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Evaluation of Luminex xTAG Fungal Analyte-Specific Reagents for Rapid Identification of Clinically Relevant Fungi[▽]

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Invasive fungal infections (IFI) remain a serious threat to immunocompromised hosts. Current diagnostic methods, including fungal culture and antigen detection, are slow and often lack specificity. Rapid diagnostic tools with increased sensitivity and specificity could improve the care of patients with IFI. Recently, Luminex Molecular Diagnostics (Toronto, Canada) developed 23 analyte-specific reagents (ASRs) for the detection of the most common clinically relevant fungi. This study's objective was to evaluate the sensitivity and specificity of a subset of these ASRs for fungal isolates and clinical specimens. Previously characterized fungal and bacterial isolates ($n = 110$), blood culture specimens ($n = 34$), and respiratory specimens ($n = 44$) were tested using either a *Candida* 7-plex panel (*Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida lusitaniae*, *Candida guilliermondii*, and *Candida krusei*) or a mold 11-plex panel (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Scedosporium prolificans*, *Scedosporium apiospermum*, *Fusarium oxysporum*/*Fusarium solani*, *Rhizopus arrhizus*, *Rhizopus microsporus*, *Mucor indicus*, and *Cunninghamella bertholletiae*). The *Candida* 7-plex panel correctly identified all *Candida* isolates as confirmed by fungal culture and biochemical tests, for a sensitivity and specificity of 100%. The mold 11-plex panel correctly identified all mold isolates tested except for *A. niger*. Fungal isolates of *Rhizopus* and *Mucor* species were not detected, either, although they could represent species other than those targeted by the ASRs. Further evaluation will be necessary to confirm the sensitivities of some of the mold ASRs. Implementation of these ASRs will allow same-day detection of fungal DNA in clinical specimens.

Invasive fungal infections (IFI) remain a major cause of morbidity and mortality in immunocompromised hosts. In patients undergoing hematopoietic stem cell transplantation (HSCT), the timing for the acquisition of an IFI differs with the type of transplant (autologous versus allogeneic HSCT) and the type of IFI (candidiasis versus aspergillosis) (24). A recent article by the Transplant-Associated Infection Surveillance Network (TRANSNET) reported that the 12-month cumulative incidence for IFI in HSCT was 3.4%, with a range of 0.9 to 13.2% and an overall 1-year survival rate ranging from 6.3% to 33.6% depending on the cause of IFI (16). The most common cause of IFI was invasive aspergillosis (most commonly *Aspergillus fumigatus*), followed by invasive candidiasis (most commonly *Candida glabrata*) and mucormycosis (formerly zygomycosis) (16). These cases included only proven and probable fungal infections as defined by the Mycoses Study Group and the Cooperative Group of the European Organization for Research and Treatment of Cancer (EORTC) (2).

Diagnosis of IFI currently relies on the recovery of molds or yeasts in culture, fungal stains, detection of antigens, including galactomannan and β-D glucans, and various radiological findings of pulmonary infiltrates (7). Although useful, these methods can lack specificity, can be time-consuming, or can result in inconclusive findings. Therefore, the search for the optimal IFI diagnostic tool continues. Several real-time PCR assays have been developed over the past few years, with various levels of sensitivity and specificity and often with a limited range, targeting only a few *Candida* or mold species (12, 13, 21, 28, 31). A recent shift toward the development of panfungal assays, reflecting the need for tools that detect most of the clinically relevant fungal pathogens in patient specimens, can be observed in the literature (6, 17, 23, 26). Recently, Luminex Molecular Diagnostics (LMD; Toronto, Canada) developed 23 analyte-specific reagents (ASRs) for the detection of the most common clinically relevant fungi. The objective of this study was to evaluate the sensitivity and specificity of a subset of these ASRs for the detection of fungi in culture and directly from clinical specimens.

MATERIALS AND METHODS

Culture isolates. The fungal and bacterial strains used to determine the assay sensitivity and specificity were archived, previously characterized clinical isolates or strains obtained from the American Type Culture Collection (ATCC) (Table 1). Yeast isolates were subcultured from frozen stock onto Sabouraud dextrose agar plates and were incubated at 30°C for 24 h. The identities of the isolates were confirmed using a combination of germ tube tests, cornmeal agar morphology, carbohydrate assimilation, and API 20C AUX yeast identification kits (bioMérieux, Durham, NC). Molds were subcultured from their stock onto Sabouraud dextrose agar plates and were incubated at 30°C for 48 to 72 h. The identities of the molds were confirmed using a combination of macroscopic morphology, microscopic examination by lactophenol cotton blue staining, and temperature tolerance. A slide culture on potato dextrose agar was also performed when necessary.

Clinical specimens. Clinical specimens ($n = 78$) were remnant specimens collected over a 9-month period from patients ($n = 46$) with suspected fungal infections. These specimens included bronchial wash samples ($n = 16$), bronchoalveolar lavage (BAL) fluids ($n = 2$), pleural fluids ($n = 2$), a tracheal secretion ($n = 1$), sputa ($n = 17$), lung biopsy specimens ($n = 5$), an appendix

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TABLE 1. Fungal and bacterial strains used in this study

Organism	Source	No. of strains
Candida albicans	ATCC 18804	1
	Clinical isolates	9
Candida glabrata	Clinical isolates	6
Candida parapsilosis	ATCC 22019	1
	Clinical isolates	5
Candida tropicalis	ATCC 750	1
	Clinical isolates	5
Candida lusitaniae	Proficiency organism	1
	Clinical isolates	6
Candida krusei	ATCC 6258	1
	Clinical isolates	4
Candida guilliermondii	ATCC 6260	1
	Clinical isolates	2
Aspergillus fumigatus	Clinical isolates	8
Aspergillus flavus	Clinical isolates	5
Aspergillus terreus	Clinical isolates	4
Aspergillus niger	ATCC 16404	1
	Clinical isolates	7
Fusarium species	Clinical isolates	3
Scedosporium apiospermum	Clinical isolates	7
Scedosporium prolificans	Clinical isolates	2
Cunninghamella bertholletiae	Clinical isolates	2
Mucor species	Clinical isolates	6
Rhizopus species	Clinical isolates	5
Rhizopus microsporus	Clinical isolate	1
Staphylococcus aureus	ATCC 29213	1
	ATCC 43300	1
Staphylococcus epidermidis	ATCC 12228	1
Streptococcus pyogenes	ATCC 19615	1
Streptococcus pneumoniae	ATCC 49136	1
Streptococcus bovis	ATCC 49147	1
Enterococcus faecalis	ATCC 29212	1
Escherichia coli	ATCC 25922	1
	ATCC 35218	1
Klebsiella pneumoniae	ATCC 35657	1
Proteus mirabilis	ATCC 35659	1
Proteus vulgaris	ATCC 49132	1
Enterobacter cloacae	ATCC 13047	1
Haemophilus influenzae	ATCC 9006	1
Micrococcus luteus	ATCC 4698	1
Moraxella catarrhalis	ATCC 8176	1

tissue ($n = 1$), and blood culture bottles ($n = 34$). Respiratory specimens were cultured by inoculating a brain heart infusion (BHI) agar plate with sheep blood supplemented with chloramphenicol (50 μg/ml) and gentamicin (40 μg/ml); tissues were inoculated onto BHI agar with 5% sheep blood agar; and positive blood cultures were subcultured on sheep blood agar. Yeasts and molds were identified as described above.

Nucleic acid extraction. Total nucleic acid extracts were prepared by placing a 1-μl loopful of organisms in a 2.0-ml microcentrifuge tube containing ceramic beads (BioExpress, Kaysville, UT) and 500 μl of yeast lysis buffer (Epicentre Biotechnologies, Madison, WI). The solution was incubated at 95°C for 10 min, followed by a 2-min mechanical lysis step on the Disruptor Genie (Scientific Industries, Bohemia, NY). Two hundred microliters was then extracted using the MagNA pure LC instrument (Roche Diagnostics, Indianapolis, IN) and was eluted in a final volume of 100 μl.

For respiratory specimens, total nucleic acids were extracted by transferring 1.5 ml of the specimen to a 2.0-ml microcentrifuge tube and centrifuging for 5 min at room temperature at maximum speed (~16,000 × *g*). The supernatant was discarded, and the pellet was resuspended in 500 μl of yeast lysis buffer (Epicentre Biotechnologies, Madison, WI) and transferred to a microcentrifuge tube containing ceramic beads (BioExpress, Kaysville, UT). The solution was treated as described above for extraction of nucleic acids from fungal isolates. The total nucleic acid extract was eluted in a final volume of 100 μl.

Blood culture bottles were processed by transferring 1.5 ml of the specimen to a 2.0-ml microcentrifuge and adding 75 μl of Luminex R/W lysis buffer. The solution was mixed by gentle inversion of the tube and was incubated at room temperature for 5 min. Following a 5-min spin at ~16,000 × *g*, the supernatant was discarded and the pellet resuspended in 1.5 ml of PCR-grade water. The centrifugation and wash steps were repeated twice, and the pellet was resuspended in 500 μl of yeast lysis buffer (Epicentre Biotechnologies, Madison, WI) and was transferred to a microcentrifuge tube containing ceramic beads (BioExpress, Kaysville, UT). The solution was treated as described above for the extraction of nucleic acids from fungal isolates. The total nucleic acid extract was eluted in a final volume of 100 μl.

Tissues were transferred to a microcentrifuge tube containing 400 μl of 1× Tris-EDTA buffer, 100 μl proteinase K (Roche Diagnostics, Indianapolis, IN), and 50 μl 10% sodium dodecyl sulfate. The tube was vortexed briefly and was incubated overnight at 55°C. The following day, 200 μl of the digested tissues was extracted on the MagNA Pure LC instrument, and total nucleic acids were eluted in 100 μl.

Multiplex PCR. Two master mixes were prepared using a subset of the following Luminex analyte-specific reagents (ASRs): xTAG *Candida albicans*, xTAG *Candida glabrata*, xTAG *Candida parapsilosis*, xTAG *Candida tropicalis*, xTAG *Candida lusitaniae*, xTAG *Candida krusei*, xTAG *Candida guilliermondii*, xTAG *Aspergillus fumigatus*, xTAG *Aspergillus flavus*, xTAG *Aspergillus terreus*, xTAG *Aspergillus niger*, xTAG *Fusarium oxysporum*/*Fusarium solani*, xTAG *Scedosporium apiospermum*, xTAG *Scedosporium prolificans*, xTAG *Cunninghamella bertholletiae*, xTAG *Mucor indicus*, xTAG *Rhizopus microsporus*, xTAG *Rhizopus arrhizus*, xTAG *Pneumocystis jirovecii*, xTAG *Histoplasma capsulatum*, xTAG *Blastomyces dermatitidis*, xTAG *Coccidioides immitis*, and xTAG *Cryptococcus neoformans*. Each reaction mixture for the *Candida* 7-plex panel included 0.5 μl (10 pmol/μl) of the primer pairs for *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, and *Tremella fuciformis* (external control), 1 μl (5 mM) of a deoxynucleoside triphosphate (dNTP) mixture (Qiagen Inc., Valencia, CA), 4.4 μl of 10× buffer (Qiagen Inc.), 0.8 μl of 25 mM $MgCl_2$ (Qiagen Inc.), and 0.3 μl of Hot Start *Taq* polymerase (Qiagen Inc.). The total volume per reaction mixture was 20 μl (15 μl master mix plus 5 μl extracted nucleic acids). Each reaction mixture for the mold 11-plex panel was set up similarly to the *Candida* 7-plex panel but used the primer pairs for the following organisms: *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *S. prolificans*, *S. apiospermum*, *F. oxysporum*/*F. solani*, *R. arrhizus*, *R. microsporus*, *M. indicus*, *C. bertholletiae*, and *Tremella fuciformis* (external control). PCR amplification was performed on a MyCycler thermocycler (Bio-Rad, Hercules, CA) using the following cycling parameters: 1 enzyme activation step at 95°C for 15 min; 35 amplification cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; 1 final cycle at 72°C for 5 min; and a hold at 4°C until the product was ready for use.

Hybridization. Each bottle of MagPlex-TAG microspheres (LMD, Toronto, Ontario, Canada) was vortexed for 10 s and was sonicated for 5 s. One microliter of each stock of MagPlex-TAG beads/reaction was combined in a microcentrifuge tube and was centrifuged for 2 min at ~16,000 × *g*. The supernatant was removed, and the bead pellet was resuspended in an amount of 1× hybridization buffer equal to the number of reactions multiplied by 20 (i.e., for 10 reactions, beads were resuspended in 200 μl of hybridization buffer). A 1:75 dilution of xTAG streptavidin and R-phycoerythrin G75 (SA-PE G75) was prepared in 1× hybridization buffer. Each hybridization reaction mixture contained 20 μl of the bead mixture, 1 μl of amplified DNA, and 75 μl of SA-PE G75 in a 96-well plate. The plate was incubated for 45 min at 45°C, followed by analysis on the Luminex 200 instrument (LMD, Toronto, Canada).

Luminex detection. A minimum of 100 beads were measured for each analyte in the *Candida* 7-plex or mold 11-plex panel. The raw median fluorescence intensity (MFI) signal was generated by the instrument, and results were interpreted using TDAS LSM software (Luminex Molecular Diagnostics) with a minimum MFI of 500 for a positive result. Positive and negative controls were added to each run.

Analytical sensitivity. Serial dilutions of amplified *C. albicans* and *A. fumigatus* DNA (10 μg/ml to 0.001 μg/ml) were tested to establish the analytical sensitivities of the assays. The starting concentration of DNA was measured using the Qubit fluorometer (Invitrogen, Carlsbad, CA), and dilution was carried out in PCR-grade water.

Sequencing of discordant results. A few isolates, which tested positive by culture but negative by the mold 11-plex assay, were sent out to the Mayo Medical Laboratories (MML; Rochester, MN) for sequencing of the D1/D2 domain of the large ribosomal subunit of fungi and to the LMD site in Toronto for sequencing of the 18S rRNA gene using previously published primer sequences (14).

TABLE 2. Comparison of the *Candida* 7-plex assay to culture for the identification of *Candida* from culture isolates and in blood culture bottles

Result by culture	No. of isolates with the following result by the *Candida* 7-plex assay:	
	Positive	Negative
Culture isolates (n = 59)		
Positive	43	0
Negative	0	16
Total	43	16
Blood bottles (n = 34)		
Positive	22	0
Negative	0	12
Total	22	12

TABLE 3. Comparison of the mold 11-plex assay to culture for the identification of mold from culture isolates and clinical specimens

Result by culture	No. of isolates and clinical specimens with the following result by the mold 11-plex assay:	
	Positive	Negative
Culture isolates (n = 51)		
Positive	32	19[a]
Negative	0	0
Total	32	19
Clinical specimens (n = 44)		
Positive	15[b]	10[c]
Negative	6[d]	19
Total	21	29

[a] Includes 8 *A. niger* isolates, 6 *Mucor* species, and 5 *Rhizopus* species.
[b] Includes multiple infections.
[c] Includes 8 *A. fumigatus* isolates, 1 *A. flavus* isolate, and 1 *Rhizopus* species.
[d] Includes 2 *A fumigatus*, 3 *A. flavus*, and 1 *S. apiospermum* isolate.

RESULTS

Sensitivity and specificity for fungal isolates. A total of 43 *Candida* strains and 16 bacterial strains were tested using the *Candida* 7-plex assay. The panel correctly identified all *Candida* isolates, as confirmed by fungal culture and biochemical tests, with no cross-reaction with any of the bacterial strains tested, for a sensitivity and specificity of 100% (43/43; 16/16) (Table 2).

A total of 51 mold species were tested using the mold 11-plex assay. The panel correctly identified all species of *Aspergillus* (8/8 *A. fumigatus*, 4/4 *A. terreus*, and 5/5 *A. flavus* isolates) except *A. niger* (0/8 isolates), as well as *S. apiospermum* (7/7), *S. prolificans* (2/2), *F. oxysporum*/*F. solani* (3/3), and *C. bertholletiae* (2/2), for a sensitivity and specificity of 100% for each of these species except *A. niger* (0%). *Mucor* (0/6 isolates) and *Rhizopus* (1/6 isolates) species were not detected, except for one isolate, which was identified both by culture and by the mold 11-plex assay as *Rhizopus microsporus* (Table 3).

Sensitivity and specificity of detection from clinical specimens. Thirty-four blood culture bottles (29 positive and 5 negative) were tested for the presence of *Candida* species using the *Candida* 7-plex assay. The 29 positive blood culture bottles included *Rhodotorula glutinis* (n = 5), *Fusarium* species (n = 2), *C. albicans* (n = 9), *C. parapsilosis* (n = 10), and *C. glabrata* (n = 3). The blood culture bottles that were positive for *Rhodotorula glutinis* and *Fusarium* species and all the negative blood culture bottles (n = 5) tested negative by the *Candida* 7-plex assay. The sensitivity of the assay was 100% for *C. albicans* (9/9 cultures), *C. parapsilosis* (10/10), and *C. glabrata* (3/3), with a specificity of 100% for each species (Table 2).

Forty-three respiratory specimens and 1 appendix tissue (23 positive and 21 negative) were tested for the presence of molds using the mold 11-plex assay. The 22 positive specimens included *S. apiospermum* (n = 1), *A. fumigatus* (n = 19), *A. flavus* (n = 2), *A. niger* (n = 1), and a mixed infection with *A. fumigatus*, *A. flavus*, *and Rhizopus* spp. (n = 1). The sensitivity and specificity of the assay were, respectively, 100% (1/1) and 98% for *S. apiospermum* (43/44), 58% (11/19) and 92% (25/27) for *A. fumigatus*, and 67% (2/3) and 93% (38/41) for *A. flavus*. The mold 11-plex assay failed to detect the one *Rhizopus* species and the *A. niger* strains detected in culture, results similar to those with fungal isolates (Table 3). Two culture-negative specimens (lung biopsy specimens) were positive for *A. fumigatus* by the mold 11-plex assay. Two bronchial wash specimens were positive by culture for *A. fumigatus* only, but by the mold 11-plex assay, they were positive for both *A. fumigatus* and *A. flavus*. One tracheal aspirate was positive for *A. fumigatus* by culture and positive for *A. fumigatus*, *A. flavus*, and *S. apiospermum* by the mold 11-plex assay (Table 3). These two bronchial wash specimens and one tracheal aspirate were from the same patient whose previous sputum sample was positive for *A. fumigatus*, *A. flavus*, and *Rhizopus* spp.

Sequencing. Four isolates (two *Rhizopus* species and two *A. niger* isolates) were sequenced at the MML. The two *Rhizopus* species were identified by sequencing as *Rhizopus* species (100%) but could not be identified beyond the genus level. The two *A. niger* isolates were identified as 100% *A. niger*.

Eighteen isolates (5 *Mucor* species, 5 *Rhizopus* species, and 8 *A. niger* isolates) were sequenced at LMD. Three of the *Mucor* isolates were identified as *Mucor* spp. other than *Mucor indicus*, and no information (i.e., poor sequences) could be obtained from the other two strains. One of the *Rhizopus* isolates was identified as *R. arrhizus*, and no information was available from the other 4 isolates. Out of the 8 *A. niger* isolates sequenced, 3 were confirmed as *A. niger*, 4 were identified as *Aspergillus tubingensis*, and 1 could not be distinguished as either *A. niger* or *A. tubingensis*.

Analytical sensitivity. The analytical sensitivity, as determined by 10-fold serial dilutions of *C. albicans* and *A. fumigatus* DNA, was 1.0 pg/ml for both organisms (data not shown).

DISCUSSION

We report the evaluation of a set of new analyte-specific reagents from Luminex Molecular Diagnostics (Toronto, Canada) for the detection of several clinically important fungal organisms. Rapid and accurate identification of fungal pathogens is critical to improving the diagnosis and management of IFI. Several PCR assays have been reported previously for the detection of fungal pathogens, often with an emphasis on *C. albicans* and *A. fumigatus* (12, 13, 21, 28, 31). However, other fungal species are becoming more common and should be

included in the effort to develop molecular assays for fungal infection. Therefore, the development of panfungal assays has become a more attractive option in terms of laboratory diagnostic tools. In a study by Schabereiter-Gurtner and colleagues, a real-time PCR assay was developed to identify 11 *Aspergillus* and *Candida* species directly in clinical specimens (26). Differentiation of species was based on each species having a different melting curve, which can be challenging in case of a single mutation or polymorphism in the target region. Another study, by Baskova et al., also targeted a series of *Aspergillus* and *Candida* species but used a single primer pair and a universal probe to detect all fungi, so that the assay served as a screening assay and required additional testing to determine the exact species (6). Other technologies, including matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS), have been successfully evaluated in several studies for the identification of yeasts from both solid media and blood culture and have shown detection rates ranging from 85% to 96%, with minimal consumables and hands-on time and a very rapid turnaround time (less than 30 min) (8, 10, 30, 33, 35). However, challenges remain for the identification of molds by MALDI-TOF MS, as well as for its use directly on clinical specimens.

In this study, we selected the ASRs to evaluate out of 23 possible choices. One of the advantages of using ASRs is that laboratories can select and create fungal panels to detect yeasts or molds commonly encountered in their individual patient populations. The presence or absence of a specific fungal organism is determined by the intensity of the fluorescent signal detected by the Luminex instrument. We created a *Candida* 7-plex panel for the identification of *Candida* species growing on solid media and in blood culture bottles. The sensitivity and specificity of the assay was 100% for all strains tested (Table 2). Although this use was not tested in our study, ideally the assay would be used directly on whole blood specimens instead of blood culture for increased benefit. However, in its current form, this assay can be used similarly to the *Candida* peptide nucleic acid (PNA) fluorescent *in situ* hybridization (FISH) assay (AdvanDx, Woburn, MA) with a longer turnaround time (i.e., 5 to 6 h versus 2.5 h) but the added advantage of detecting seven unique *Candida* species.

It should be noted that each *Candida* ASR, like current phenotypic methods, reports the *Candida* species identified without distinguishing between members of the complexes. For example, *C. parapsilosis*, a genetically heterogenous species, is composed of three phenotypically indistinguishable groups, *C. parapsilosis* groups I, II, and III; groups II and III have recently been renamed *Candida orthopsilosis* and *Candida metapsilosis* on the basis of multilocus sequence typing of several genes (32). Although *C. parapsilosis* sensu stricto (group I) is the most common isolate in the laboratory, a recent study has shown that as many as 10.9% of laboratory isolates identified as *C. parapsilosis* were in fact *C. orthopsilosis* or *C. metapsilosis* (19). A large study of *C. parapsilosis* isolates from Portugal showed low incidences of *C. orthopsilosis* and *C. metapsilosis*, and comparison of antifungal susceptibility patterns revealed that azole resistance was detected only among *C. parapsilosis* sensu stricto isolates, not among *C. orthopsilosis* or *C. metapsilosis* isolates, suggesting a possible benefit in specific identification of members of the complex (27). As with the *C. parapsilosis* complex, the *Candida* ASRs do not differentiate between members of the *C. glabrata* complex (*C. glabrata*, *Candida nivariensis*, and *Candida bracarensis*) (18) or the *C. guilliermondii* complex (*C. guilliermondii*, *Candida carpophila*, and *Candida fermentati*) (20, 34). Although they are rare and are not currently associated with any differences in antifungal susceptibilities (18, 20), knowledge of the presence of these cryptic species in clinical isolates could potentially have clinical benefits. Interestingly, due to sequence similarity, the PNA FISH probes for *C. parapsilosis* cross-react with *C. orthopsilosis*, and those for *C. glabrata* cross-react with *Nakaseomyces delphensis*, *C. bracarensis*, and *C. nivariensis*, resulting in false-positive PNA FISH results (*Candida* PNA FISH assay product insert; AdvanDx, Woburn, MA). Additional testing of these cryptic species from each of these complexes to determine if they can be detected by the Luminex ASRs will be necessary, and clinical studies will established if routine laboratory discrimination of members of these complexes is warranted.

Our second panel was designed to identify molds from culture and directly from respiratory specimens. The sensitivity and specificity of the mold 11-plex assay from culture were excellent for all targets except *A. niger*, *M. indicus*, *R. arrhizus*, and *R. microsporus*. The detection of *A. fumigatus* and *A. flavus* in clinical specimens was not optimal, which could be attributed to low organism burdens in clinical specimens. Increasing the specimen input for extraction might increase the sensitivity of the assay, but this approach also carries the risk of copurifying inhibitory substances. All culture isolates of *A. fumigatus* were detected by the mold 11-plex assay; however, the same limitation described above for *Candida* species applies to the ability of the Luminex ASRs to distinguish among members of the *A. fumigatus* complex, including *A. fumigatus*, *Aspergillus lentulus*, *Aspergillus udagawae*, and *Neosartorya pseudofischeri* (3–5). Although rare, as demonstrated by molecular screening of a large collection of *A. fumigatus* complex isolates (4, 9), the other members of the *A. fumigatus* complex showed differential susceptibility to antifungal drugs (4, 29), suggesting that there could possibly be a clinical benefit in developing an assay that could routinely identify them.

Sequencing of the 18S rRNA gene revealed that a few *A. niger* strains isolated in the laboratory could actually represent strains of *A. tubingensis*, a black mold belonging to the *Aspergillus* species of the *Nigri* group (1). Phenotypically indistinguishable from each other, *A. niger* and *A. tubingensis* can be differentiated only by molecular methods, including sequencing of the β-tubulin gene, the calmodulin gene, or the internal transcribed spacers of rDNA units (1, 11). In this study, only sequencing of the 18S rRNA and not that of the D1/D2 domain was able to distinguish some of the *A. niger* strains from *A. tubingensis*. Since these two species are not routinely distinguished, the clinical significance and necessity of identifying them to the species level remains to be determined. The results of our study suggest that the sequences targeted by the *A. niger* ASR might be too specific and that it would be more beneficial for mycology laboratories to be able to detect all members of the *Aspergillus* species of the *Nigri* group. Of note, the one clinical specimen that was positive by culture for *A. niger* and negative by the mold 11-plex assay was detected as a low positive signal when tested using only the *A. niger* primers and beads in a singleplex assay (data not shown). This suggests that

some interference might be occurring in the multiplex reaction, warranting further studies to determine the impact of multiplexing on sensitivity for each of the analytes.

The performance of the mold 11-plex assay was more difficult to assess for the Mucormycetes (formerly Zygomycetes). In our laboratory, Mucormycetes are usually identified only to the genus level (i.e., *Mucor* species, *Rhizopus* species), except for *C. bertholletiae.* The Luminex ASRs were designed to specifically detect *M. indicus*, *R. arrhizus*, and *R. microsporus*. Because of their highly conserved sequences, the development of molecular methods for the identification of various members of the Mucormycetes can be difficult, and attempting to identify specific species might be even more challenging. As suggested by other previously published studies, a better approach would be to focus on detecting the most common genera isolated in most clinical laboratories, including *Lichtheimia* (formerly *Absidia*) spp., *Mucor* spp., *Rhizopus* spp., and *Rhizomucor* spp. (12, 15, 22). Furthermore, due to the lack of clinical studies, the choice of therapy in the treatment of mucormycosis is based mostly on experience and generally consists of the use of liposomal amphotericin B or, more recently, posaconazole without special regard for the specific genus (i.e., *Mucor* spp. versus *Rhizopus* spp.) (25).

In conclusion, the Luminex fungal ASRs represent one of the first commercial attempts to produce reagents for the detection of several yeasts and molds of clinical importance. The availability of such reagents would facilitate the standardization of molecular assays for the diagnosis of invasive fungal infections. Furthermore, implementation of laboratory-developed tests using these ASRs will shorten the time to detection from several days or weeks to as little as 24 h (depending on laboratories' staffing capacities), and this molecular technology could be used as an adjunct to culture.

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