 vs. non-specific *C. parapsilosis* probe-2 of 274). Otherwise, probes demonstrated good specificity with a low background signal (Table 2). For *C. glabrata* and *C. albicans* the LOD was 1000 genome equivalents/ μ l and for *A. fumigatus* the LOD was 10000 genome equivalents/ μ l ($p < 0.05$, Figure 1). The average correlation coefficient among the LOD studies was 0.71 for *C. glabrata*, 0.82 for *C. albicans* and 0.99 for *A. fumigatus* probes 1 and 2.

The assay correctly detected all multiplexed samples (Figure 2). With the exception of *C. parapsilosis*, likely due to a non-specific *C. krusei* cross-reaction in the 10-plex samples, mean FGC did not differ significantly between 10-plex and 8-plex samples. In our study, *A. flavus*-1, *A. terreus*-1 and *A. nidulans*-1 probes demonstrated high intra-assay variability. Additionally, the *C. parapsilosis* probe demonstrated cross reactivity with the closely related species, *C. metapsilosis* (van Asbeck, et al., 2009), and *C. krusei* probes. Thus, more robust probe design will be necessary prior to clinical application. The detection limit for the current assay suggests that the nCounter may not have the capability of detecting low copy number events such as a fungemia due to a mould infection. Future clinical study is needed to define the detection sensitivity and clinical applicability of the nCounter system. In addition, we did not evaluate effects of potential inhibitors, although others have reported using the nCounter assay in urine and paraffin embedded tissue with acceptable detection capacity (Barczak, et al., 2012; Tang, et al., 2012).

Our results are the first to demonstrate that an amplification-free technology can detect multiple fungal pathogens at the species level with acceptable specificity, sensitivity and reproducibility within a 24-hour turnaround time. These results should now lead to studies of spiked clinical specimens (e.g., in human serum or tissue homogenates), and of specimens where DNA contents are determined by other methods (such as PCR) for comparison. As the assay has the capacity to simultaneously detect >800 targets, it has the potential for the detection of other pathogens, and genes of drug resistance. Simultaneous determination of multiple fungal pathogens without amplification, using a commercially available platform with standardized methodology, could be a substantial step forward in managing IFDs.

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References

- Arnaud MB, Cerqueira GC, Inglis DO, Skrzypek MS, Binkley J, Chibucos MC, Crabtree J, Howarth C, Orvis J, Shah P, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G, Wortman JR. The Aspergillus Genome Database (AspGD): recent developments in comprehensive multispecies curation, comparative genomics and community resources. *Nucleic Acids Res.* 2012; 40:D653–659. [PubMed: 22080559]
- Barczak AK, Gomez JE, Kaufmann BB, Hinson ER, Cosimi L, Borowsky ML, Onderdonk AB, Stanley SA, Kaur D, Bryant KF, Knipe DM, Sloutsky A, Hung DT. RNA signatures allow rapid identification of pathogens and antibiotic susceptibilities. *Proc Natl Acad Sci U S A.* 2012; 109:6217–6222. [PubMed: 22474362]
- Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol.* 2008; 26:317–325. [PubMed: 18278033]
- Hsu JL, Ruoss SJ, Bower ND, Lin M, Holodniy M, Stevens DA. Diagnosing invasive fungal disease in critically ill patients. *Crit Rev Microbiol.* 2011; 37:277–312. [PubMed: 21749278]
- Tang W, Morgan DR, Meyers MO, Dominguez RL, Martinez E, Kakudo K, Kuan PF, Banet N, Muallem H, Woodward K, Speck O, Gulley ML. Epstein-Barr virus infected gastric adenocarcinoma expresses latent and lytic viral transcripts and has a distinct human gene expression profile. *Infect Agent Cancer.* 2012; 7:21. [PubMed: 22929309]
- van Asbeck EC, Clemons KV, Markham AN, Stevens DA. Correlation of restriction fragment length polymorphism genotyping with internal transcribed spacer sequence, randomly amplified polymorphic DNA and multilocus sequence groupings for *Candida parapsilosis*. *Mycoses.* 2009; 52:493–498. [PubMed: 19207835]
- Wapinski I, Pfeffer A, Friedman N, Regev A. Automatic genome-wide reconstruction of phylogenetic gene trees. *Bioinformatics.* 2007; 23:i549–558. [PubMed: 17646342]

Figure 1. Limit of Detection for *Candida glabrata*, *Candida albicans* and *Aspergillus fumigatus* probes 1 and 2 (n = 5 replicates per probe), serial 10-fold dilutions, starting concentration of 1,000,000 genome equivalents/μl. Fluorescent gene counts on a log 10 scale vs. concentration on a log 10 scale. Error bars represent ± standard deviation. The correlation coefficient (R^2) of a linear fit to the average signal for *C. glabrata* (0.71), *C. albicans* (0.81) *A. fumigatus* probe-1 (0.99) and *A. fumigatus* probe-2 (0.99) The minimum detection limit for *C. glabrata* and *C. albicans* was 1000 genome equivalents/μl (* $P < 0.05$) and for *A. fumigatus*-1, 2 was 10,000 genome equivalents/μl (** $P < 0.05$).

Figure 2. Multiplex simultaneous detection

DNA of 10 fungal species or 8 fungal species (excluding *Aspergillus fumigatus* and *Candida krusei*) was mixed. DNA detection is shown by mean fluorescent gene counts ($\pm$ standard deviation, $n = 2$) on a log 10 scale against fungal probes. Probes designated as “-1”, “-2” represent distinct probe sequences for each strain.

Table 1

Comparison of current diagnostic assays for fungal detection *

Assay	Multiplex capacity	Species level specificity	Advantages	Disadvantages
<i>Microbiological Culture</i>	–	+	<ul style="list-style-type: none"> • “Gold standard” for defining fungal infection 	<ul style="list-style-type: none"> • Poor sensitivity • Long turnaround time
<i>(1-3)-βD glucan</i>	–	–	<ul style="list-style-type: none"> • Not impacted by treatment • Rapid turnaround time (e.g., hours) 	<ul style="list-style-type: none"> • False positives common
<i>Aspergillus Galactomannan</i>	–	–	<ul style="list-style-type: none"> • Good for surveillance in high risk populations 	<ul style="list-style-type: none"> • False positives common • Variable performance according to patient population
<i>Polymerase Chain Reaction (PCR)</i>	+ (>20 fungal species)	+	<ul style="list-style-type: none"> • Low limit of detection • Rapid turnaround time (e.g., minutes to hours) 	<ul style="list-style-type: none"> • Amplification-based: false positives--external contamination, lack of standardization, uncertain reliability in certain settings • No FDA approved assay
<i>nCounter Assay</i>	+ (>800 targets per assay)	+	<ul style="list-style-type: none"> • Standardized assay • Non-amplification technology • Turnaround time (<24 hours) 	<ul style="list-style-type: none"> • Detection limit not yet validated in clinical samples • Probes require further optimization

* Modified from Hsu, et al. 2011

Table 2

Characteristics of Singlet Assays

Target Fungal Organism	Target Sequence for Probes	Mean Positive Signal (FGC) ± SEM	Mean Non-specific Signal (FGC)	Intra-assay CV%
<i>A. fumus</i> -1 *	CCAGGAAGCAGGTCTCTCTGATGCGGCTATTGAAAGTCCATGATCTCAATCAGBGACTCACATAGACTTTGTATACAGCTTATCTCTTTGGCTTGGCGAAT	376 ± 30	4	26
<i>A. fumus</i> -2 *	AAACGGAAGAAGCCCTTCAGCGCTTTTACGGCGCTAAATATATCAGAAGAAATAGCACTCTACCAAGAACAATCTTACTGATGAGGGCGATAGATCTGCT	361 ± 34	7	9
<i>A. fumigatus</i> -1 *	ATCGCTCACATGGGGTTCTATAGCGGTCACCTCTTATTTCCCTCTGTACTTCACTCTCTCTCTGTGAGTGCGTATTTTCTTTCCTCTCGTGGAGCGCAAC	1139 ± 244	5	3
<i>A. fumigatus</i> -2 *	CGCAACGAAGAAGACGAGATCCGGAAGGCTTATCGCAAGGTTCGAAAAAACGCAACACGCTTCTCATCTCCAGCGCGAAACTTAACAGAGATTACTGACTCGATG	1802 ± 476	4	3
<i>A. nidulans</i> -1 *	GTACATGACCATCAAAAGATCACCAAGAACGTGGCTTCTGCAATCCCTCTGACTGTACTTTGCCACGATGCTGAGTGGGTTCTATACAATGAATTCGTCTCACG	33 ± 12	4	22
<i>A. nidulans</i> -2 *	TCACGCTGTATGATGATAGCTTTGCCGTTTGGGCTGATCAATGCCAGGAGGCTGACGCCCTTTTCGAACGGCGCTAACGACTACGATGATATTCACAACAA	55 ± 1	1	10
<i>A. terreus</i> -1 *	ATGAAAAAGCCAGGCCATGGAAATGTTGTGGAGAACCTGAAACAGCTGTATCATTGGTCCACTGGAGCTTACAAAGAAGAACCCCGTCTCTCGCTGTGGGATGACC	184 ± 16	1	28
<i>A. terreus</i> -2 *	CTGTTCTCCGTCCGAGAACTTCTCTTAATGTTGGCCGTGTGTGCCGGTGATGGCTGGTGTTTCTTGTGGCGCAAGATGCCAGCGTTCCGCGTTTGGGAGCGA	425 ± 28	6	6
<i>C. parapsilosis</i> -1 *	CCTCAGGAAGAAGTTACCCATAGTGATGTGGAAATATATACATATATAAGGGAAGCTTGTGCTCATACGGTGCATATGGCGGGTTGTGTGCGTTATGTGGCA	2148 ± 145	242	7
<i>C. parapsilosis</i> -2 *	ATCGTTACCAAGTTTGGCAATCTCAGCACGCCCTCAACTTTGTTAGAGGATTAGCTGCTTACAAATCTGTGCMAAACCCACATGTCAATGTGGGAACCTATCGGT	2584 ± 230	332	12
<i>C. albicans</i>	CAATGGCTTAGGTCTAACCAAAAAACATTTGTTGGCGGTAAAGTCTACACGTATATCTTCAAACTTTGACCTCAAAATCAGGTAGGACTACCCGCTGAA	14290 ± 93	2	12
<i>C. glabrata</i>	TTGATTTTGTCTGAGCTCGGAGAGAGACATCTCTGGGGAGGACCAGGTAGACACTCAGAGGGCTCCTTAAAAATATTTTCTCTGTGTGAATGCTATTTCTC	152384 ± 22578	2	7
<i>C. lusnei</i>	GACTTTTTCAGGGAGCGCTTGGCGCGCGAGAGCGAGTGTTCGAGACAAACAAAAGCTCGACCTCAAAATCAGGTAGGAATAACCCGCTGMACTTAAGCAT	3056 ± 153	2	6
<i>C. neoformans</i>	GGTTTGTGAAAGAAGGGGGAGTATAAACTAATGGATAGGTTTTTTTTTTCACCTCATTTGGTACAAACTCCAAACATCTTCCAAATTCGACCTCAAA	3837 ± 185	6	3
<i>C. tropicalis</i>	GTGTTTTTATATGAACAAAATTTCTTTGGTGGCGGGAGCAATCTCTACGCCCGAGAGGTTATACTTAAACCAAACTTTTATTTACAGTCAAAACTTGTGATAT	147795 ± 10396	3	5

Mean non-specific signal for negative control probes were as follows **HIN1**: 1.62 FGC, **HCV genotype 2a**: 1.77 FGC and **HCV genotype 2b**: 1.81 FGC.

SEM: Standard error of the mean

CV%: Coefficient of variation

FGC = Fluorescent gene count.

* Single copy probes, “-1, -2” denotes two separate probes created for each fungal species

** Mean positive signal and mean non-specific signal differed (p<0.05)