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MAJOR ARTICLE

A Novel Giant Magnetoresistance–Enabled Multiplex Polymerase Chain Reaction Assay for the Diagnosis of Invasive Fungal Infection

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Background. Despite advances in clinical microbiology, the diagnosis of invasive fungal infections remains challenging. Giant magnetoresistance (GMR) is a novel technology that enables the detection of trace amounts of cell-free DNA (cfDNA). We evaluated a high-multiplex molecular diagnostic assay coupled with GMR-enabled lab-on-a-chip technology that can detect 18 different fungal species.

Methods. Analytical performance was evaluated in spiked plasma samples. After amplification, cfDNA was digested. Residual single-stranded DNA was flowed over a GMR sensor that was surface-coated with probes specific to different fungal species. After hybridization, magnetic beads bound to the probe complexes produced a GMR signal that was detected by the sensors. Clinical performance was determined using residual serum samples collected before the initiation of antifungal treatment from 20 patients with infection.

Results. The limit of detection of the assay ranged from 5 to 50 copies per polymerase chain reaction (PCR) reaction. Nonspecific signals were not observed in the spiked samples. Fungal cfDNA was detected in 80% of patients with invasive candidiasis (3/4 with candidemia, 5/6 with invasive candidiasis without candidemia), all 3 cases of invasive pulmonary aspergillosis, and all 3 cases of disseminated histoplasmosis. cfDNA was not detected in 2 patients with cryptococcosis (both had negative blood cultures) and 2 patients with *Pneumocystis* pneumonia.

Conclusions. We developed a novel GMR-enabled multiplex PCR assay detecting fungal pathogens that have been prioritized for public health action. Clinical sensitivity was highest in cases of presumed angioinvasion and dissemination. This technology has the potential for use in the clinical microbiology laboratory setting.

Keywords. aspergillosis; candidiasis; cell-free DNA; giant magnetoresistance; PCR.

Invasive fungal infections pose a significant threat, particularly among critically ill and immunocompromised patients [1]. Diagnosis remains challenging, often resulting in delayed or missed detection, leading to significant morbidity and mortality. In recent studies, the 90-day mortality of invasive candidiasis is approximately 40% [2, 3], and the 6-week mortality of primary invasive aspergillosis reaches 20% [4, 5]. Conventional diagnostic methods, such as culture and antibody testing, have significant limitations, including prolonged turnaround time and low sensitivity. For invasive candidiasis, blood culture is regarded as the gold standard; however, the prolonged incubation period (sometimes >48–72 hours) may lead to delays in instituting effective antifungal therapy. The interpretation of a positive β-D-glucan test is constrained by its lack of specificity. Consequently, there is an urgent need for the development and implementation of novel diagnostic methods. Evaluation of these methods is challenging when a "gold standard" is lacking [6].

Significant advances in molecular-based diagnostics have been achieved facilitating the diagnosis of fungal infections by detecting minute quantities of target DNA [7]. These assays offer several advantages over traditional methods, including rapid detection, high sensitivity, independence from culturing requirements, and the ability to identify specific fungal pathogens at the species level. In recent years, standardized polymerase chain reaction (PCR) assays have become commercially available [8]. However, these assays exhibit restricted multiplexing capabilities, allowing them to detect only a limited range of fungal genera and species. The integration of high-

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multiplex assays into syndromic testing protocols offers several advantages. By having the ability to detect a wide range of fungal pathogens within a single sample, diagnosis can be significantly expedited, leading to prompt initiation of effective treatment. The potential to identify coinfections in a single analysis offers additional guidance to the treating clinician. Moreover, multiplex testing can be cost-effective by consolidating several tests into a single assay. Ultimately, high-throughput capacity ensures efficient utilization of resources and laboratory infrastructure.

Pathogen-derived cell-free DNA (cfDNA) is released from tissue during episodes of acute infection and can be detected in plasma and other body fluids [9]. Detection of cfDNA offers the potential for early and accurate diagnosis of infection, sometimes even before symptom onset [10]. The 2 main methods for detecting cfDNA are PCR and next-generation sequencing (NGS). Untargeted metagenomic NGS offers the benefit of a pathogen-agnostic approach. However, it is associated with high cost, bioinformatic analysis, and complex interpretation [11]. Therefore, it can only be performed in centralized laboratories.

Giant magnetoresistance (GMR) is a novel technology that has been applied in medical diagnostics [12–15]. GMR involves a drastic change in electrical resistance, which occurs in the presence of a magnetic field, particularly in thin multilayer structures composed of ferromagnetic and nonmagnetic layers. The exceptionally sensitive GMR sensor enables the detection of minute levels of biological markers, rendering it highly efficient for diagnostics, especially in detecting trace amounts of cfDNA. In terms of cost and automation, GMR technology is notable for its potential to consolidate all essential components onto a single cartridge, which diminishes the need for numerous distinct procedures.

Herein, we describe the use of a high-multiplex molecular diagnostic assay coupled with GMR-enabled microfluidic technology for the detection of fungal cfDNA. Specific capture probes that match pathogen-specific genetic sequences are printed on a sensor array to achieve multiplex detection. After sample DNA extraction and amplification, the amplicon flows over the sensor for hybridization and subsequent GMR signal detection. The assay is designed to simultaneously detect cfDNA from 18 fungal organisms in a single test. These fungal species represent the most prevalent pathogens responsible for invasive fungal infection [1].

MATERIALS AND METHODS

Analytical Performance

The analytical performance of the assay was evaluated using spiked samples. Plasma from healthy humans was used as the sample matrix. Standard PCR technology was used to amplify extracted cfDNA. After amplification, cfDNA was divided into 4 groups, which were combined and digested with lambda exonuclease to remove 5′-phosphorylated DNA strands from the double-stranded DNA amplicons. The residual biotin-labeled single-stranded DNA was then flowed over a GMR sensor, which was surface-coated with oligonucleotide probes specific to different fungal species. As the sample flowed over the sensor, the biotin-labeled DNA hybridized with the probes. After hybridization, magnetic beads labeled with streptavidin selectively bound to the amplicon-probe complexes through streptavidin–biotin binding (Figure 1). These magnetic beads then produced a detectable GMR signal, with the signal strength corresponding to the variable levels of hybridizations on the GMR sensor surface. To verify further the validity of the signal of specific fungal targets, the instrument increased the temperature on the sensor surface after the introduction of magnetic beads to induce thermal stress onto the hybridized complex. Mismatched DNA exhibited less preferential binding under thermal stress, leaving only the perfectly matched probe–target DNA complexes (Figure 2).

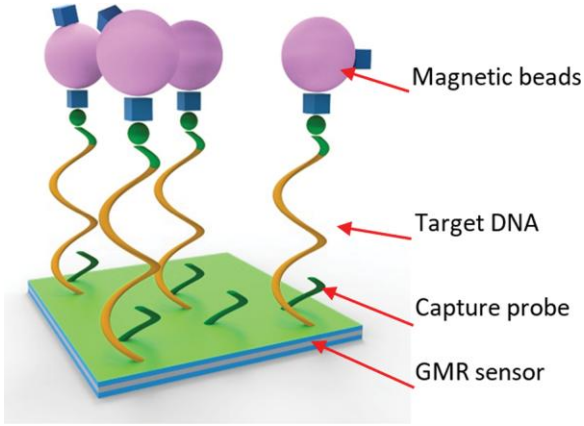

Figure 1. The formation of the complex occurs stepwise: The target DNA first binds to the capture probe. This is followed by the introduction of streptavidin-labeled magnetic beads, which then bind to the hybridized probe-DNA complex. Abbreviation: GMR, giant magnetoresistance.

The analytical sensitivity of the assay was validated using species-specific synthetic templates for each on-panel target (Supplementary Table 1). The assay detects 18 fungal species. It cannot differentiate between the various *Coccidioides* and *Fusarium* species. Synthetic templates were spiked into pooled plasma at serially diluted concentrations and tested using only its multiplexed group until the GMR signal was undetectable. The limit of detection was then determined at the concentration in which all 4 replicates showed positive signals for the target. The specificity of the assay was determined by running 4×10^4 copies per PCR reaction of synthetic template for each on-panel target, using all multiplex PCR groups. Exclusivity was similarly determined by running 4×10^4 copies per PCR reaction of synthetic template in the

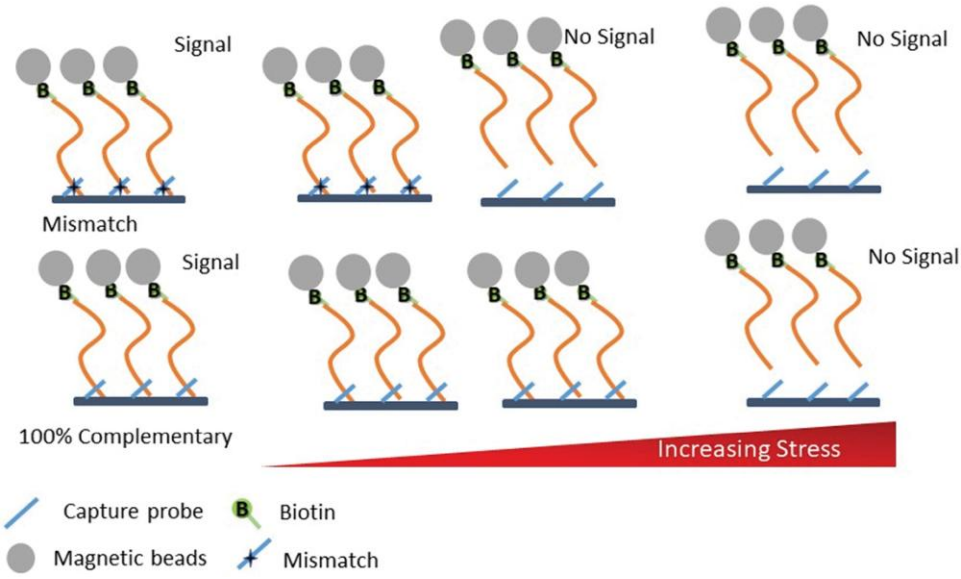

Figure 2. When thermal stress is introduced to the sensor surface, the less preferential binding of the mismatched complex dissociates, leaving only the optimally matched complexes on the surface.

presence of sheared genomic DNA from 24 off-panel microorganisms using all multiplexed PCR groups.

Clinical Performance

Using electronic health records and microbiology data, we identified patients with a new diagnosis of invasive fungal infection. Two investigators (P. V. and E. E. O.) independently confirmed the diagnosis of proven or probable infection based on consensus definitions by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium [16].

Residual serum samples collected before the initiation of antifungal treatment were recovered and stored at −4°C for 3–9 days before being sent to the processing laboratory (Zepto Life Technology, Saint Paul, Minnesota). If not tested on the day of arrival, delivered samples were promptly stored at temperatures below −70°C. Immediately before testing, frozen serum samples were thawed at room temperature. cfDNA was extracted from 1 mL of serum sample using the MagMax cfDNA Isolation Kit (Applied Biosystems, Waltham, Massachusetts). cfDNA was divided into 4 reactions, each mixed with 1 of 4 multiplex primer groups. Within these groups, the forward primers were labeled with 5′-biotin tags (IDT DNA, Coralville, Iowa), and the reverse primers were labeled with 5′-phosphate. PCR was performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California). To evaluate clinical sensitivity, DNA detection rates were compared to standard-of-care assays. Patients without evidence of infection or with infection caused by pathogens not included in the detection panel were used as negative controls to evaluate the specificity of the assay.

Patient Consent Statement

All patients provided research authorization, as required by Minnesota law. The study was approved by the Mayo Clinic Institutional Review Board (ID 23-000032). The study did not include factors necessitating patient consent.

RESULTS

Analytical Performance

A summary of the validation results can be found in Table 1. The limit of detection of the assay ranged from 5 to 50 copies per PCR reaction. In terms of specificity and exclusivity, nonspecific GMR signals were not observed for any on-panel or off-panel targets.

Clinical Performance

We evaluated 20 patients with proven or probable infection: invasive candidiasis (n = 10), invasive pulmonary aspergillosis (n = 3), disseminated histoplasmosis (n = 3), cryptococcosis (n = 2), and *Pneumocystis jirovecii* pneumonia (n = 2). Sites of infection, conventional diagnostic method and cfDNA detection rate are summarized in Table 2 (detailed information is provided in Supplementary Table 2). cfDNA was detected in 80% of patients with invasive candidiasis (3/4 cases of candidemia, 5/6 cases of invasive candidiasis without candidemia). Fungal cfDNA was

Table 1. Results From Assay Validation

Assay Validation	Template Species	Number of Replicates	Limit of Detection	Positive Results
Sensitivity	*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Coccidioides posadasii/immitis*, *Cryptococcus neoformans*, *Pneumocystis jirovecii*	4 replicates per concentration	5 copies per reaction	4/4[a]
	Aspergillus flavus	4 replicates per concentration	10 copies per reaction	4/4[a]
	Candida auris, *Histoplasma capsulatum*	4 replicates per concentration	20 copies per reaction	4/4[a]
	Candida krusei, *Candida tropicalis*, *Aspergillus niger*, *Fusarium verticilloides/oxysporum/solani*	4 replicates per concentration	50 copies per reaction	4/4[a]
Specificity	18 on-panel species[b]	2 replicates per species	~4×10^4 copies per reaction	2/2[c]
Exclusivity	24 off-panel microorganisms[b]	2 replicates per species	~4×10^4 copies per reaction	0/2[d]

[a]Results generated using templates at lowest concentration.

[b]Eighteen on-panel and 24 off-panel species are listed in Supplementary Table 1.

[c]All spiked on-panel samples yielded expected positive results. No other signal was observed during specificity testing.

[d]No off-panel organisms generated signals during exclusivity testing.

Table 2. Clinical Characteristics, Conventional Diagnostic Method, and Cell-Free DNA Detection Rate

Invasive Fungal Infection	Site of Infection	Conventional Diagnostic Method	Serum cfDNA Detection	DNA Detection Rate
Candidemia (4)	Bloodstream infection (4) Pyelonephritis (1)	Blood culture: • *Candida albicans* (1) • *Candida glabrata* (2) • *Candida parapsilosis* (1) • *Candida tropicalis* (1)	*C albicans* (1) *C glabrata* (1) *C parapsilosis* (1) *C tropicalis* (1)	3/4
Invasive candidiasis without candidemia (6)	Intra-abdominal abscess (4) Liver abscess with peritonitis (1) Peritonitis without abscess (1)	Aspirate fluid culture: • *C albicans* (3) • *C glabrata* (2) • *C tropicalis* (2) • *Candida krusei* (1)	*C albicans* (2) *C glabrata* (2) *C krusei* (1)	5/6
Aspergillosis (3)	Invasive pulmonary aspergillosis (3)	BAL culture: *Aspergillus fumigatus* (3) Positive BAL *Aspergillus* Ag (3) Positive serum *Aspergillus* Ag (2)	*A fumigatus* (3)	3/3
Histoplasmosis (3)	Fungemia with multiorgan involvement (2) Fungemia with prosthetic valve endocarditis (1)	Cultures: • *Histoplasma* spp complex (blood, 3) • *Histoplasma* spp complex (BAL/sputum, 2) • *Histoplasma* spp complex (bone marrow, 1) • Positive *Histoplasma* Ag (serum, 3)	*Histoplasma capsulatum*	3/3
Cryptococcosis (2)	Pneumonia (1) Chronic meningitis (1)	Culture: *Cryptococcus neoformans* spp complex (CSF, 1; BAL, 1)	Negative	0/2
Pneumocystis infection (2)	Pneumonia (2)	*Pneumocystis* PCR (sputum, 1; BAL, 1)	Negative	0/2

Abbreviations: Ag, antigen; BAL, bronchoalveolar lavage; cfDNA, cell-free DNA; CSF, cerebrospinal fluid; PCR, polymerase chain reaction.

detected in all cases of invasive pulmonary aspergillosis and histoplasmosis. The test was negative in 2 patients with cryptococcosis (both had negative blood cultures) and 2 patients with *Pneumocystis* pneumonia (both had negative serum β-D-glucan).

We obtained control samples from 4 patients with infection caused by organisms not on the detection panel (bloodstream infection caused by *Candida dubliniensis*, *Streptococcus gallolyticus*, or *Staphylococcus aureus* and urinary tract infection caused by *Escherichia coli*), and 5 patients without evidence of infection. Fungal cfDNA was detected in 2 of the 9 control samples. *Candida albicans* was detected in a patient with *C dubliniensis* bloodstream infection who had a malignant biliary obstruction and multiple hepatic abscesses. Sampling of these abscesses was not pursued. A low-positive signal for *Fusarium* was detected in the patient with *S gallolyticus* bacteremia and transcatheter aortic valve replacement–associated endocarditis. No remaining samples were available to verify the result by confirmatory testing.

DISCUSSION

Fungal infections pose a significant diagnostic challenge. Given the limitations of standard microbiologic methods and serologic assays, there has been a growing interest in the development of rapid, sensitive, specific, simple, and cost-effective diagnostics. GMR is a technology that has the potential to detect minute levels of biological markers. We developed a GMR-enabled multiplex PCR assay that can detect cfDNA of 18 different fungal species using lab-on-a-chip technology. We determined the limit of detection and analytical specificity of the assay using spiked samples. We also studied the clinical performance of the assay. We were able to detect fungal cfDNA in 80% of patients with candidemia/invasive candidiasis and all cases of invasive pulmonary aspergillosis and disseminated histoplasmosis. The technology can integrate several molecular diagnostic processes into a miniaturized device and can potentially be used in any clinical microbiology laboratory.

GMR biosensors have the potential for use in a wide variety of biomedical applications, such as diagnostics, genotyping, and food and drug regulation as well as brain and cardiac mapping [17]. The GMR technology has been applied to detect *Mycobacterium tuberculosis* [18] as well as severe acute respiratory syndrome coronavirus 2 [19], influenza A virus [20], and hepatitis B virus [21]. In the current study, we established and demonstrated the ability of a GMR platform to detect minuscule levels of fungal cfDNA in human serum. After hybridization with specific probes on the biosensor, magnetic beads were bound to the biotin-labeled hybridization complex. The magnetic bead linked to the biosensor created a change in magnetoresistance, which was measured by the GMR detection system. Within this platform, we were able to combine conventional molecular methods with the GMR technology to optimize sensitivity. In addition, we applied thermal stress to increase specificity by detecting only optimally matched probe–DNA complexes. Lab-on-a-chip technology allows for standardization, small reagent volumes, fast turnaround time, and reduced risk of DNA contamination. The device can be further refined for clinical use.

This novel technology has several benefits. It can rapidly identify fungal pathogens within hours. The actual turnaround time will be determined as we refine the assay and the system. cfDNA detection in serum or plasma may obviate the need for an invasive procedure. The multiplexed assay offers the advantage of differential detection of multiple pathogens. Interpretation of those results does not require advanced training or expertise. Finally, we anticipate that the assay will be cost-effective, allowing it to be used in a wide spectrum of clinical settings and possibly as a point-of-care test.

The analytical sensitivity of the assay ranges from 5 to 50 copies of DNA per reaction. The high sensitivity is due to the nature of GMR detection and the relative absence of noise at baseline, which is distinct from optical-based detection systems. Our analytical evaluation also showed 100% specificity. The high specificity of the assay is enhanced through 3 distinct processes: (1) the primer set for selective amplification, (2) the use of dual detection probes and magnetic beads, and (3) an end-of-run thermal stress verification. The analytical performance of the assay, leveraging lab-on-a-chip technology, exhibits promising potential for high sensitivity and multiplexed testing within an automated, user-friendly workflow.

The GMR-enabled multiplex assay can detect 6 different *Candida* species. Delaying effective antifungal treatment has been associated with increased mortality in invasive candidiasis [22]. Therefore, early pathogen detection and identification at the species level can guide the selection of appropriate therapy based on the anticipated resistance patterns. In the cases of invasive candidiasis without candidemia (intra-abdominal abscess or peritonitis), the assay may establish a microbiologic diagnosis without the need for an invasive procedure for fluid or tissue sampling.

Our assay detected *Aspergillus fumigatus* DNA in all 3 cases of invasive pulmonary aspergillosis. These cases probably had a high fungal burden and angioinvasive disease based on the extent of radiographic abnormalities. Serum *Aspergillus* galactomannan was negative in only 1 of those cases, a nonneutropenic kidney transplant recipient. In a large retrospective study, *Aspergillus* plasma cfDNA detection by PCR was indeed more sensitive compared to serum galactomannan in patients with a new diagnosis of invasive aspergillosis [23]. That same study demonstrated that PCR sensitivity was higher in patients with hematologic malignancies and hematopoietic cell transplant recipients compared to other immunosuppressed individuals. Combining PCR and galactomannan testing could potentially offer higher diagnostic accuracy.

Our assay detected *Histoplasma* DNA in the 3 cases of disseminated histoplasmosis. All patients had fungemia. In future studies, we plan to investigate the performance of the assay in disease limited to the lungs or cases of dissemination without documented fungemia. Our assay did not detect DNA in 2 patients with cryptococcal infection (pneumonia and meningitis). Both had negative blood cultures. We suspect that the lack of detectable serum DNA in the above cases may be related to the stage in the natural history of the disease. Finally, the assay did not detect DNA in 2 cases of *Pneumocystis* pneumonia. Both cases had clinical and radiographic abnormalities consistent with pneumonia, positive *Pneumocystis* PCR in respiratory samples, and a negative serum β-D-glucan. The negative serum biomarker probably reflects a lower fungal burden. In future studies, we plan to include cases of *Pneumocystis* pneumonia with positive serum β-D-glucan. Differences in detection rates across fungal species may depend on the level of circulating DNA for each type of infection.

In our study, we included 9 control samples, of which 7 had negative GMR signals and 2 had positive signals. Of the 2 samples with positive signals, 1 was collected from a patient with malignant biliary obstruction and hepatic abscesses who had polymicrobial bloodstream infection caused by enterococci and *C dubliniensis*, a fungal species not included in our detection panel. In this patient, we were able to detect *C albicans* DNA, likely due to the polymicrobial nature of the infection. Sampling of the liver abscesses was not pursued to confirm coinfection with 2 *Candida* species. The other control sample with a positive signal was from a patient with *S gallolyticus* endocarditis. In this patient, we detected a low-level positive signal of *Fusarium* DNA, despite the absence of risk factors or radiographic evidence of an invasive fungal infection. This could be explained by low-grade fungal coinfection, contamination during sample collection or processing, or false positivity. Similar to other diagnostic tests, our assay must be interpreted within the relevant clinical context.

Based on these findings, we believe that the assay will demonstrate its highest potential in diagnosing (1) candidemia/invasive candidiasis in at-risk individuals and (2) pulmonary fungal infection in patients with consistent radiographic abnormalities. In October 2022, the World Health Organization (WHO) published a fungal priority pathogens list to guide research and development efforts worldwide [24]. Thirteen species that can be detected by our GMR-enabled PCR assay are included in this list, with 4 belonging to the critical-priority group (*Cryptococcus neoformans*, *A fumigatus*, *Candida auris*, and *C albicans*), 6 to the high-priority group (*Candida glabrata*, *Fusarium* spp, *Candida parapsilosis*, *Histoplasma capsulatum*, and *Candida tropicalis*), and 3 to the medium-priority group (*Coccidioides* spp, *Candida krusei*, and *P jirovecii*). These pathogens are considered to have the greatest public health impact and risk for emerging antifungal resistance. Focusing our efforts on the early detection of these organisms will significantly improve healthcare delivery and clinical outcomes at the global level.

Our study has several limitations. We evaluated a small number of clinical samples for a broad array of fungal pathogens included in the PCR panel. The volume of each clinical sample (1 mL) prohibited verification studies. Using a larger sample volume could also have increased cfDNA detection rates. Each on-panel pathogen can be associated with variable clinical manifestations depending on the host immune response and the extent of infection. We observed high sensitivity in cases of presumed angioinvasion and dissemination. The performance of the assay in disease limited to the lungs or other body sites (such as the peritoneal cavity or central nervous system) will be further characterized in future studies. Serum samples were used for this study. The performance of the assay on plasma will also be characterized in future studies. Another limitation is that serum was refrigerated several hours after residual clinical sample collection. This may have impacted DNA stability and, consequently, the sensitivity of the assay. Handling of these residual samples may also have increased the risk of DNA contamination, which can potentially cause false-positive signals. Finally, all samples were collected prior to the initiation of antifungal therapy. It remains to be determined how the assay performs in patients receiving empiric antifungal therapy.

In conclusion, we developed a novel GMR-enabled multiplex PCR assay that can detect up to 18 different species, most of which are included in the WHO fungal priority pathogens list. We evaluated the analytical sensitivity, specificity, and exclusivity of the assay and its clinical performance in a small number of human samples. We expect the final product to offer a short turnaround time and the capability to test multiple samples simultaneously. This technology has the potential for use in the clinical microbiology laboratory setting and may facilitate the early diagnosis of invasive fungal infection.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. Investigators from Zepto Life Technology were directly involved in design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, and approval of the manuscript; and decision to submit the manuscript for publication.

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Potential conflicts of interest. P. V. reports research funding from Mundipharma, Scynexis, F2G, and Ansun; serves as consultant for AbbVie and Scynexis; and reports receiving honoraria from the Merck Manuals. J.-A. H. Y. reports research funding from Mundipharma/Cidara, Scynexis, F2G, Pulmocide, Zepto, and Basilea. All other authors report no potential conflicts.

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