Diagnostic Microbiology and Infectious Disease 78 (2014) 137–140

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mycology

Application of a non–amplification-based technology to detect invasive fungal pathogens ☆

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ARTICLE INFO

Article history:
Received 8 July 2013
Received in revised form 8 November 2013
Accepted 11 November 2013
Available online 21 November 2013

Keywords:
Invasive fungal disease
Diagnosis
Aspergillus
Candida
nCounter analysis system

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.

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 Food and Drug Administration (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, USA) to quantify and differentiate 10 clinically relevant fungal species. This assay is an amplification-free, fluorescence-based technology that captures and counts nucleic acid molecules (Geiss, et al., 2008).

We designed Internal transcribed spacer (ITS)/5.8S probes for *Candida glabrata*, *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida metapsilosis*, Hepatitis C virus (HCV), and 2009 H1N1 influenza from GenBank sequences. We designed *Candida 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.

C. 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 cm^2 of culture was added to 200-μL phosphate-buffered saline. Beads (garnet matrix with ¼ inch ceramic sphere; MP Biomedicals, Solon, OH, USA) were added and samples shaken (5000 rpm) for 3 minutes using a Mini bead beater (Cole Parmer, Vernon Hills, IL, USA). DNA was extracted with QiAmp DNA Mini Kit (Qiagen Inc, Valencia, CA, USA) 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, USA) 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 10× buffer (1× buffer: 50 mmol/L potassium acetate, 20 mmol/L Tris acetate, 10 mmol/L magnesium acetate, 1 mmol/L Dithiothreitol) for 2 hours at 37 °C. DNA was denatured at 95 °C for 5 minutes, then incubated at 4 °C for 2 minutes. Ten microliters 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

☆ Funding/support: JLH, DAS, and KVC were funded in part through the National Institutes of Health grant (R21 AI 85566–01). The work was also supported by a grant to MH from the Department of Veterans Affairs.

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http://dx.doi.org/10.1016/j.diagmicrobio.2013.11.013

Table 1
Comparison of current diagnostic assays for fungal detection.[a]

Assay	Multiplex capacity	Species level specificity	Advantages	Disadvantages
Microbiological Culture	−	+	• "Gold standard" for defining fungal infection	• Poor sensitivity • Long turnaround time
(1–3)-β-D glucan	−	−	• Not impacted by treatment • Rapid turnaround time (e.g., hours)	• False positives common
Aspergillus Galactomannan	−	−	• Good for surveillance in high risk populations	• False positives common • Variable performance according to patient population
PCR	+ (>20 fungal species)	+	• Low limit of detection • Rapid turnaround time (e.g., minutes to hours)	• Amplification-based: false positives—external contamination, lack of standardization, uncertain reliability in certain settings • No FDA approved assay
nCounter Assay	+ (>800 targets per assay)	+	• Standardized assay • Non-amplification technology • Turnaround time (<24 hours)	• Detection limit not yet validated in clinical samples • Probes require further optimization

[a] Modified from Hsu et al. (2011).

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 5 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 $P < .05$ level, using a 2-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 *Aspergillus 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.

Table 2
Characteristics of singlet assays.

Target fungal organism	Target sequence for probes	Mean positive signal (FGC)[b] ± SEM	Mean non-specific signal (FGC)[b]	Intra-assay CV%
A. flavus-1[a]	CCAGGAAGCAGTCTCCTCTGATGCGCCTATTGAAGTCCATGATCTCAATC AGGGACTCACCATAGACTTTGTATCAGCTTATCTCTTTGGCTTGGCGAAT	376 ± 30	4	26
A. flavus-2[a]	AAACGGAAGAAGCCTTCAGCCGTTTAACGGCGCTAAATAATCAGAAGAA TAGCACTCTACCAGAAGAACATCTTATTACTGATGAGGCGATAGATCTGCT	361 ± 34	7	9
A. fumigatus-1[a]	ATCGCTCACATGGGGTTCTATAGCCGTCCATCCTTATTTCCTCTGTTACT TCACTCTCTCCTTGAAGTCGCTATTTTCTTTCCCTCTCGTGGAGCGCAAC	1139 ± 244	5	3
A. fumigatus-2[a]	CGCAACGAAAGACGAGATCCGGAAGGCTTATCGCAAGGTCGAAAAAA CGCACACCGTTCTCATTCCAGCCGGAAACTTAACAGAGATTACTGACTCGATG	1802 ± 476	4	3
A. nidulans-1[a]	GTACATGACCATCAAAGATCACCAGAACGTGCTTCTGCATCCCTCGACTGTA CTTGCCCACGATGCTGAGTGGGTTCTATACAATGAATTCGTCCTCACG	33 ± 12	4	22
A. nidulans-2[a]	TCACGCTGTATGATGATGATAGCTTGCCGTTTGGGCTGATCAATGCCG AGGAGCTGACGCCCTTTCGAACGGCGCTAACGACTACGATGATATTCAACAA	55 ± 1	1	10
A. terreus-1[a]	ATGAAAGGCCAGGCATGGAATGTTGTGGAGACCCTGAAACAGCCTGATCA TGGTCCACTGGAGCTTACAAGAAGAACCCGTGTCTGCGTGTGGGATGACC	184 ± 16	1	28
A. terreus-2[a]	CTGTTCTCCGTCGCAGAACTTCTCTAATGGTGGCCGTGTGTCCGGTGATGG CTGGGTGTTTTCTGGTGGCCAAGATGCCAGCGTTCGCGTTTGGGAGCGA	425 ± 28	6	6
C. parapsilosis-1[a]	CCTCAGGAAGAAGTTACCCATAGTGATGTGGAAATATTACATATAAGG GAAGCTTGTGCTCATACGGTGCAATATGGCGGGTTGTGTGCGTTATGTGGCA	2148 ± 145	242	7
C. parapsilosis-2[a]	ATCGTTACCACGTTTGGCAATCTCAGCACGCCCTCAACTTGTTAGAGGATT AGCTGCTTACAATCGTGCAAAACCACATGTCAATGTGGGAACTATCGGT	2584 ± 230	332	12
C. albicans	CAATGGCTTAGGTCTAACCAAAAACATTGCTTGCGGCGGTAACGTCTA CCACGTATATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAA	14290 ± 93	2	12
C. glabrata	TTGATTTGTCTGAGCTCGGAGAGAGACATCTCTGGGGAGGACCAGTGTAG ACACTCAGGAGGCTCCTAAAATATTTTCTCTGCTGTGAATGCTATTTCTC	152384 ± 22578	2	7
C. krusei	GACTTTTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAA CAAAAAGCTCGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCAT	3056 ± 153	2	6
C. metapsilosis	GGTTTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTT TTTCCACTCATTGGTACAAACTCCAAACATTCTTCCAAATTCGACCTCAAA	3837 ± 185	6	3
C. tropicalis	GTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATCCTACCGCC AGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATNTAT	147795 ± 10396	3	5

Mean non-specific signal for negative control probes were as follows H1N1: 1.62 FGC, HCV genotype 2a: 1.77 FGC and HCV genotype 2b: 1.81 FGC.
[a] Single copy probes, "-1, -2" denotes 2 separate probes created for each fungal species.
[b] Mean positive signal and mean non-specific signal differed ($P < .05$).