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ARTICLE

Evaluation of a digital microfluidic real-time PCR platform to detect DNA of *Candida albicans* in blood

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Abstract Species of *Candida* frequently cause life-threatening infections in neonates, transplant and intensive care unit (ICU) patients, and others with compromised host defenses. The successful management of systemic candidiasis depends upon early, rapid diagnosis. Blood cultures are the standard diagnostic method, but identification requires days and less than half of the patients are positive. These limitations may be eliminated by using real-time polymerase chain reaction (PCR) to detect *Candida* DNA in the blood specimens of patients at risk. Here, we optimized a PCR protocol to detect 5–10 yeasts in low volumes of simulated and clinical specimens. We also used a mouse model of systemic candidiasis and determined that candidemia is optimally detectable during the first few days after infection. However, PCR tests are often costly, labor-intensive, and inconvenient for routine use. To address these obstacles, we evaluated the innovative microfluidic real-time PCR platform (Advanced Liquid Logic, Inc.), which has the potential for full automation and rapid turnaround. Eleven and nine of 16 specimens from individual patients with culture-proven candidemia tested positive for *C. albicans* DNA by conventional and microfluidic real-time PCR, respectively, for a combined sensitivity of 94%. The microfluidic platform offers a significant technical advance in the detection of microbial DNA in clinical specimens.

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Introduction

Invasive candidiasis is a major complication among patients receiving long-term, broad-spectrum antibacterial therapy, transplant recipients, patients with hematological dyscrasias, intensive care unit (ICU) patients, neonates, and others with compromised innate host defenses. In adults, candidemia is costly, increasing in frequency, and accompanied by mortality rates that may exceed 40% [1–5]. For example, 5% to 10% of all ICU-acquired infections are invasive candidiasis [5]. In extremely low birth weight infants, the prevalence of systemic candidiasis is 10%, mortality is 20% to 30%, and most survivors exhibit severe neurological impairment [6]. In both adults and neonates, early, rapid diagnosis and administration of appropriate therapy are essential to obtain an optimal outcome [7, 8]. Candidiasis is usually caused by an opportunistic species of *Candida* normally present among the microbiota of the epithelial mucosa. The most common cause is *C. albicans*. Other significant species are

C. parapsilosis, which is relatively more prevalent in neonates and in association with foreign bodies, and *C. glabrata*, which is important because many strains are inherently resistance to antifungal azoles [9]. Because the response to treatment varies, it is essential to identify the causative species of *Candida*.

The universal procedure for diagnosing invasive candidiasis relies on blood cultures to detect candidemia. Unfortunately, this approach is plagued by the need for large volumes of blood, low sensitivity, and the lengthy time period required for the growth, isolation, and identification of *Candida*. Real-time polymerase chain reaction (PCR)-based assays have the potential to eliminate each of these shortcomings. Amplification of fungal-specific regions of the ribosomal RNA (rDNA) gene has been shown to detect fungi in clinical specimens [10–13]. In addition, hypervariable regions of fungal rDNA contain species-specific sequences that permit the identification of hundreds of species of pathogenic fungi. In contrast to blood cultures, diagnostic PCR tests offer the potential of rapid, specific, and sensitive diagnosis of candidemia. However, they have not replaced conventional blood cultures because they suffer from other drawbacks, especially high equipment costs, labor-intensive sample preparation, and the need for molecularly trained technologists.

To improve these deficiencies in the PCR diagnosis of candidemia, we developed and evaluated a novel digital microfluidic real-time PCR platform to detect rDNA of *C. albicans*. This technology was recently introduced by Advanced Liquid Logic, Inc. (http://www.liquid-logic.com) [14, 15], and it satisfies the criteria of low cost, rapid turnaround, automation, and applicability for low blood sample volumes. The microfluidic on-chip platform has the capability of being multiplexed to detect multiple pathogens. In brief, the digital microfluidic real-time PCR platform uses a disposable chip or cartridge (86 mm × 86 mm) containing electrodes and a cover plate with ports for loading fluid. The electrodes are arranged and programmed to split, move, and mix nanoliter-scale droplets within microchannels on the cartridge [14, 16, 17]. The cartridge is inserted into a cartridge deck that contains heaters and magnets at fixed locations. Liquid-handling operations are controlled by a computer and program that switches the cartridge's electrodes on or off as needed. Droplets from the extracted specimen are automatically mixed with PCR reagents and cycled between two fixed temperature zones that are controlled by heaters beneath the cartridge. Amplicons are detected using an LED–photodiode fluorimeter module that is mounted directly above the cartridge deck opposite the zone of DNA annealing and extension. Because the droplets can be rapidly transported between thermal zones and their volumes are on orders of magnitude smaller than the volumes used in conventional PCR, temperature changes are rapid and cycle times are dramatically shortened [14].

In this report, we used simulated clinical blood samples seeded with *C. albicans* to develop methods of DNA extraction, optimized a protocol for conventional real-time PCR, and evaluated its ability to detect limiting numbers of fungal cells in a murine model of experimental candidemia. We then adapted this protocol to the microfluidic real-time PCR platform and compared the performance of each platform by testing clinical blood specimens from patients with culture-proven candidemia.

Materials and methods

Candida strains

The following reference strains were used: *C. albicans* (DUMC 117.00=ATCC 36082), *C. glabrata* (DUMC 135.08=ATCC 750=CBS 138), *C. krusei* (DUMC 104.07=ATCC 6258=CBS 573), *C. metapsilosis* (MMRL 2456=ATCC 96144), *C. orthopsilosis* (MMRL 2453=ATCC 96139), *C. parapsilosis* (DUMC 136.00=ATCC 22019=CBS 604), and *C. tropicalis* (DUMC 106.06=ATCC 2001=CBS 94). Multiple strain numbers reflect the availability of these reference strains in different culture collections: Duke University Medical Center (DUMC), American Type Culture Collection (ATCC), Medical Mycological Research Laboratory (MMRL), and Centraalbureau voor Schimmelcultures (CBS). For experimental use in preparing simulated blood samples, pure cultures were grown overnight on Sabouraud agar plates at 37°C, suspended in sterile water, enumerated by a hemocytometer (confirmed by viable plate counts), and adjusted to the appropriate concentration of colony-forming units (CFU)/mL. For preparing inocula for murine infections, pure cultures were grown overnight at 37°C in Sabouraud broth shaken at 250 rpm and washed three times in sterile phosphate-buffered saline (PBS) at pH 7.4, enumerated, and adjusted to the appropriate concentration.

Experimental candidiasis

Systemic infections were established in mice by intravenous challenge with exponentially grown yeast cells of *C. albicans* [18]. In each experiment, CD-1 adult male mice were infected by tail vein injection of 2×10^5 CFU *C. albicans* in 100 μL sterile PBS. At subsequent time points, the mice were euthanized by carbon dioxide, EDTA-anticoagulated blood samples were obtained by cardiac puncture, and these specimens were aliquoted for quantitative culturing on YPD agar plates and conventional real-time PCR testing. In addition, the kidneys were removed aseptically and cultured

on YPD agar plates to confirm that the mice had developed systemic candidiasis. In follow-up experiments, mice were infected, but at 24 h postinfection, they were given daily intraperitoneal injections of 20 mg/kg or 100 mg/kg fluconazole. Control mice were infected but untreated. At day 7, the mice were euthanized and bled, and the blood samples of similarly treated mice were combined to yield 10 pooled blood samples of 1.1 to 1.8 mL. As above, each blood sample was split, half was cultured and half was tested for *C. albicans* DNA by conventional PCR. In addition, one kidney from each mouse was cultured.

Clinical and control blood specimens

In compliance with approved institutional review board protocols, samples of EDTA-anticoagulated blood were obtained from hospitalized patients at Duke Hospital at risk for candidemia and banked at −80°C until thawed for use. They included samples from patients with positive blood cultures for species of *Candida* as well as many blood culture-negative controls. Based on clinical records, patients were categorized as suspected positive or suspected negative for candidemia. From several hundred enrolled patients with multiple blood cultures, we selected 16 banked blood specimens suspected to harbor *C. albicans* DNA for comparative testing on conventional and microfluidic real-time PCR platforms. Each of the 16 PCR-tested blood specimens was collected within 24 h of a specimen from the same patient that later became culture-positive for *C. albicans*. Thirteen of these patients had received antifungal therapy at the time of specimen collection: three were treated for four days (caspofungin), 21 days (micafungin), and 17 days (caspofungin) each prior to breakthrough candidemia, and ten patients had received fluconazole (n=9) or caspofungin (n=1) for ≤48 h prior to sampling (mean antifungal exposure time, 18.8 h; range 0.25–48 h). For normal human blood controls, we collected blood from a healthy human volunteer. Simulated clinical specimens were prepared by spiking samples of normal human whole blood with yeast cells of *C. albicans* or *C. parapsilosis* at ten-fold concentrations of 10^5 through 10^0 CFU/mL. These simulated specimens were used to develop methods of extraction, purification, and testing, and they served as external positive controls.

Design of oligonucleotides

GenBank sequences of fungal rDNA were analyzed to design a capture probe in the 18S small subunit rRNA that would hybridize to the highly conserved sequence of *C. albicans* rRNA adjacent to the internal transcribed spacer 1 (ITS1) region. This biotinylated oligonucleotide capture probe (5′-[Biotin]CTC CGG ATT GGT TTA GGA AAG GGG GCA ACT CCA TTC TGG AAC CGA GAA GC-3′) was employed with a paramagnetic extraction and purification system, as previously described [14, 15]. Using GenBank sequences, we designed primers that are anchored in the 5.8S and 26S regions to non-specifically amplify *Candida* rDNA spanning the ITS2 region (forward primer: CaG fw: 5′-CTGTTTGAGCGTCGTTTC-3′; reverse primer CaG rv: 5′-ATGCTTAAGTTCAGCGGGTAG-3′). In addition, we designed hydrolysis probes to detect the closely related species *C. albicans* and *C. dubliniensis* [FAM] CTGGGTTTGGTGTTGAGCAATACG[BHQ]), as well as *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis* ([TET]TCGGGTTTGGTGTTGAGCGATAC[BHQ1]).

DNA extraction

To test limited volumes, 1-mL samples of simulated and clinical blood specimens were split into 500-μL or 200-μL aliquots for separate processing. As blood specimens had been frozen, there was no need for a red cell lysis step, but all simulated human and murine samples were subjected to freezing to replicate the storage conditions of clinical specimens. In preliminary experiments, we evaluated enzymatic and thermal methods of releasing fungal DNA, but milling with glass beads proved to be more efficient, less expensive, and faster (data not shown), as recently reported by White et al. [19]. We also determined that extracting candidal DNA from whole blood was superior to serum. Aliquoted specimens were milled for 2 min (Mini-Beadbeater-16, BioSpec Products Cat. No. 607) using 0.3-g glass beads (710–1,180 μm diameter, Sigma G1152). Tubes were then pulse-minifuged to remove drops from inside the caps, 40 μL protease K (Qiagen, Valencia, CA) was added, and the tubes were mixed by pulse-vortexing for 10 s. After adding 400 μL lysing buffer (AL buffer, Qiagen), the tubes were mixed by pulse-vortexing for 15 s, incubated for 15 min at 56°C, and, once again, pulse-minifuged to remove drops from inside the caps. The content of each tube was then transferred to a new 1.5-mL tube without glass beads.

As previously reported, we initially used paramagnetic beads with attached biotinylated capture probes to concentrate and purify the DNA [14, 15]. However, blood sample volumes of 500 μL or less failed to achieve acceptable precision for the detection of low concentrations of *C. albicans* DNA (ca. 5 CFU/mL), and greater volumes reduced the efficiency of DNA extraction with paramagnetic beads (see the supplementary material). Consequently, 1-mL blood samples were extracted using silica membrane spin columns, which did not adversely affect the PCR tests. After transferring specimens from bead beating tubes to new tubes, DNA was purified using silica membrane spin columns (QiaAmp, Qiagen), as indicated by the manufacturer's instructions, except that the volume of each specified

reagent was doubled. The elution of co-purified human and fungal DNA was performed by adding 10 μL AE buffer (Qiagen), of which 6 μL was aliquoted, frozen at −20°C, and then tested in replicate by conventional PCR using an ABI 7700 real-time PCR thermocycler (Applied Biosystems, Inc., Foster City, CA). The remaining 4 μL DNA eluate was frozen at −20°C and subsequently analyzed using the R100 digital microfluidic real-time PCR instrument (Advanced Liquid Logic, Inc., Research Triangle Park, NC).

Positive controls, non-template controls, and external standards

To evaluate the efficiency and potential contamination during extraction and processing, we analyzed positive controls and negative non-template controls, which consisted of healthy human blood with or without the addition of *C. albicans* at 10^3 CFU/mL. To determine the efficiency of real-time PCR, purified genomic *C. albicans* DNA was quantified using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE) and ten-fold dilutions from 140 fg to 140 pg were tested. Conventional real-time PCR efficiencies ranged from 89% to 105%.

Conventional real-time PCR

To minimize contamination and artifactual results, PCR tubes were prepared and amplified in different physical locations, and we used the uracil N-glycosylase technology (AmpErase®, Applied Biosystems) to prevent the re-amplification of amplicons. Each PCR was run in a volume of 20 μL containing 2 μL template DNA (or sample), 10 μL ABI master mix (ABI Cat No. 4352046), 0.2 μL 250 nM of each generic fungal primer, 0.05 μL 250 nM species-specific hydrolysis probe , 0.116 μL AmpErase®, and 7.6 μL PCR-grade water. An ABI 7900HT thermocycler (Applied Biosystems, Inc.) was programmed as follows: 2 min at 50°C, 10 min at 95°C, then 45 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension). All conventional real-time PCR tests were performed in triplicate. The clinical samples were tested on two different days, which provided six replicate tests per specimen. A PCR test was defined as positive for the target DNA if the amount of amplicon exceeded the threshold for amplification, which was calculated using SDS2.3 software [14]. A specimen was considered to be positive if any replicate PCR test was positive.

Digital microfluidic real-time PCR

The remaining DNA spin column eluate of each 1.0-mL blood specimen was thawed and introduced into the cartridge after capture on magnetic beads as follows. Up to 4 μL of DNA specimen was mixed with a 0.34-μL suspension of 10 mg beads/mL (Ademtech, Inc., France) in 10 μL DNA binding buffer and incubated at room temperature for 10 min. Beads were then pulled down by magnetism, supernatant was removed, 20 μL of wash buffer were added, and the beads were resuspended by pipetting up and down several times. The wash process was repeated two more times, and the beads were then resuspended in 3.2 μL PCR master mix and loaded into the reservoir of a microfluidic real-time PCR cartridge. Primers and probes were identical to those used in the conventional real-time PCR testing. The thermocycling program consisted of 2 min at 95°C, followed by 50 cycles of 10 s at 95°C and 45 s at 58°C [14]. After PCR completion, product droplets were retrieved from the cartridge and analyzed by agarose gel electrophoresis to confirm the presence of the 211-bp *C. albicans* amplicon. A sample was considered to be positive if an amplification curve was generated on the cartridge and a band of the correct size was demonstrated by agarose gel electrophoresis. In the current configuration, each microfluidic real-time PCR platform cartridge allows four real-time PCR tests. The PCR tests were run in daily batches. The first and last cartridge of each day's tests included two clinical specimens and a positive and negative control sample. All the other cartridges were loaded with three specimens and a positive control. The positive control consisted of *C. albicans* DNA spiked into human genomic DNA and captured on magnetic beads as described above. The negative controls were normal human genomic DNA captured on beads.

Results

Detection of *C. albicans* in human blood by conventional real-time PCR

Conventional real-time PCR was used to evaluate the detection of *C. albicans* DNA in samples of normal human blood seeded with yeast cells of *C. albicans*, and samples were tested in triplicate. For blood seeded with 1 CFU/ml (four experiments), 10 CFU/ml (18 experiments) or 100 CFU/ml (15 experiments), *C. albicans* DNA was detected in 25%, 83% and 100% of the samples, respectively. The hydrolysis probes for *C. albicans*/*C. dubliniensis* and the *C. parapsilosis* species complex differed by six bases but exhibited incomplete species specificity (data not shown). However, these PCR tests did not amplify DNA from *C. glabrata*, *C. krusei*, or *C. tropicalis*. Using this optimized protocol for conventional real-time PCR, we conducted preliminary tests on 24 clinical specimens from patients suspected to harbor *C. albicans* or *C. parapsilosis* DNA (data not shown). Preliminary results confirmed the stochastic nature of

detecting DNA in small volumes of blood with low concentrations of *C. albicans* and suggested that PCR-based assays for candidemia should utilize blood specimen volumes of at least 1 mL (see the supplementary material).

Detection of *C. albicans* in blood of mice with systemic candidiasis by conventional real-time PCR

To further explore the relationship between fungal burden in blood and PCR assays for candidemia, systemic candidiasis was established in CD-1 mice by intravenous challenge. At various times postinfection, one to three mice were euthanized, blood was pooled, and half was tested for *C. albicans* DNA and the other half was quantitatively cultured to determine the census of CFU/mL. As indicated in Fig. 1 and the legend, blood cultures and PCR tests were positive on days 1, 2, and 4, and, with one exception, both assays were negative on days 7 through 14. The exception was one of the three mice sacrificed on day 7, which had a negative PCR but a positive culture of less than 1 CFU/mL. Kidney cultures of all the mice sacrificed between day 7 and 14 revealed confluent growth of *C. albicans*, confirming systemic candidiasis in these mice. Another experiment was conducted to determine the potential effects of antifungal therapy with fluconazole on the ability to detect *C. albicans* DNA. Mice were infected as above, but beginning 24 h postinfection, they received daily intraperitoneal injections of 20 mg/kg or 100 mg/kg fluconazole. Control mice were infected but untreated. At day 7, the mice were euthanized and bled, and half of each pooled blood sample was cultured and half was PCR tested for *C. albicans* DNA. The kidneys from all but one mouse yielded heavy growth of *C. albicans*. However, all cultures and PCR assays of blood were negative. Thus, despite significant renal candidiasis and treatment with fluconazole for seven days, there was no detectable *Candida* DNA in their blood.

Detection of *C. albicans* in patient blood by conventional and digital microfluidic real-time PCR

Clinical blood samples of 16 patients with culture-proven candidemia were extracted with spin columns and tested for *C. albicans* DNA using both conventional and microfluidic real-time PCR platforms. Each patient had a positive blood culture for *C. albicans* within 24 h of the time the sample was collected for DNA testing. The conventional and microfluidic real-time PCR tests had sensitivities of 69% and 56%, respectively. Agreement between the two platforms was 37.5%, but 94% (15/16) of the patients had at least one positive PCR test (Table 1). The conventional and microfluidic positive samples were detected with a mean Ct± standard deviation (SD) of 36.3±5.3 and 34.8±2.9, respectively. All positive controls (n>20 on each platform) had 10^3 CFU/mL and amplified with average Cts of 32.8±0.6 (conventional) and 32.4±0.3 (microfluidic). All of the null extraction controls and negative PCR controls were negative. Thus, the analytical specificity of both platforms was 100%. In this sample, there was no correlation of the PCR result or positive Ct with the clinical data or prior antifungal treatment.

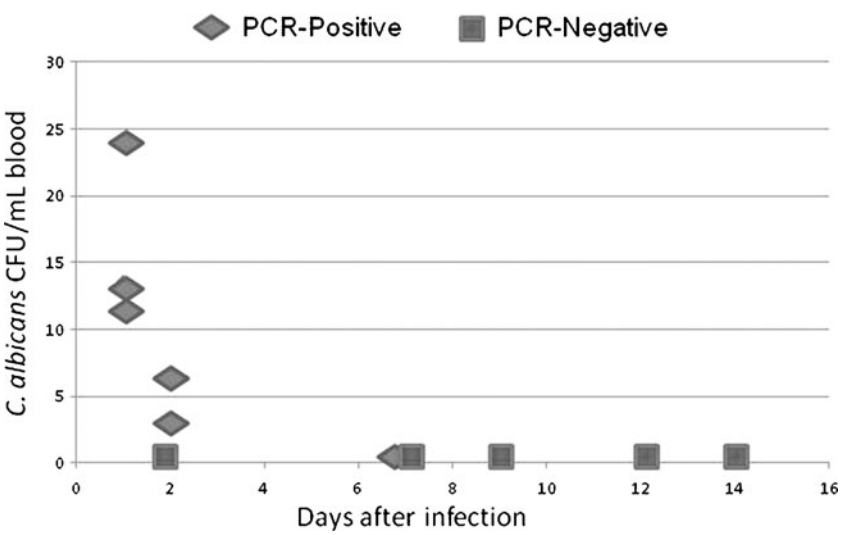

Fig. 1 CD-1 adult male mice were infected by tail vein injections with 2×10^5 colony-forming units (CFU) *Candida albicans*. On the indicated days, mice were euthanized and bled. Blood samples of at least 1 mL were split for quantitative blood cultures and polymerase chain reaction (PCR) testing. With one exception, blood cultures and PCR tests were positive only on days 1, 2, and 4, and negative on days 7 through 14, when the experiment was terminated. The datum point for day 4 is not shown because that mouse had an exceptionally high yeast census of 360 CFU/mL blood. Despite the negative blood cultures and PCR tests, the kidneys, which were cultured from all mice between days 7 and 14, revealed abundant growth of *C. albicans*

Table 1 Comparison of conventional and microfluidic real-time polymerase chain reaction (PCR) for the detection of *Candida albicans* DNA in 16 clinical blood specimens from patients with culture-proven candidemia. Combined sensitivity is 94%

		Microfluidic PCR		Total
		+	−	
Conventional PCR	+	5	6	11
	−	4	1	5
	Total	9	7	16

Discussion

This investigation confirmed that the digital microfluidic real-time PCR platform matches conventional real-time PCR in its capability to diagnose candidemia by detecting *C. albicans* DNA in blood sample volumes as low as 1 mL. We first optimized the real-time PCR protocol to detect limiting numbers of yeast cells, adapted this protocol to the microfluidic platform, and then compared both platforms by testing 16 clinical blood specimens from patients with culture-proven candidemia due to *C. albicans*. Using precautions to minimize contamination and false-positive amplification (e.g., uracil glycosylase), tests of non-candidemic blood samples indicated that the specificity was 100%. Although we did not test blood from patients with other fungal infections, searches of genomic databases indicated that the capture probe and primers were specific for *C. albicans*. The sensitivity was 69% and 56%, respectively, for the conventional and microfluidic platforms, and the combined sensitivity of both tests on these specimens was 94%. As documented in recent reviews, these results are similar to those of most other PCR-based systems, which have employed a variety of target DNA sequences and extraction methods [10, 20–23]. These studies have also reported that candidal DNA is not detected in some patients with proven or probable candidiasis. As with our cohort, candidemic patients are likely to have received antifungal therapy at the time of testing. Nevertheless, the sensitivity of real-time PCR exceeds that of blood cultures, which a recent meta-analysis indicated were positive in only 29% to 46% of patients with invasive candidiasis [10].

To accommodate high-risk patients, such as neonates, transplant recipients, and ICU patients, real-time PCR protocols for the diagnosis of candidiasis and other invasive mycosis must be able to detect fungal DNA in low specimen volumes [22, 24]. Tests that only require small volumes also permit more frequent testing to increase the probability of detecting candidiasis. Using simulated specimens, we optimized the extraction protocol to detect 5 CFU *C. albicans* in 500 μL blood; however, testing low CFU in small blood volumes confirmed the stochasticity of this procedure (see the supplementary material). This problem could be ameliorated by sampling at least 1.0 mL blood and/or performing multiple tests. These results suggest that negative PCR tests in patients with candidiasis could be attributed to the absence, transient presence, and/or low census of *C. albicans* in the circulation of patients with systemic candidiasis. (The reproducibility of our simulated and banked EDTA-treated blood specimens indicated that the fungal DNA was not degraded during storage at −80°C for up to two years.)

We tested this explanation by infecting mice intravenously with *C. albicans*. As shown in Fig. 1, the mice were both culture- and PCR-positive during the initial days after infection, but after this period, the tests were negative, despite the development of renal candidiasis. The few other studies of experimental systemic candidiasis that compared PCR tests for *C. albicans* DNA with quantitative blood cultures reported similar results [25, 26]. Both blood cultures and PCR tests are positive early in infection, but within a week, invasive candidiasis becomes localized. Blood culture-negative samples with positive PCR tests probably reflect the detection of DNA from non-viable *C. albicans* cells. In subsequent experiments, we treated mice with fluconazole, and after one week, candidal lesions persisted in the kidneys, but blood cultures and PCR tests became negative because the infections were sequestered. There is some experimental evidence that neutropenia and treatment with amphotericin B may increase the positivity of PCR tests of blood because normal clearance of the yeasts is compromised and amphotericin B may release DNA [26].

This intravenous murine model only partially simulated the natural history of systemic candidiasis. Many patients acquire their *Candida* infection via contaminated indwelling catheters, in which biofilms often develop, and the inoculum is gradually introduced. In patients at risk, such as those with impaired or depleted neutrophils, the ensuing candidemia may seed multiple organs and lead to chronic systemic candidiasis (e.g., hepatosplenic candidiasis) [9]. Once chronic, localized candidiasis is established, blood cultures are frequently negative. Thus, our murine data reflected the typically transient initial candidemia and the development of chronic infection in the kidneys and other organs [27]. In addition, the observation that fluconazole-treated mice had negative blood culture and PCR results suggests that these diagnostic tests may not be reliable indicators of clinical recovery.

The cumulative results suggest that the inconsistent sensitivity of both blood cultures and PCR tests in detecting systemic candidiasis in the early stages of disease is due to the low numbers of *C. albicans* in the circulation. This conclusion was recently corroborated by a retrospective analysis of quantitative blood cultures from patients with proven or probable systemic candidiasis [28]. Analyzing data obtained in 1987–1991 by the lysis centrifugation

method of culturing and quantifying fungi in blood specimens [29, 30], Pfeiffer et al. documented a median fungal burden of 1 CFU/mL among 152 primary episodes of candidemia [28]. Notably, 73% (61/86) of *C. albicans* culture-positive blood specimens contained <5 CFU/mL. Thus, the number of yeast cells is near the limit of detection of PCR-based assays for *C. albicans* DNA. These data underscore the observation that a single positive PCR test can be diagnostic in a high-risk patient, and this situation could be countered by frequent, perhaps daily, PCR testing.

The digital microfluidic platform for real-time PCR, pioneered by Advanced Liquid Logic, Inc. (http://www.liquid-logic.com), has the potential to revolutionize the diagnosis of infectious diseases by offering rapid, automated, economical, and point-of-care detection of pathogenic DNA in clinical samples [31]. Preliminary reports of this innovative technology have demonstrated its exceptional performance in detecting DNA of methicillin-resistant *Staphylococcus aureus*, *C. albicans*, and *Mycoplasma pneumoniae* in respiratory specimens of patients with community-acquired pneumonia [14, 15]. The microfluidic real-time PCR has all the diagnostic advantages of conventional real-time PCR, including the capacity for multiplexing and detecting multiple, specific pathogens. However, compared to conventional PCR, the microfluidic real-time PCR platform offers significant enhancements, such as eliminating the need for a molecularly trained technologist, using smaller sample elution volumes, and performing faster than conventional real-time PCR because the thermocycling times are shorter. As highlighted in Table 2, the microfluidic platform is more economical because the PCR cartridges are inexpensive, disposable, and use smaller reagent quantities. With the implementation of an automated pre-cartridge sample processing step, the microfluidic real-time PCR platform will be fully portable for point-of-care testing. With regard to sample processing, the detection of fungal DNA in specimens of blood is the most challenging, but this report has confirmed the value of the microfluidic platform for this purpose. We are currently developing protocols for rapid, low-tech pre-cartridge extraction and concentration of microbial DNA from a variety of clinical samples. Indeed, specimens with smaller amounts of blood and extraneous human DNA, such as urines, respiratory fluids, and nasopharyngeal washes, require minimal processing [15, 32]. In particular, the model R100 microfluidic real-time PCR platform used here is being modified to include a specimen DNA extraction step, increase the number of samples per cartridge, and accommodate multiplexing to identify key species of *Candida*.

Microfluidics has emerged as an attractive and promising technology for lab-on-a-chip applications. Early microfluidic systems employed fabricated chips with microchannels for the conveyance of liquid reagents. This prototype was improved by the use of droplets that reduced the dispersal of liquid during transport. The digital microfluidic system developed by Advanced Liquid Logic, Inc. has the advantage of droplet-based flow but eliminates the need for prefabricated channels because the droplets are directed and transported on the chip by the process of electrowetting and directed by electrodes.

The compelling advantage of digital microfluidics technology is its versatility. The same cartridge deck that is used to program cartridges for real-time PCR can also be used for an expanding repertoire of other diagnostic tests, including clinical chemistry, enzymes, immunoassays, cell membrane antigens, and pathognomonic biomarkers [31, 33, 34]. For example, non-PCR digital microfluidic cartridges have been developed to screen infants for congenital enzymatic defects to diagnose lysosomal storage diseases [34] and to assay blood levels of insulin, troponin I, and interleukin 6 [31]. The digital microfluidics real-time PCR platform has been shown to detect diagnostic levels of *M. pneumoniae* DNA in respiratory specimens [15], and this report has validated its potential application for the diagnosis of candidemia.

The ideal diagnostic PCR test should be minimally invasive, specific and sensitive, provide early detection, amenable to frequent testing, effective with minimal sample sizes, and predictive of infection in the target population, as well as allow quantification, identification of multiple pathogens,

Table 2 Comparison of conventional and microfluidic real-time PCR platforms for critical features of *C. albicans* DNA detection in specimens of blood

Feature	Conventional real-time PCR	Microfluidic real-time PCR
Specificity	Excellent	Excellent
Sensitivity	Good	Good
Flexibility (multiplexing)	Yes	Yes
Low sample volumes	Yes	Yes
Minimal turnaround time	Good	Excellent
PCR run time (45 cycles)	≈70 min	≈45 min
Reagent cost per PCR	Moderate	Inexpensive
Specialized training required	Extensive	Minimal
Ease of daily or stat testing	Cumbersome	Routine
Portable, point-of-care testing	No	Yes

have a low cost, and not require special expertise to perform [20]. The digital microfluidic real-time PCR platform is on track to satisfy all of these criteria.

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Conflict of interest Co-authors Benton, Poore, Rouse, Boles, V. K. Pamula, Eckhardt, and Pollack are employed by Advanced Liquid Logic, Inc. Co-authors Smith, Johnson, Alexander, Benjamin, Perfect, and Mitchell declare no conflict of interest. Co-author Schell has a small equity ownership in Advanced Liquid Logic, Inc.

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