Broad-Range Direct Detection and Identification of Fungi by Use of the PLEX-ID PCR-Electrospray Ionization Mass Spectrometry (ESI-MS) System

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The PLEX-ID system is a novel technology that couples PCR amplification and electrospray ionization-mass spectrometry to identify pathogens directly in clinical specimens. The analytical performance of the PLEX-ID Broad Fungal assay was compared with that of traditional culture identification by using 91 characterized fungal culture isolates (64 manufacturer-claimed and 27 nonclaimed organisms) and directly by using 395 respiratory specimens. Discordant results were resolved by D2 large-subunit ribosomal DNA fungal sequencing. Environmental studies were performed to monitor for potential contamination. The PLEX-ID Broad Fungal assay correctly identified 95.6% (87/91) and 81.3% (74/91) of the culture isolates to the genus and species levels, respectively. Of the manufacturer-claimed organisms, 100% (64/64) and 92.2% (59/64) were correctly identified to the genus and species levels, respectively. Direct analysis of respiratory specimens resulted in 67.6% (267/395) and 66.6% (263/395) agreement with culture results to the genus and species levels, respectively, with 16.2% (64/395) of the results discordant with culture and 16.2% (64/395) not detected by the system. The majority (>95%) of the isolates not detected directly by the PLEX-ID system ultimately grew in low quantities in culture (≤ 20 colonies). In 20.3% (35/172) of the respiratory specimens where no growth was observed in culture, the PLEX-ID system identified a fungus, suggesting a potential increase in sensitivity over culture in some instances. The PLEX-ID system provides a rapid method for the detection of a broad array of fungi directly in respiratory specimens and has the potential of impacting turnaround times and patient care by reducing the need to wait for the growth of an organism in culture.

Invasive fungal infections (IFIs) have increased in parallel with the escalating number of immunosuppressed patients seen in health care facilities over the past several decades. IFIs are a major cause of morbidity and mortality in high-risk populations, including those individuals who are at the extremes of age, who are undergoing solid organ or hematopoietic stem cell transplantation, who are undergoing prescribed immunosuppressive therapy, or who have an immunosuppressive disease (e.g., AIDS) (1). The most common fungal causes of IFIs are *Candida* species, *Aspergillus* species, and *Cryptococcus* species. However, the spectrum of fungal agents causing IFIs has broadened to include more atypical agents, including opportunistic yeasts (e.g., *Trichosporon* species and *Rhodotorula* species), the mucormycetes (e.g., *Lichtheimia*, *Rhizopus*, and *Mucor* species), the hyalohyphomycetes (e.g., *Fusarium* and *Scedosporium* species), the phaeohyphomycetes (e.g., *Alternaria*, *Scedosporium*, and *Bipolaris* species), and the causes of endemic mycoses (e.g., *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis*/*C. posadasii*) (1, 2). Early diagnosis and prompt therapy of IFIs are instrumental in the successful treatment of immunosuppressed populations, but conventional methods for the diagnosis of fungal disease, such as culture, histology, and serology, are time-consuming and often lack sensitivity (3).

Recently, molecular methods such as real-time PCR and peptide nucleic acid fluorescent hybridization probes have become important tools for the detection of fungal pathogens in the clinical microbiology laboratory (4–7). Although these methods can significantly reduce the turnaround time (TAT) for results, they require the clinician to make an educated guess as to the potential cause of infection since they often target only a single pathogen or a small group of related pathogens (8).

A novel technology, the PLEX-ID system, uses broad-range PCR amplification coupled with electrospray ionization-mass spectrometry (ESI-MS) for the direct detection of pathogens without the need to wait for growth in culture. The Broad Fungal assay kit for the PLEX-ID system allows the identification and semi-quantitation of many clinically significant fungal agents in a single assay within 6 h (9, 10). This system measures the mass-to-charge ratio of PCR amplicons generated from several different target loci, focusing on both conserved and species-specific regions, to identify base compositions that can then be compared to a database of fungal signatures. By using base composition as a unique molecular signature (“fingerprint”), the PLEX-ID system is able to identify single or multiple organisms at trace levels in a variety of specimen sources.

The purpose of this study was to evaluate the performance of the PLEX-ID Broad Fungal assay for the detection and identification of fungi in previously characterized culture isolates and directly in respiratory specimens without the need to wait for growth in culture.

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FIG 1 Workflow for the processing of respiratory specimens with the PLEX-ID system. The time required to process six samples (one Broad Fungal assay plate) on the PLEX-ID system from start to finish is approximately 6 h, with approximately 1 h of hands-on time. Following a strict unidirectional workflow, the overall TAT to reporting of results was 24 h, as the plates were placed on the PLEX-ID system at the end of the work day and the data were analyzed the following morning.

MATERIALS AND METHODS

Samples. The analytical performance of the PLEX-ID Broad Fungal assay was compared to that of traditional culture identification by testing (i) 91 previously characterized fungal culture isolates and (ii) 395 respiratory specimens from patients suspected of having a fungal infection and for which routine mycology testing had been requested.

(i) Fungal culture isolates. Ninety-one previously characterized culture isolates were analyzed, including 64 manufacturer-claimed organisms and 27 nonclaimed organisms that were added to assess assay specificity. The isolates included type strains (e.g., ATCC) and culture isolates that were identified by routine phenotypic methods, with the phenotypic identification confirmed by D2 large-subunit ribosomal DNA (rDNA) fungal sequencing as previously described (11). The isolates were freshly subcultured onto inhibitory mold agar (IMA; BBL, Sparks, MD) and processed by placing a 1- μ l loopful of the organism into a 2.0-ml tube containing 500 μ l of sterilized water, 0.1-mm silica glass beads, and 2.4-mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK). The tubes were heated at 95°C for 10 min and then placed on a Disruptor Genie (Scientific Industries, Bohemia, NY) for 2 min to mechanically lyse the organisms and release the nucleic acid. The nucleic acid was extracted and eluted into 200 μ l of elution buffer on the MagNA Pure instrument (Roche Diagnostics, Indianapolis, IN) by using the total nucleic acid isolation kit (Roche Diagnostics, Indianapolis, IN). Prior to analysis, the concentration of double-stranded DNA (dsDNA) was determined by using the Quant-iT PicoGreen dsDNA kit (Life Technologies, Carlsbad, CA) on the LightCycler 2.0 instrument (Roche Diagnostics, Indianapolis, IN) and diluted to 100 genomes/ μ l with sterile distilled water. If a discordance between the phenotypic/sequencing and PCR-ESI-MS system results was identified, the PLEX-ID testing was repeated in duplicate.

(ii) Respiratory specimens. From October to December 2011, 395 respiratory specimens with sufficient excess volume (>1 ml) remaining after all other testing was completed were analyzed by using the PLEX-ID Broad Fungal assay. Specimen types included bronchoalveolar lavage (BAL) fluid samples ($n = 186$), bronchial wash samples ($n = 112$), sputum samples ($n = 51$), and tracheal secretion samples ($n = 46$). Figure 1 summarizes the workflow required for the processing of respiratory specimens with the PLEX-ID system.

Genomic extraction. Genomic extraction of the respiratory specimen was performed as recommended by Abbott Molecular (Des Plaines, IL). Mechanical lysis was performed by adding 1 ml of respiratory specimen, 25 μ l of proteinase K (Abbott Molecular, Des Plaines, IL), 250 μ l of 10% bovine serum albumin (Abbott Molecular, Des Plaines, IL), and 142 μ l of 20% SDS/EC (Abbott Molecular, Des Plaines, IL) reagent to a process tube with beads (Abbott Molecular, Des Plaines, IL). In order to perform mechanical lysis, the tube was placed on a Disruptor Genie (Scientific Industries, Bohemia, NY) for 90 s in triplicate to prevent excessive heating. Lysis was followed by protease digestion at 56°C for 15 min (Abbott

Molecular, Des Plaines, IL). The lysed samples were placed on the PLEX-ID SP KingFisher extractor (Abbott Molecular, Des Plaines, IL), and nucleic acid was extracted by using magnetic bead technology and placed into 280 μ l of elution buffer.

PCR-ESI-MS analysis. PCRs were assembled by adding 10 μ l of extracted nucleic acid to each well of the 96-well microtiter Broad Fungal assay plate by using the Corbett Robotics CAS 1200 (QUIagility, Qiagen, Valencia, CA) fluid handling robotic platform. The Broad Fungal assay targets and primers have been previously described (12). The plates were sealed with PLEX-ID PCR seals (Abbott Molecular, Des Plaines, IL) and loaded onto an Eppendorf Mastercycler ProS Thermal Cycler (Eppendorf, Hamburg, Germany). The PCR was carried out by using the manufacturer's recommended thermocycler protocol of 95°C for 10 min, followed by eight cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s. This reaction was followed by 37 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 20 s, followed by 72°C for 2 min and 99°C for 20 min.

Sample analysis. Sample analysis was performed following PCR amplification by manually loading the plate onto the PLEX-ID system where the PCR product was desalted and analyzed by using mass spectrometry (Abbott Molecular, Des Plaines, IL). The base count of each amplicon was determined from each primer pair, which was used to identify the fungi present with database version S1.4.1.0-A6.390.426.330.1-IVD01_6.26.27.21.1. The PLEX-ID Broad Fungal assay utilizes signal thresholds (cutoffs) designed to limit the reporting of irreproducible detections. Cutoffs are applied to two measurements. The first, termed the "level," is an indication of the amount of the amplicon present in the sample, reported as the number of genome equivalents (GE)/well. This is calculated with reference to the internal calibrant and has been described previously (13). The linear range for the reporting of these levels is between $0.1 \times$ and $10 \times$ the levels of the internal controls in the assay, which in the case of the Broad Fungal assay represents a working range of ~ 2 to 200 GE/well. The second is the quality (Q) score, which represents a relative measure of the strength of the data supporting identification. The Q score is a rating between 0 (low) and 1 (high) based on parameters including an indicator of how well the hypothesized organisms, as a group, represent the observed data, an indicator of how significant the contribution of a single organism is to the solution; the fraction of missed detections, which represents the percentage of primers for a detected organism that should have produced known base count compositions but did not; and finally, the percentage of primers for a detected organism for which no known data exist within the PLEX-ID system. Q score cutoffs are designed to prevent the reporting (positive identification) of specific organisms when the information obtained is not sufficient to confidently resolve the organism's identity. For the PLEX-ID Broad Fungal assay, a Q score of ≥ 0.85 is considered a reportable result. Any result defined as a multiple match is a single detection with multiple database matches associated with a single Q score and level. Any result defined as multiple organisms is the detection of multiple organisms with the association of a Q score and level with each organism identified.

Precision studies. Three respiratory specimens each with a volume of >10 ml were used for precision studies. The analysis, including extraction, was repeated 10 times for each specimen by using three different Broad Fungal assay plates on 3 different days to assess the repeatability and reproducibility of the assay. The mean, standard deviation (SD), and percent coefficient of variation (CV) were calculated for both the Q score and the level.

Identification of yeast isolates from nonsterile respiratory sources. Common mycology laboratory practice for yeast isolated from nonsterile respiratory sources is to identify them phenotypically from culture as "Yeast not *Cryptococcus* sp." (14, 15). Thus, there was no genus and/or species identification available to use for comparison with the PLEX-ID results for those specimens. In an effort to provide a full identification for comparison with the PLEX-ID results, any residual specimen remaining after PLEX-ID analysis was cultured on an IMA plate and any yeast that grew was identified by an alternate method of matrix-assisted laser de-

sorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis that has previously been validated in our laboratory (16, 17).

Environmental testing. Environmental testing was performed routinely during this study to assess the potential for amplicon contamination because the PLEX-ID system is an open amplification system with multiple processing steps (outlined in Fig. 1). Environmental testing was performed upon the installation of the PLEX-ID system, after the installation of an exhaust adaptor from the PLEX-ID pump to the exterior building ventilation, and on 4 different days when the PLEX-ID system was in use. Monitoring was conducted by swabbing the environment at 10 different sites (e.g., the biosafety cabinet and benchtop surface where samples were processed, the PLEX-ID SP KingFisher extractor, the Eppendorf Mastercycler ProS Thermal Cycler, and the PLEX-ID instrument), including 4 sites in the specimen-processing area (designated sites 1 to 4), 2 sites in the thermocycling room (sites 5 and 6), and 4 sites in the PLEX-ID analysis room (sites 7 to 10). Sterile 2-ml screw-cap tubes were filled with 700 μ l of sterile water. At each site, the swab was dipped in the sterile water, an area of 10 cm² was sampled, and then the swab was twirled in the water inside the screw-cap tube. Two negative controls (water only) were included for each set of environmental samples taken. Ten microliters of each environmental sample was amplified on the Broad Fungal assay plate and analyzed on the PLEX-ID system.

Biologic safety precautions. As some of the culture isolates and respiratory specimens used in this study contained biosafety level 3 (BSL3) organisms (*Blastomyces dermatitidis*, *Coccidioides immitis*/*Coccidioides posadasii*, *Histoplasma capsulatum*, etc.), appropriate safety precautions were taken. All respiratory specimens were processed by using BSL2 practices prior to inactivation by heating and mechanical lysis. Any BSL3 organism cultures were processed in accordance with BSL3 practices until inactivation.

RESULTS

Fungal culture. The PLEX-ID Broad Fungal assay correctly identified 95.6% (87/91; 95% CI, 89.1 to 98.8%) and 81.3% (74/91; 95% CI, 71.8 to 88.7%) of the culture isolates to the genus and species levels, respectively (Table 1). The remaining 4.4% (4/91; 95% CI, 1.2 to 10.9%) were misidentified by the PLEX-ID system, as the phenotypic identification was confirmed to be correct by D2 large-subunit rDNA fungal sequencing. Of the 91 culture isolates tested, 64 were manufacturer-claimed organisms; of those, 100% (64/64; 95% CI, 94.4 to 100%) and 92.2% (59/64; 95% CI, 82.7 to 97.4%) were correctly identified to the genus and species levels, respectively (Table 1). Of the remaining 27 nonclaimed organisms added to this study to assess assay specificity, 85.2% (23/27; 95% CI, 66.3 to 95.8%) and 55.6% (15/27; 95% CI, 35.3 to 74.5%) of the culture isolates were identified to the genus and species levels, respectively (Table 1). Four of the nonclaimed organisms (14.8%; 95% CI, 4.2 to 33.7%) were misidentified by the PLEX-ID system. One of the four was identified as having No Match in the database.

Respiratory specimens. Of the 395 respiratory specimens tested directly by the PLEX-ID system, 223 (56.5%) had growth present and 172 (43.5%) had no growth observed in fungal culture initiated from the same specimen used for the PLEX-ID analysis. Direct analysis of respiratory specimens resulted in 67.6% (267/395; 95% CI, 62.7 to 72.2%) and 66.6% (263/395; 95% CI, 61.7 to 71.2%) agreement with culture results to the genus and species levels, respectively, with 16.2% (64/395; 95% CI, 12.7 to 20.2%) of the results discordant with culture and 16.2% (64/395; 95% CI, 12.7 to 20.2%) not detected by the PLEX-ID assay (Table 2). The majority (>95%) of the isolates not detected directly by the PLEX-ID system ultimately grew in low quantities in culture ($\leq$ 20 colonies). In 20.3% (35/172) of the respiratory specimens where no growth was observed in culture, the PLEX-ID system identified

a fungal species, indicating a potential increase in sensitivity over culture in some instances. In 73/172 (42.4%) respiratory cultures, multiple organisms grew, with up to four organisms seen in a single culture. The PLEX-ID system detected multiple organisms present in 5/73 specimens (6.8%) and just one of the multiple organisms present in 36/73 (49.3%) cultures (Table 2). The PLEX-ID result was considered correct if it identified at least one of the multiple organisms that grew in culture, as the majority of the time, the most abundant and clinically significant pathogen that grew in culture was detected. Breakdown of the respiratory specimen into individual sources (BAL fluid samples versus bronchial wash samples versus tracheal secretion samples versus sputum samples) revealed a similar picture, with no statistically significant difference between the specimen types. A subset of 18 yeast isolates identified by culture as “Yeast not *Cryptococcus* sp.” from nonsterile respiratory sources were further identified by MALDI-TOF MS and also compared to the PLEX-ID results. One hundred percent (18/18) of those further identified by MALDI-TOF were in agreement with the PLEX-ID results (Table 2). There was one isolate identified as *Candida parapsilosis* by MALDI-TOF MS that was identified as *C. parapsilosis* and *C. albicans* by the PLEX-ID system. Discordances between the culture and PLEX-ID system results for respiratory specimens are listed in Table 3. These disagreements could not be arbitrated by a third method because of the lack of additional specimens. In addition, it was difficult to discern a cutoff level to determine the importance of the discordant results, as the levels of discordant results were variable, ranging from 2 to 304.

Precision studies. Interassay and intra-assay precision was determined by testing three respiratory specimens with a volume of $\geq$ 10 ml, repeated 10 times for each specimen on three different Broad Fungal assay plates on 3 different days. The respiratory specimens were positive for *C. albicans*, *C. parapsilosis*, and *C. albicans* on all occasions, in agreement with culture. For *C. albicans*, the Q score was 0.97 ± 0.02 (mean $\pm$ SD; CV, 2.1%), with a level of 92.7 ± 30.3 (CV, 32.7%); for *C. parapsilosis*, the Q score was 0.98 ± 0.02 (CV, 2.0%), with a level of 32.2 ± 48.9 (CV, 151.9%); and for *C. albicans*, the Q score was 0.97 ± 0.02 (CV, 2.1%), with a level of 138.3 ± 29.9 (CV, 21.6%).

Environmental testing. Environmental testing was performed six times during the study period, and the results are summarized in Table 4. During initial training on the PLEX-ID system, prior to the installation of the exhaust adaptor and the implementation of a strict unidirectional workflow, environmental studies revealed the presence of amplicon contamination with the detection of the internal positive control on all surfaces in the PLEX-ID room. After the installation of the exhaust adaptor and the adoption of a very strict one-way workflow, no amplicon contamination was detected. However, environmental fungi were detected throughout the study at various sites.

DISCUSSION

To our knowledge, this is the first reported study of the PLEX-ID system in which the system was installed in a clinical laboratory with analysis performed solely by the end user. Previous published studies with the PLEX-ID system have used specimens sent to the manufacturer's facility for analysis by the personnel there (12). Thus, this study is the first to assess the performance of the PLEX-ID system in a clinical mycology laboratory. In addition, this is one of the first evaluations of the PLEX-ID Broad Fungal

TABLE 1 Identification of fungal culture isolates with the PLEX-ID Broad Fungal assay

		PLEX-ID Broad Fungal assay result agreement level (Q score ^a ≥ 0.85)			
Phenotypic identification	No. of isolates tested	Genus	Species	Misidentification	Discordant identification
Manufacturer-claimed organisms					
<i>Aspergillus flavus</i>	3	1	2		<i>Aspergillus sclerotiorum</i>
<i>Aspergillus fumigatus</i>	3		3		
<i>Aspergillus nidulans</i>	1		1		
<i>Aspergillus niger</i>	3		3		
<i>Aspergillus terreus</i>	2		2		
<i>Bipolaris hawaiiensis</i>	3		3		
<i>Blastomyces dermatitidis</i>	3		3		
<i>Candida albicans</i>	3		3		
<i>Candida dubliniensis</i>	3		3		
<i>Candida glabrata</i>	3		3		
<i>Candida guilliermondii</i>	1		1		
<i>Candida krusei</i>	3		3		
<i>Candida parapsilosis</i>	3		3		
<i>Candida tropicalis</i>	2		2		
<i>Clavispora lusitaniae</i>	3		3		
<i>Coccidioides immitis</i>	3		3		
<i>Cryptococcus gattii</i>	3		3		
<i>Cryptococcus neoformans</i>	3		3		
<i>Exophiala dermatitidis</i>	3	1	2		<i>Exophiala xenobiotica</i>
<i>Fusarium</i> species	1	1			<i>Fusarium solani</i>
<i>Fusarium oxysporum</i>	3		3		
<i>Fusarium solani</i>	1	1			<i>Fusarium proliferatum</i> / <i>Fusarium oxysporum</i>
<i>Histoplasma capsulatum</i>	3		3		
<i>Penicillium marneffei</i>	1		1		
<i>Rhizopus</i> species	1	1			<i>Rhizopus microsporus</i>
<i>Scedosporium apiospermum</i>	3		3		
Total (%)	64	64 (100)	59 (92.2)		
Nonclaimed organisms					
<i>Arthroderma persicolor</i>	1		1		
<i>Aspergillus versicolor</i>	1		1		
<i>Bipolaris spicifera</i>	2	2			<i>Bipolaris hawaiiensis</i>
<i>Candida africana</i>	1		1		
<i>Candida famata</i>	1		1		
<i>Candida inconspicua</i>	1		1		
<i>Candida kefyr</i>	1		1		
<i>Candida metapsilosis</i>	1	1			<i>Candida parapsilosis</i>
<i>Candida sphaerica</i>	1		1		
<i>Cladophialophora bantiana</i>	1		1		
<i>Cryptococcus laurentii</i>	2		2		
<i>Cryptococcus uniguttulatus</i>	1		1		
<i>Curvularia pallescens</i>	1	1			<i>Curvularia affinis</i>
<i>Emmonsia pasteuriana</i>	1			1	<i>Chrysosporium carmichaelii</i>
<i>Fonsecaea pedrosoi</i>	1		1		
<i>Fusarium moniliforme</i>	1	1			<i>Fusarium solani</i>
<i>Geotrichum candidum</i>	1		1		
<i>Paecilomyces variotii</i>	1	1			<i>Paecilomyces formosa</i>
<i>Penicillium</i> species	1	1			<i>Penicillium</i> species
<i>Rhizomucor</i> species	1	1			<i>Rhizomucor pusillus</i>
<i>Sepedonium chrysospermum</i>	1			1	<i>Fusarium proliferatum</i> / <i>Fusarium oxysporum</i>
<i>Scedosporium prolificans</i>	1		1		
<i>Sporothrix schenckii</i>	1		1		
<i>Trichosporon mucoides</i>	1			1	No match
<i>Trichophyton mentagrophytes</i>	1			1	<i>Trichophyton tonsurans</i>
Total (%)	27	23 (85.2)	15 (55.6)	4 (14.8)	
All organisms (total = 91) (%)		87 (95.6)	74 (81.3)	4 (4.4)	

^a The Q score is the level of confidence associated with the results. Any Q score of ≥0.85 was considered significant on the basis of the manufacturer's cutoff.

TABLE 2 Direct fungal identification with the PLEX-ID Broad Fungal assay from respiratory specimens compared to culture results

Fungal culture result (no. of isolates)	Avg time (days) to growth in culture	No. (%) of PLEX-ID Broad Fungal assay results		
		Agreement ^a	Discordance	Not detected
No growth (172)	NA ^d	137 (79.7)	35 (20.3)	NA
Yeast, not <i>Cryptococcus</i> sp. (82)	2–5	62 (75.6)	5 (6.1)	15 (18.3)
Subset ^e				
<i>C. albicans</i> (11)	2–3	11 (100)	0	0
<i>C. glabrata</i> (5)	2–3	5 (100)	0	0
<i>C. parapsilosis</i> (1)	3	1 (100) ^b	0	0
<i>C. tropicalis</i> (1)	3	1 (100)	0	0
Multiple organisms (73)	3–21	41 (56.2) ^c	12 (16.4)	20 (27.4)
<i>Candida</i> species (38)	2–5	17 (44.7)	4 (10.6)	17 (44.7)
<i>Penicillium</i> species (11)	2–5	0	5 (45.5)	6 (54.5)
<i>Aspergillus</i> species (8)	2–5	6 (75.0)	1 (12.5)	1 (12.5)
<i>Histoplasma capsulatum</i> (6)	15–21	4 (66.7)	0	2 (33.3)
Other (5)	5	0	2 (40.0)	3 (60.0)
Total (395)		267 (67.6)	64 (16.2)	64 (16.2)

^a Agreement to the genus level; 66.6% (263/395) were in agreement with culture results to the species level.

^b From a single specimen, the MALDI-TOF MS identified *C. parapsilosis* whereas the PLEX-ID identified both *C. parapsilosis* and *C. albicans*.

^c Identification of at least one of the multiple organisms detected by culture was considered agreement.

^d NA, not applicable.

^e A subset of the isolates in the “Yeast, not *Cryptococcus* sp.” category ($n = 18$) were further identified by MALDI-TOF MS as indicated.

assay kit that have used the PLEX-ID platform with well-characterized culture isolates and directly with respiratory specimens.

The Broad Fungal Database was developed by the manufacturer by analyzing more than 500 reference culture isolates (manufacturer-claimed isolates) and was further extended by an *in silico* survey of existing fungal entries in the GenBank *nr* database (nonclaimed organisms) that were not verified by the manufacturer, to create a more robust database. Culture isolates were included in this study to assess the specificity of the assay for both manufacturer-claimed and nonclaimed organisms. Overall, the PLEX-ID Broad Fungal assay correctly identified 95.6% (87/91; 95% CI, 89.1 to 98.8%) and 81.3% (74/91; 95% CI, 71.8 to 88.7%) of the culture isolates to the genus and species levels, respectively. Not surprisingly, the PLEX-ID system performed well in the identification of manufacturer-claimed organisms, with 100% (64/64; 95% CI, 94.4 to 100%) and 92.2% (59/64; 95% CI, 82.7 to 97.4%) agreement to the genus and species levels, respectively. However, the nonclaimed organisms added to assess assay specificity had lower levels of agreement of 85.2% (23/27; 95% CI, 66.3 to 95.8%) and 55.6% (15/27; 95% CI, 35.3 to 74.5%) at the genus and species levels, respectively. It was concerning that 11.1% of the nonclaimed organisms in this study were misidentified by the PLEX-ID system with measurable Q scores. The data were originally analyzed with an earlier version of the database (S1.4.0.1-A6.346.390.305.1-IVD01_6.21.22.16.1), and this resulted in some significant misidentifications of both manufacturer-claimed and nonclaimed organisms. These original misidentifications by the PLEX-ID system (e.g., *Cryptococcus gattii* was identified as *Coccidioides immitis*/*Coccidioides posadasii*, *Candida famata* was identified as *Penicillium marneffeii*, and *Emmonsia pasteuriana* was identified as *Histoplasma capsulatum*) had the potential to negatively impact patient care. During this study, a database upgrade was performed by the manufacturer of the PLEX-ID system to address the misidentifications noted with the earlier database. Following

the database upgrade (now database S1.4.1.0-A6.390.426.330.1-IVD01_6.26.27.21.1), we reanalyzed our spectral files and the identification of fungi from culture isolates improved, reflecting our present results. The updated database was able to correctly identify those culture isolates with significant misidentifications as described above. Although the major errors were corrected with the updated database, these types of errors could still be possible with other organisms not included in the present study and vigilance must be used when interpreting identifications of nonverified organisms. Thus, it appears that although the PLEX-ID system had substantial levels of agreement with culture for the identification of fungi, a more robust database with additional entries and diversification may enhance fungal identification.

To date, there is only one other published study that has compared characterized fungal culture isolates by using the PCR-ESI-MS technology. That study, by Gu et al., used the BAC assay, which identifies bacteria, antibiotic resistance markers, and *Candida* species, to identify characterized *Candida* species isolates. The study compared results from the Ibis T5000 instrument (the precursor of the PLEX-ID system) to repetitive sequence PCR and the reference standard, sequencing of the internal transcribed spacer rRNA gene. In that study, the T5000 instrument was similar to repetitive sequence PCR, identifying 51 of the 61 *Candida* species isolates (83.6%) to the species level (18). In our study, the PLEX-ID Broad Fungal assay correctly identified all manufacturer-claimed *Candida* isolates to the species level (18/18) and 5 (83.3%) of 6 nonclaimed *Candida* species to the species level. Although the results of the studies are difficult to compare because of differences between the kits (BAC versus Broad Fungal) and the platforms (T5000 versus PLEX-ID) used, they both demonstrate good performance of the technology in identifying the claimed *Candida* species.

Direct analysis of respiratory specimens resulted in 67.6% (267/395; 95% CI, 62.7 to 72.2%) and 66.6% (263/395; 95% CI,

TABLE 3 Direct respiratory specimen discordant culture and PLEX-ID results

Culture ID	PLEX-ID result with Q score ^a of ≥ 0.85 (no. of isolates)	Level ^b (range)
No growth (35)	<i>Cladosporium cladosporioides</i> (6)	2–199
	<i>Candida glabrata</i> (5)	2–134
	<i>Pneumocystis jirovecii</i> (4)	29–170
	<i>Candida albicans</i> (4)	1–166
	Multiple matches (1) (<i>Stephanonectria keithii</i> , <i>Corollospora maritima</i>)	1
	Multiple matches (1) (<i>Cercostigmina punctata</i> , <i>Pseudocercospora ocimicola</i>)	3
	Multiple matches (1) (<i>Candida tropicalis</i> , <i>Saccharomyces cerevisiae</i>)	97
	Multiple matches (1) (<i>Fusarium oxysporum</i> , <i>Trichoderma reesei</i>)	2
	<i>Cryptococcus luteolus</i> (2)	1–3
	<i>Spiromastix warcupii</i> (2)	2
	<i>Aspergillus wentii</i> (1)	7
	<i>Botryotinia fuckeliana</i> (1)	1
	<i>Neosartorya fischeri</i> (1)	2
	<i>Penicillium freii</i> (1)	304
	<i>Pichia jadinii</i> (1)	79
	<i>Aspergillus aculeatus</i> (1)	1
	<i>Aspergillus penicillioides</i> (1)	2
	<i>Coccidioides immitis</i> / <i>Coccidioides posadasii</i> (1)	1
Multiple organisms (12)		
Yeast, not <i>Cryptococcus</i> species, <i>A. fumigatus</i> , <i>Penicillium</i> species	Multiple matches (<i>Gibberella moniliformis</i> , <i>Fusarium oxysporum</i> , <i>Gibberella zeae</i>)	143
<i>C. albicans</i> , <i>Cladosporium</i> species	Multiple matches (<i>Stylodothis puccinioides</i> , <i>Dothidea sambuci</i>)	3
<i>C. albicans</i> , <i>Cryptococcus neoformans</i>	<i>Candida dubliniensis</i>	3
<i>C. albicans</i> , <i>A. fumigatus</i> , basidiomycete	<i>Aspergillus nidulans</i>	19
<i>Bispora</i> species, <i>Penicillium</i> species	<i>Alternaria alternata</i>	11
Yeast, <i>Aspergillus terreus</i>	<i>Aspergillus nidulans</i>	34
<i>C. albicans</i> , <i>C. glabrata</i> , <i>C. tropicalis</i>	<i>Cladosporium cladosporioides</i>	167
<i>A. terreus</i> , <i>A. niger</i> , <i>Paecilomyces</i> species	<i>Saccharomyces cerevisiae</i>	4
<i>Penicillium</i> species, <i>Aspergillus</i> species, <i>A. versicolor</i>	<i>Physconia muscigena</i>	26
<i>A. niger</i> , <i>C. dubliniensis</i> , <i>Penicillium</i> species	<i>Malassezia pachydermatis</i>	23
<i>Penicillium</i> species, <i>A. versicolor</i>	<i>Mucor hiemalis</i>	2
<i>Bispora</i> species, <i>Penicillium</i> species	<i>Alternaria alternata</i>	11
<i>Penicillium</i> species (5)	<i>Pneumocystis jirovecii</i>	136
	<i>Saccharomyces cerevisiae</i> (2)	2–5
	<i>Physconia muscigena</i>	26
	<i>Nectria haematococca</i>	64
Yeast, not <i>Cryptococcus</i> species (5)	Multiple matches (<i>Neosartorya fischeri</i> , <i>Aspergillus flavus</i> , <i>Aspergillus oryzae</i>)	71
	<i>Hamigera avellanea</i>	4
	<i>Pneumocystis jirovecii</i>	152
	<i>Aspergillus fumigatus</i>	45
	<i>Coccidioides immitis</i>	2
<i>Candida</i> species (4)	Multiple matches (1) (<i>Gibberella moniliformis</i> , <i>Fusarium oxysporum</i> , <i>Gibberella zeae</i>)	136
	Multiple matches (1) (<i>Pichia stipitis</i> , <i>Meyerozyma guilliermondii</i>)	102
	<i>Cladosporium cladosporioides</i>	7
	<i>Flavoparmelia caperata</i>	2
<i>Aspergillus versicolor</i> (1)	<i>Coccidioides immitis</i> / <i>Coccidioides posadasii</i>	3
<i>Paecilomyces</i> species (1):	<i>Malassezia pachydermatis</i>	184
<i>Cryptococcus neoformans</i> (1)	<i>Candida albicans</i>	2

^a The Q score is the level of confidence associated with the results. Any Q score of ≥ 0.85 was considered significant on the basis of the manufacturer's cutoff.

^b The level is a semiquantitative estimate of the concentration of the target present in comparison to the known concentration of the internal calibrant in each well.

TABLE 4 Summary of environmental testing results from the PLEX-ID fungal study

Time period	Organism found (site[s])		
	Specimen processing area ^a	Thermocycling room ^b	PLEX-ID room ^c
During training	<i>Boeremia exigua</i> (1), <i>Cladosporium cladosporioides</i> (2, 3)	<i>Cercostigmina punctata</i> (5), <i>Pseudocercospora ocimicola</i> (5), <i>Malassezia pachydermatis</i> (6)	IPC ^d (7–10), <i>Cladosporium cladosporioides</i> (9), <i>Malassezia pachydermatis</i> (9)
Postadaptor ^f times:			
Wk 1	<i>Candida tropicalis</i> (1)	None	NT ^e
Wk 2	<i>Candida</i> species (4)	None	NT
Wk 4	<i>Cladosporium cladosporioides</i> (2), <i>Cryptococcus luteolus</i> (3)	None	NT
Wk 9	None	None	None
Wk 25	None	<i>Cercostigmina punctata</i> (5)	None

^a Sites 1 to 4.^b Sites 5 and 6.^c Sites 7 to 10.^d IPC, internal positive control.^e NT, not tested.^f After installation of exhaust adaptor and implementation of strict unidirectional workflow.

61.7 to 71.2%) agreement with culture results to the genus and species levels, respectively, with 16.2% (64/395; 95% CI, 12.7 to 20.2%) of the results discordant with culture and 16.2% (64/395; 95% CI, 12.7 to 20.2%) not detected by the PLEX-ID assay. Overall, the TAT was reduced from 2 to 20 days for growth in culture to a maximum of 1 day by using the PLEX-ID system. The majority (>95%) of the isolates not detected directly by the PLEX-ID system ultimately grew in low quantities in culture (≤ 20 colonies). Even though the PLEX-ID system did not detect 16.2% of the fungi observed in culture, this technology is likely not to replace culturing of fungi but rather to serve as an adjunct test that provides rapid identification of fungi with a TAT of days to weeks prior to their growth in culture. In 20.3% (35/172) of the respiratory specimens where no growth was observed in culture, the PLEX-ID system identified a fungal species, indicating a potential increase in sensitivity over culture in some instances. Among those, the nonculturable fungus *Pneumocystis jirovecii* was identified in four specimens by the PLEX-ID system. This assay provides the advantage of being able to pick up nonculturable organisms such as *P. jirovecii*. An additional concerning discordance between culture and the PLEX-ID system was the identification of *Coccidioides immitis*/*Coccidioides posadasii* in two separate incidences by the PLEX-ID system that was not identified by culture. Unfortunately, there was no method available to arbitrate this discrepancy because of the lack of a residual specimen. In addition, further review of the patients' clinical histories was unrevealing. Although the PLEX-ID system potentially demonstrates increased sensitivity over culture, it is difficult to predict the clinical significance of these results, as some of the isolates (e.g., *Cladosporium cladosporioides*, *Cercostigmina punctata*, *Pseudocercospora ocimicola*, etc.) may be considered environmental saprobes possibly representing contaminants and could also represent nonviable DNA. Likewise, it is probable to hypothesize that these organisms are present at low levels as colonizers or transient contaminants in the body without causing disease and therefore their detection by the PLEX-ID system may accurately reflect their presence in the specimen.

Our results are comparable to those of a study performed by Shin et al. that examined 691 nonduplicate BAL fluid specimens by using the Broad Fungal assay on the PLEX-ID platform (12).

Their study revealed 81.3% and 62.7% agreement to the genus and species levels, respectively. These results are slightly better than our BAL fluid results, which identified 65.1% to the genus level and 63.4% to the species level, respectively. In contrast, our study rarely had multiple detections and never had any identifications as “Fungus detected—no ID can be provided” on the PLEX-ID system, which were both common in the study by Shin et al. Differences in results may be due to the use of different databases, different BAL fluid collection procedures, and the collection of isolates from a single urban hospital versus a reference laboratory.

As the PLEX-ID system is an open platform, there were initial concerns about amplicon contamination issues and so environmental studies were performed (9). These studies were performed prior to and after the installation of an exhaust adaptor from the PLEX-ID pump to the exterior ventilation. Prior to installation of the exhaust adaptor, amplicon contamination did occur in the immediate environment surrounding the PLEX-ID system. A very strict unidirectional workflow was implemented, and plates were loaded onto the PLEX-ID system only at the end of the work day, with the technologist not returning to other areas of the laboratory after entering the PLEX-ID room. After installation of the exhaust adaptor and ventilation from the pump to the exterior was established, no amplicon contamination was detected during environmental sampling. Thus, although contamination is a concern with the PLEX-ID system, as it is with any other molecular amplification assay, proper safeguards may be put in place to greatly decrease the risk of contamination.

The PLEX-ID system provides clinical laboratories with the ability to perform broad-range, direct identifications of fungi without the need to grow organisms in culture first and without the need to have *a priori* knowledge of what the organisms might be, as is the case with many PCR assays. This is a tremendous advancement in mycology, but it comes with a significant cost, with the PLEX-ID system priced at in excess of \$500,000, not including the associated equipment and service contract fees. A cost analysis conducted in our laboratory estimated that the cost for the analysis of a single sample on the PLEX-ID system (excluding overhead and full-time employee costs) would be about \$160.00 (data not presented). This is higher than the estimated value of the kits of \$50 to \$100/sample quoted by Wolk et al., as

our estimated value included maintenance reagents (cleanup reagent kits, the Analyzer Check Plate system for quality control, etc.), kit reagents and consumables (process tubes, extraction consumable, Broad Fungal assay plates, etc.), and extras (tips, ethanol, etc.) (9). The significant advantage of the PLEX-ID system is that it can be adapted to varied applications (bacteria, mycobacteria, fungi, parasites, and viruses), and the ability to perform a broad-range analysis of a variety of organism types without the need for culture or prior knowledge of the target may make the PLEX-ID system a more financially feasible option than it appears at first glance, especially among reference or public health laboratories. At this point, the PLEX-ID system is not an FDA-approved platform and is classified as not for diagnostic use (19).

Implementation of the Broad Fungal assay in the clinical microbiology laboratory requires several considerations, including utility, workflow, and quality control. The Broad Fungal assay may not be practical to use for all patients or for all specimen types because of the associated labor and costs. However, for patients who are severely immunocompromised (e.g., hematology-oncology patients, transplant patients, patients with traumatic wounds), the ability of the PLEX-ID system to rapidly identify a broad range of fungal organisms well before growth in culture provides the clinician with a rapid diagnosis and the ability to initiate the appropriate targeted antifungal therapy as soon as possible. Other potential niches would include patients whose specimens are culture negative despite a strong suspicion of a fungal infection or patients for whom cultures were not ordered. Although it has a shorter TAT than culture, culture will still be necessary if antifungal susceptibility determination is required. The costs of a PLEX-ID-based rapid identification will likely be insignificant compared with the associated downstream costs of hospitalization of a patient in whom a causative agent cannot be identified.

The workflow for the Broad Fungal assay requires approximately 6 h for the processing of six samples (one Broad Fungal assay plate), of which approximately 1 h is hands-on time. Thus, the workflow can be incorporated into the molecular component of a clinical microbiology laboratory with a core set of personnel experienced in molecular testing.

A limitation of our study included the absence of a method to arbitrate the discrepancies between culture and the PLEX-ID system for the respiratory specimen.

Overall, the PLEX-ID system provides a rapid and sensitive method for the detection of a broad array of fungi directly in respiratory specimens and has the potential of positively impacting TAT and patient care by reducing the need to wait for the growth of an organism in culture. The algorithm for identification appears scientifically sound, providing valuable and accurate results. Planned future improvements of the database and the instrument architecture should enhance its utility in the clinical laboratory.

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REFERENCES

1. Azie N, Neofytos D, Pfaller M, Meier-Kriesche HU, Quan SP, Horn D. 2012. The PATH (Prospective Antifungal Therapy) Alliance® registry and

- invasive fungal infections: update 2012. *Diagn. Microbiol. Infect. Dis.* 73: 293–300.
2. Pfaller MA, Pappas PG, Wingard JR. 2006. Invasive fungal pathogens: current epidemiological trends. *Clin. Infect. Dis.* 43(Suppl):S3–S14.
3. Alexander BD, Pfaller MA. 2006. Contemporary tools for the diagnosis and management of invasive mycoses. *Clin. Infect. Dis.* 43(Suppl):S15–S27.
4. Hall L, Le Febvre KM, Deml SM, Wohlfiel SL, Wengenack NL. 2012. Evaluation of the Yeast Traffic Light PNA FISH probes for identification of *Candida* species from positive blood cultures. *J. Clin. Microbiol.* 50:1446–1448.
5. Farina C, Perin S, Andreoni S, Conte M, Fazio P, Lombardi G, Manso E, Morazzoni C, Sanna S, Medical Mycology Committee (CoSM) 'Fungemia Diagnosis Study Group' Italian Society of Clinical Microbiology (AMCLI). 2012. Evaluation of the peptide nucleic acid fluorescence *in situ* hybridisation technology for yeast identification directly from positive blood cultures: an Italian experience. *Mycoses* 55:388–392.
6. Babady NE, Buckwalter SP, Hall L, Le Febvre KM, Binnicker MJ, Wengenack NL. 2011. Detection of *Blastomyces dermatitidis* and *Histoplasma capsulatum* from culture isolates and clinical specimens by use of real-time PCR. *J. Clin. Microbiol.* 49:3204–3208.
7. Hata DJ, Buckwalter SP, Pritt BS, Roberts GD, Wengenack NL. 2008. Real-time PCR method for detection of zygomycetes. *J. Clin. Microbiol.* 46:2353–2358.
8. Kourkoupetis TK, Fuchs BB, Coleman JJ, Desalermos A, Mylonakis E. 2012. Polymerase chain reaction-based assays for the diagnosis of invasive fungal infections. *Clin. Infect. Dis.* 54:1322–1331.
9. Wolk DM, Kaleta EJ, Wysocki VH. 2012. PCR-electrospray ionization mass spectrometry: the potential to change infectious disease diagnostics in clinical and public health laboratories. *J. Mol. Diagn.* 14:295–304.
10. Ecker DJ, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, Hofstadler SA. 2008. Ibis T5000: a universal biosensor approach for microbiology. *Nat. Rev. Microbiol.* 6:553–558.
11. Hall L, Wohlfiel S, Roberts GD. 2004. Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. *J. Clin. Microbiol.* 42: 622–626.
12. Shin JH, Ranken R, Sefers SE, Lovari R, Quinn CD, Meng S, Carolan HE, Toleno D, Li H, Lee JN, Stratton CW, Massire C, Tang Y. 2013. Detection, identification, and distribution of fungi in bronchoalveolar lavage specimens by use of multilocus PCR coupled with electrospray ionization/mass spectrometry. *J. Clin. Microbiol.* 51:136–141.
13. Hofstadler SA, Sampath R, Blyn LB, Eshoo MW, Hall TA, Jiang Y, Drader JJ, Hannis JC, Sannes-Lowery KA, Cummins LL, Libby B, Walcott DJ, Schink A, Massire C, Ranken R, Gutierrez J, Manalili S, Ivy C, Melton R, Levene H, Barrett-Wilt G, Li F, Zapp V, White N, Samant V, McNeil JA, Knize D, Robbins D, Rudnick K, Desai A, Moradi E, Ecker DJ. 2005. TIGER: the universal biosensor. *Int. J. Mass Spectrom.* 242:23–41.
14. Barenfanger J, Arakere P, Cruz RD, Imran A, Drake C, Lawhorn J, Verhulst SJ, Khardori N. 2003. Improved outcomes associated with limiting identification of *Candida* spp. in respiratory secretions. *J. Clin. Microbiol.* 41:5645–5649.
15. Murray PR, Van Scoy RE, Roberts GD. 1977. Should yeasts in respiratory secretions be identified? *Mayo Clin. Proc.* 52:42–45.
16. Dhiman N, Hall L, Wohlfiel SL, Buckwalter SP, Wengenack NL. 2011. Performance and cost analysis of matrix-assisted laser desorption ionization-time of flight mass spectrometry for routine identification of yeast. *J. Clin. Microbiol.* 49:1614–1616.
17. Theel ES, Schmitt BH, Hall L, Walchak RC, Patel R, Wengenack NL. 2012. Formic acid-based direct, on-plate testing of yeast and *Corynebacterium* species by Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 50:3093–3095.
18. Gu Z, Hall TA, Frinder M, Walsh TJ, Hayden RT. 2012. Evaluation of repetitive sequence PCR and PCR-mass spectrometry for the identification of clinically relevant *Candida* species. *Med. Mycol.* 50:259–265.
19. Caliendo AM. 2011. Multiplex PCR and emerging technologies for the detection of respiratory pathogens. *Clin. Infect. Dis.* 52(Suppl 4):S326–S330.