New Panfungal Real-Time PCR Assay for Diagnosis of Invasive Fungal Infections

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The diagnosis of invasive fungal infections (IFIs) is usually based on the isolation of the fungus in culture and histopathological techniques. However, these methods have many limitations often delaying the definitive diagnosis. In recent years, molecular diagnostics methods have emerged as a suitable alternative for IFI diagnosis. When there is not a clear suspicion of the fungus involved in the IFI, panfungal real-time PCR assays have been used, allowing amplification of any fungal DNA. However, this approach requires subsequent amplicon sequencing to identify the fungal species involved, increasing response time. In this work, a new panfungal real-time PCR assay using the combination of an intercalating dye and sequence-specific probes was developed. After DNA amplification, a melting curve analysis was also performed. The technique was standardized by using 11 different fungal species and validated in 60 clinical samples from patients with proven and probable IFI. A melting curve database was constructed by collecting those melting curves obtained from fungal species included in the standardization assay. Results showed high reproducibility (coefficient of variation [CV] < 5%; $r > 0.95$) and specificity (100%). The overall sensitivity of the technique was 83.3%, with the group of fungi involved in the infection detected in 77.8% of the positive samples with IFIs covered by molecular beacon probes. Moreover, sequencing was avoided in 67.8% of these "probe-positive" results, enabling report of a positive result in 24 h. This technique is fast, sensitive, and specific and promises to be useful for improving early diagnosis of IFIs.

nvasive fungal infections (IFIs) remain a major cause of morbidity and mortality in immunocompromised patients, and diagnosis continues to be problematic (1). Laboratory diagnosis of IFIs is based on classical methods such as fungus isolation in culture and histopathological examination; however, these methods present several limitations. Fungal cultures are frequently slow growing, and such assays are low in sensitivity as well as in specificity because fungi are habitual laboratory contaminants and part of the saprophytic human flora (2). Furthermore, histopathological studies show low sensitivity, require skilled personnel, and do not distinguish among fungal species (3), which is problematic since such distinctions are crucial in order to define an appropriate antifungal therapy, due to the differences in antifungal susceptibility exhibited by different fungal species (4).

In recent years, molecular methods, such as PCR assays, have emerged as a suitable alternative to conventional methods for the diagnosis of IFIs. These assays have a higher sensitivity as they allow the detection of small amounts of DNA in clinical samples. In addition, those protocols based on quantitative real-time PCR (qPCR) have the benefit of quantifying the fungal burden in clinical specimens (5). Several qPCR protocols have been developed for the diagnosis of IFIs, mainly for *Aspergillus* and *Candida* species (6) but also for less frequent fungal species (7, 8, 9, 10). However, the utility of a species or genus-specific approach is limited when there is not a clear suspicion of the fungus involved in the IFI. To solve this limitation, panfungal or broad-range fungal PCR assays have been described as an appropriate alternative. However, these techniques also present the inconvenience of the requirement of sequencing after amplification, which involves a delay in definite diagnosis. To date, several studies based on panfungal PCR showing suitable results (11, 12, 13) and demonstrating its usefulness in certain groups of patients, such as hemato-oncology patients (14) and immunocompromised pediatric patients (15), have been described.

Melting curve analysis has been reported by several authors as a fast, reliable, and cost-effective method to identify fungal species while avoiding sequencing (16, 17, 18, 19, 20). Moreover, a combination of probe detection and high-resolution melting analysis has been already used for detecting and identifying *Aspergillus* spp. from clinical samples (21). However, to our knowledge, this is the first method developed for IFI diagnosis that combines panfungal and species-specific detection with species identification by using melting curve analysis in the same run.

In this work, a panfungal qPCR assay combining a DNA binding dye and specific molecular beacon probes followed by a melting curve analysis has been designed in order to detect a wide range of fungal species and decrease response time by avoiding sequencing as far as possible. The technique has been standardized and validated for clinical strains and clinical samples from patients with proven and probable IFI. The aim of this study was to improve early diagnosis of IFIs in patients when there is not a clear suspicion of the fungus involved in the infection.

MATERIALS AND METHODS

Control strains. All strains included in the assay belonged to the fungal collection of the Spanish National Centre for Microbiology. DNA from the following organisms (strains in parentheses) was used to standardize

TABLE 1 Sequences of primers and probes designed for the new panfungal qPCR assay[a]

Primer or probe[b]	Sequence
Primers	
ITS1 (f)	5′-TCCGTAGGTGAACCTGCGG-3′
ITS2Deg2 (r)	5′-GCT**R**CGTTCTTCATCGAT**R**C-3′
Probes	
PanAspDeg	5′-ROX-<u>CGCGATC</u>AACCTCCCACCCGTG**W**CTA**YY**GTACC<u>GATCGCG</u>-BHQ2-3′
PanRhiz	5′-HEX-<u>CGCGAT</u>TTCTGGGGTTTGATCGATGCCA<u>ATCGCG</u>-BHQ1-3′
PanMuc	5′-HEX-<u>CGCGATC</u>GGCTTGGTATCCTATTATTATTTACCAAAAAGAATT <u>GATCGCG</u>-BHQ1-3′
PanHBP	5′-Cy5-<u>CGCGAT</u>TCGGCGGGCCTGCAGCG<u>ATCGCG</u>-BHQ2-3′
PanCocci3	5′-Cy5-<u>CGCGATC</u>GCGCCTGCCAG**Y**GGATCAATT<u>ATCGCG</u>-BHQ2-3′

[a] f, forward; r, reverse; BHQ1, black hole quencher 1; BHQ2, black hole quencher 2. Degenerated positions are in bold, and stem regions are underlined.
[b] Primers were used to amplify the ITS1 region from the fungal rDNA. Molecular beacon-specific probes were designed to detect each group of fungi, as follows: MB-PanAspDeg, *Aspergillus* species; MB-PanRhiz, *Rhizopus* species; MB-PanMuc, *Mucor* species; MB-PanHBP, *Histoplasma*, *Blastomyces*, and *Paracoccidioides* species; MB-PanCocci3, *Coccidioides* species.

the new panfungal qPCR assay: *Rhizopus oryzae* (CNM-CM 3020), *Rhizopus microsporus* (CNM-CM 4244), *Mucor circinelloides* (CNM-CM 2437), *Aspergillus flavus* (CNM-CM 3509), *Aspergillus terreus* (CNM-CM 3508), *Aspergillus fumigatus* (CNM-CM 2580), *Histoplasma capsulatum* var. *capsulatum* (CNM-CM 2721), *Histoplasma capsulatum* var. *duboisii* (CNM-CM 4626), *Coccidioides immitis* (CNM-CM 7056), *Coccidioides posadasii* (CNM-CM 2911), *Paracoccidioides brasiliensis* (CNM-CM 2908), and *Blastomyces dermatitidis* (CNM-CM 3114).

To assess the specificity of the technique, the following yeast and mold strains were used: *Scedosporium apiospermum* (CNM-CM 3169), *Scedosporium prolificans* (CNM-CM 1627), *Fusarium solani* (CNM-CM 3530), *Fusarium oxysporum* (CNM-CM 3197), *Cunninghamella elegans* (CNM-CM 7046), *Penicillium commune* (CNM-CM 7192), *Cryptococcus gattii* (CNM-CL 5007), *Cryptococcus neoformans* (CNM-CL 5801), *Candida albicans* (CNM-CL 8701), *Candida krusei* (CNM-CL 7057), *Candida parapsilosis* (CNM-CL 5683), *Candida glabrata* (CNM-CL 7523), *Candida tropicalis* (CNM-CL 8796), *Candida guilliermondii* (CNM-CL 7127), *Rhizomucor pusillus* (CNM-CM 2751), *Actinomucor elegans* (CNM-CM 1722), *Lichtheimia corymbifera* (CNM-CM 7053), and *Lichtheimia ramosa* (CNM-CM 7130). With the aim of evaluating the detection of *Aspergillus* spp. and *Mucor* spp., the following strains were also included: *Aspergillus lentulus* (CNM-CM 6069), *Aspergillus niger* (CNM-CM 4352), *Aspergillus viridinutans* (CNM-CM 5623), *Neosartorya udagawae* (CNM-CM 6056), *Neosartorya pseudofischeri* (CNM-CM 2270), *Aspergillus novofumigatus* (CNM-CM 6098), *Aspergillus ustus* (CNM-CM 4212) *Mucor plumbeus* (CNM-CM 5245), *Mucor velutinosus* (CNM-CM 6560), and *Mucor irregularis* (CNM-CM 7301).

Primer and probe design. Primers used in the assay were the universal primer ITS1 and a modified universal primer, ITS2 (22), which was redesigned with two degenerated positions to avoid deviation in the amplification efficiency among species (ITS2Deg2). Five molecular beacon probes labeled with different fluorescent dyes were designed to specifically target the ITS1 region of the rDNA from different groups of fungal species (*Aspergillus* spp., Mucorales, and fungi involved in endemic mycoses) based on a large database of internal transcribed spacer (ITS) sequences (containing more than 10,000 distinct sequences). Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA, USA) was used for probe design. The primers and probes designed were subjected to a BLAST search within the GenBank sequence database (http://blast.ncbi.nlm.nih.gov /Blast.cgi) to avoid cross-homology with other microorganisms. Sequences of primers and probes used in this assay are shown in Table 1.

Panfungal real-time PCR assay. The amplification assay was carried out in an LC480 unit (Roche Diagnostics, Mannheim, Germany). PCRs were performed in a 20-μl final volume containing 2× SensiMix II Probe No-ROX (Bioline, Ecogen, Madrid, Spain), 0.8 μM each primer, 0.2 μM MB-PanAspDeg, 0.2 μM MB-PanMuc, 0.1 μM MB-PanRhiz, 0.3 μM MB-PanHBP, 0.3 μM MB-PanCocci3, and 1× Resolight binding dye

(Roche Diagnostics, Mannheim, Germany) diluted 16 times. Finally, 2 μl of genomic DNA from control strains or 2 or 4 μl of DNA extracted from samples was added to PCRs in standardization and validation experiments, respectively. PCR conditions were as follows: an initial step of 10 min at 95°C, followed by 50 cycles at 95°C for 10 s, 54°C for 30 s, and 72°C for 30 s and a cooling phase of 30 s at 40°C. Melting curves were generated by increasing the temperature from 65°C to 99°C at 0.29°C/s. Results were considered positive when the fluorescent signal above the baseline was detected as determined by second-derivative analysis and were expressed in terms of the quantification cycle (C_q). Each experiment included quantification standards of five species of fungi as well as negative controls. Subsequently, a color compensation experiment was performed to prevent cross talk between dyes.

Standardization. Standard curves for the fungal species mentioned above in "Control strains" were obtained based on the result of five PCR repetitions with 10-fold serial dilutions of genomic DNA ranging from 1 ng to 0.1 fg/μl of reaction mixture. Two replicates of each dilution were included in each PCR repetition to evaluate the intrareproducibility of the technique. Regression lines were obtained by plotting the logarithm of the initial template concentration versus the corresponding C_q, and the standard curve was then used to determine the sensitivity, primer efficiencies, and reproducibility of the assay. In addition, the coefficient of variation (CV) was determined in each case.

To evaluate the specificity of the technique, 0.1 ng/μl of genomic DNA from 18 fungal species as well as mouse and human DNA (Promega, Madrid, Spain) was included in duplicate in the PCR assay.

Melting curve database construction. Melting curves of each PCR product were obtained by measuring the fluorescence of the Resolight Dye at different temperatures (from 65°C to 99°C) and analyzed by using the melting curve genotyping analysis included in LightCycler 480 Software v. 1.5 (Roche Diagnostics, Mannheim, Germany). Melting curves were automatically displayed as a melting curve chart (fluorescence [*F*] versus temperature [T_i]) and plotted by representing the first negative derivative melting curve ($-df/dt$) versus temperature, where the center of the melting peak corresponded to the point of inflection of the melting curve. This point of inflection represents the melting temperature of the PCR product. A melting curve database was generated by collecting, as external melting standards, those melting curves generated for each species included in the standardization assay presenting the best quality.

Validation assay in clinical samples. The usefulness of the new panfungal qPCR assay was evaluated using 60 clinical samples. Thirty-seven of the 60 clinical samples belonged to patients with proven IFI, and the remaining 23 belonged to patients with probable IFI, classified according to the EORTC/MSG criteria (23). Of the IFI samples, 17 samples belonged to patients with invasive aspergillosis, 11 with mucormycosis, 10 with infection by endemic mycoses, 9 with candidiasis, and 13 with IFIs caused by emerging or rare fungal species (Table 2). The origins of the samples

TABLE 2 Species distribution and sensitivity of the new panfungal qPCR assay for 60 clinical samples included in the validation assay[a]

Clinical group and fungal organism (no. of samples)	No. of organisms identified to species or genus level by:		
	Panfungal qPCR[b]	Molecular beacon[c]	Melting curve[d]
Proven IFI (37)	29	18 (62.1%)	15 (51.7%)
Aspergillus spp. (6)	6	6	4
Aspergillus fumigatus (2)	2	2	1
Aspergillus flavus (3)	3	3	2
Aspergillus penicilloides (1)	1	1	1
Mucormycetes (10)	7	6	5
Rhizopus oryzae (7)	5	5	3
Rhizopus microsporus (1)	1	1	1
Lichtheimia ramosa (1)	1	0	1
Rhizomucor pusillus (1)	0	0	0
Endemic mycoses (10)	6	6	4
Histoplasma capsulatum (8)	4	4	2
Coccidioides immitis (1)	1	1	1
Paracoccidioides brasiliensis (1)	1	1	1
Candida spp. (3)	3	0	2
Candida albicans (2)	2	0	1
Candida glabrata (1)	1	0	1
Other (8)	7	0	0
Fusarium sp. (1)	1	0	0
Cryptococcus neoformans (1)	1	0	0
Phoma exigua (1)	0	0	0
Bipolaris spicifera (1)	1	0	0
Microsphaeropsis arundinis (1)	1	0	0
Pythium insidiosum (1)	1	0	0
Cladophialophora bantiana (1)	1	0	0
Emmonsia crescens (1)	1	0	0
Probable IFI (23)	21	10 (47.6%)	7 (33.3%)
Aspergillus spp. (11)	10	9	7
Aspergillus fumigatus (6)	6	6	4
Aspergillus flavus (3)	3	2	2
Aspergillus terreus (1)	1	1	1
Aspergillus nomius (1)	0	0	0
Mucormycetes (1)	1	1	0
Rhizopus oryzae (1)	1	1	0
Candida spp. (6)	6	0	0
Candida albicans (2)	2	0	0
Candida parapsilosis (2)	2	0	0
Candida tropicalis (2)	2	0	0
Other (5)	4	0	0
Scedosporium sp. (1)	1	0	0
Aureobasidum pullulans (1)	1	0	0
Acremonium sp. (1)	1	0	0
Exophiala sp. (1)	0	0	0
Schizophyllum commune (1)	1	0	0
Total (60)	50	28 (56%)	22 (44%)

[a] Values in last three columns are numbers (percentage relative to positive samples by panfungal qPCR) of samples in which the organism was identified.
[b] Overall identification of fungal species by the new panfungal qPCR assay by positive signal of Resolight dye followed by sequencing.
[c] Identification of species or genus by positive signal of molecular beacon probes.
[d] Identification of fungal species by melting curve analysis.

were as follows: 44 were biopsy specimens (35 being fresh and the remaining paraffin-embedded biopsy specimens), 8 were respiratory samples (5 bronchoalveolar lavage fluids, 2 bronchoaspiration specimens, and 1 tracheal aspirate), 5 were cerebrospinal fluids (CSF), 1 was aqueous humor

fluid, and 1 was a nail sample. DNA extraction from clinical samples was performed manually by using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and as already described by Buitrago et al. (24) without any preprocessing step. Biopsy samples embedded in paraffin were deparaffinized by lavage with 1.5 ml of xylene (100%) followed by two lavages with 1.2 ml of ethanol (96 to 100%) and an incubation of the tissue at 37°C to evaporate the remains of the ethanol. Volumes of 2 and 4 μl of DNA extracted from each sample were used for each PCR assay.

All PCR products obtained from clinical samples were run in 2% ethidium bromide-stained agarose gels (Sigma-Aldrich Química, Madrid, Spain) to verify amplification and the quality of the amplified products. Then, PCR products were purified by using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) and sequenced (ABI 3730 XL; Applied Biosystems, Madrid, Spain) with ITS1 and ITS2Deg2 primers to verify the results. The sequences obtained were compared with those available in the GenBank database (http://www.ncbi.nih.gov/GenBank/) and the sequence database belonging to the Mycology Department, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain.

RESULTS

***In vitro* standardization of the qPCR assay.** The newly designed panfungal qPCR assay detected the DNAs from 11 fungal species included in the standardization assay. Each species was detected in the panfungal detection channel (FAM, 483 to 533 nm) and in the specific channel depending on the species tested (HEX, 523 to 568 nm, for Mucorales species; ROX, 558 to 610 nm, for *Aspergillus* spp.; or Cy5, 615 to 670 nm, for fungi causing endemic mycoses). Detection limits were established between 0.1 and 100 fg of DNA per μl, and CV values were within the acceptable limit of <5% in all cases with the exception of *C. posadasii*. Quantification was linear for all fungal species included in the assay, and the standard curve generated showed a coefficient of determination between 0.95 and 0.99 in all cases (Table 3).

No cross-reactivity to DNA from other fungi or human or mouse was detected. Moreover, as expected, the MB-PanAspDeg probe and MB-PanMuc probes were available to detect species belonging to *Aspergillus* and *Mucor* genera, respectively.

Melting curves were obtained for all species included in the standardization assay and checked visually for those with the best quality. All representative melting curves were collected as external melting standards, and the means of melting temperature values were noted (Table 4) with the aim to create a melting curve database, which was subsequently used to assess fungal species identification. In addition, melting curves of important fungal pathogens such as different species of *Candida* spp., *Fusarium* spp., and *Scedosporium* spp., which were not detected by the molecular beacon probes designed, were also included in the database (Fig. 1).

Validation of the panfungal qPCR assay in clinical samples. In order to assess the utility of this new panfungal qPCR assay, 60 clinical samples were tested. The assay was positive for 50 of the 60 clinical samples included in the validation assay (83.3%) with amplicon sequences confirming the species involved in the infection. Of the 36 cases in which causative species were susceptible to be recognized by any molecular beacon probe, we were able to identify the group of species involved in the IFI in 28 of them (77.8%). When the melting curve was analyzed in these cases, sequencing was avoided for 67.8% of them (19/28). Moreover, in the remaining 24 cases caused by species not covered by molecular probes, we were able to define the fungal species involved in the infection by

TABLE 3 Overview of standardization results of the 12 clinical strains belonging to 11 fungal species included in the new panfungal qPCR assay[a]

Species	FAM (panfungal channel)				HEX (Mucorales channel)				ROX (*Aspergillus* sp. channel)				Cy5 (endemic mycosis fungus channel)			
	S	R^2	CV (%)	DL (fg/µl)	S	R^2	CV (%)	DL (fg/µl)	S	R^2	CV (%)	DL (fg/µl)	S	R^2	CV (%)	DL (fg/µl)
Rhizopus oryzae	−3.6	0.99	2.45	0.1	−3.6	0.99	2.05	0.1								
Rhizopus microsporus	−3.6	0.98	1.54	0.1	−3.8	0.98	3.05	0.1								
Mucor circinelloides	−3.4	0.98	3.76	0.1	−3.7	0.98	4.67	1								
Aspergillus fumigatus	−3.5	0.97	2.72	1					−4.1	0.95	3.73	1				
Aspergillus flavus	−3.8	0.98	3.69	1					−4.1	0.98	3.05	1				
Aspergillus terreus	−3.5	0.98	1.52	1					−4.1	0.98	3.6	1				
Histoplasma capsulatum var. *capsulatum*	−3.5	0.98	2.23	1									−3.5	0.98	1.98	1
Histoplasma capsulatum var. *duboisii*	−3.4	0.98	2.21	10									−3.3	0.98	2.16	10
Coccidioides immitis	−3.5	0.96	2.9	10									−4.6	0.96	4.4	10
Coccidioides posadasii	−5.1	0.95	3.98	10									−6.6	0.97	5.69	100
Blastomyces dermatitidis	−3.4	0.98	1.93	1									−3.2	0.98	1.67	1
Paracoccidioides brasiliensis	−2.8	0.98	0.73	1									−2.8	0.98	0.47	1

[a] S, slope of standard curve; R^2, coefficient of determination; CV, coefficient of variation; DL, detection limit.

melting curve analysis in two cases of infection by *Candida* spp. and one by *Lichtheimia ramosa* (Fig. 2). The assay detected fungal DNA in 29 of 37 (78.4%) samples of patients with proven IFI, 62.1% (18/29) of them with a positive signal of molecular beacon probes and 51.7% (15/29) with positive species identification achieved by melting curve analysis. In patients with probable IFI, the assay was positive for 20 of 23 of samples (87%) with 47.6%

(10/23) of them detected by molecular beacon probes and 33.3% (7/23) reporting species identification by melting curve analysis. By species, the technique detected 94% of cases containing *Aspergillus* sp. DNA, 73% of cases caused by Mucorales species, 70% of cases in which endemic fungi caused the infection (7/10), 100% of cases harboring DNA from *Candida* spp. (9/9), and 85.5% of the cases containing DNA of rare and emerging species (11/13).

TABLE 4 Melting temperature values obtained for each clinical fungal strain used in the standardization assay of the new panfungal qPCR assay

| Strain	Species	T_m value (mean ± SD)		
		T_m1	T_m2	T_m3
CNM-CM 2580	*A. fumigatus*	83.37 ± 0.13	89.18 ± 0.75	93.37 ± 0.38
CNM-CM 3509	*A. flavus*	83.68 ± 0.69	88.06 ± 0.82	92.64 ± 0.08
CNM-CM 3508	*A. terreus*	83.61 ± 0.63	87.89 ± 0.89	93.06 ± 0.24
CNM-CM 3020	*R. oryzae*	78.73 ± 0.23	83.47 ± 0.19	
CNM-CM 4244	*R. microsporus*	83.67 ± 0.22		
CNM-CM 2437	*M. circinelloides*	80.68 ± 0.43		
CNM-CM 2721	*H. capsulatum* var. *capsulatum*	92.57 ± 0.31		
CNM-CM 4626	*H. capsulatum* var *duboisii*	92.05 ± 1.41		
CNM-CM 7046	*C. immitis*	82.97 ± 0.70	92.89 ± 0.08	
CNM-CM 2911	*C. posadasii*	82.37 ± 0.45	92.68 ± 0.47	
CNM-CM 3114	*B. dermatitidis*	91.76 ± 0.81		
CNM-CM 2908	*P. brasiliensis*	90.85 ± 0.31		
CNM-CM 3169	*S. apiospermum*	83.68 ± 0.07	87.74 ± 0.06	
CNM-CM 1627	*S. prolificans*	81.85 ± 0.06	85.84 ± 0.05	
CNM-CM 3530	*F. solani*	81.30 ± 0.07	84.50 ± 0.1	87.66 ± 0.1
CNM-CM 3197	*F. oxysporum*	81.05 ± 0.06	86.80 ± 0.08	
CNM-CM 7046	*C. elegans*	82.84 ± 0.02		
CNM-CM 2751	*R. pusillus*	81.75 ± 0.13		
CNM-CM 7192	*P. commune*	81.75 ± 0.03	87.62 ± 0.01	92.21 ± 0.01
CNM-CM 1722	*A. elegans*	83.81 ± 0.18		
CNM-CM 7130	*L. ramosa*	84.09 ± 0.01		
CNM-CM 7053	*L. corymbifera*	83.42 ± 0.06		
CNM-CL 8701	*C. albicans*	82.88 ± 0.03		
CNM-CL 5683	*C. parapsilosis*	80.68 ± 0.03		
CNM-CL 8796	*C. tropicalis*	80.79 ± 0.03		
CNM-CL 7523	*C. glabrata*	80.20 ± 0.05	86.53 ± 0.07	
CNM-CL 7057	*C. krusei*	80.87 ± 0.01		
CNM-CL 7127	*C. guilliermondii*	82.75 ± 0.01		
CNM-CL 5801	*C. neoformans*	80.69 ± 0.01		
CNM-CL 5007	*C. gattii*	81.40 ± 0.03		
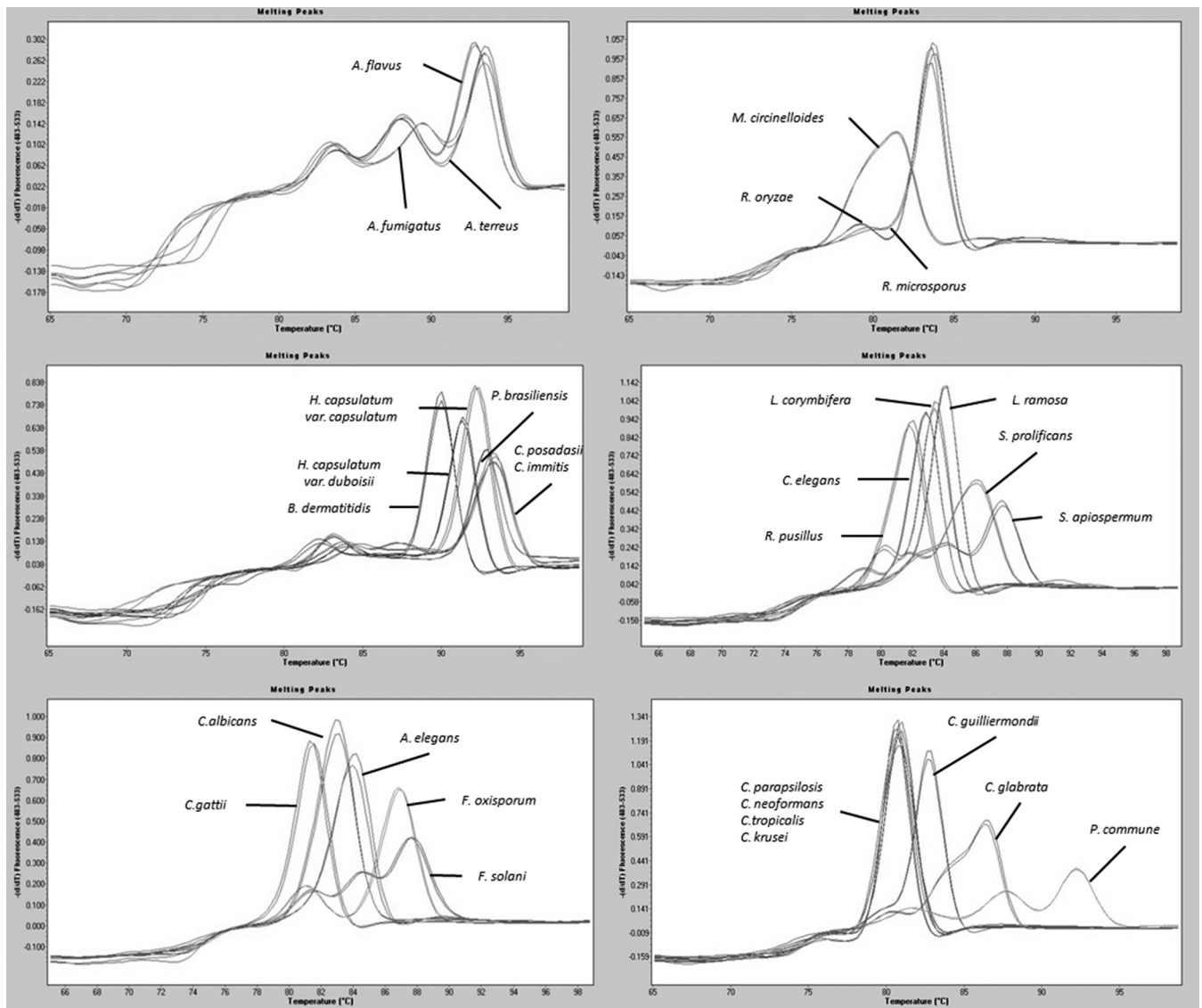
FIG 1 Melting curves for species included in the melting curve database constructed for species identification in the new panfungal qPCR assay.

Finally, 34 of the 44 biopsy specimens showed a positive result while all respiratory samples (8/8), all CSF samples (5/5), the aqueous humor fluid, and the nail sample gave a positive result. Results from the 60 clinical samples included in the study are detailed in Table 2. In Fig. 3, results for a positive sample are represented for each channel of detection of the real-time equipment.

DISCUSSION

The aim of this work was to standardize and validate a new panfungal qPCR assay to improve diagnosis of IFIs when there is not a clear suspicion of the fungus involved in the disease. The usefulness of molecular methods based on PCR for the proper detection of fungal species in IFIs has been proven (25, 26). However, several limitations exist when the fungus causing the infection is unknown. In these cases, panfungal PCR assays have been used (27, 28), but they involve a delay in the response time because they require sequencing the amplicon. As recently described, in 30% of

the samples studied from patients with IFI there was no evidence concerning the fungus implicated in the infection (24), highlighting the need for new methods. To overcome these limitations, several authors described the differentiation among fungal species by using melting curve analysis with panfungal primers (29, 30), demonstrating the capability of this technique to replace sequencing strategies, although sensitivity in these assays was limited. This new technique has been designed for the detection in one single tube of any fungal species and combines (i) panfungal detection by using a binding dye, (ii) specific detection by using molecular beacon probes, and (iii) species identification by using melting curve analysis. Merging all these techniques in the same assay eliminates the need for sequencing the amplicon and allows for an earlier identification of the species causing the disease. Figure 4 shows a diagram that explains the procedures to perform when using this new technique and also the time savings. Those samples with positive panfungal detection, positive probe detection, and a successful melting curve analysis may be reported as positive re-
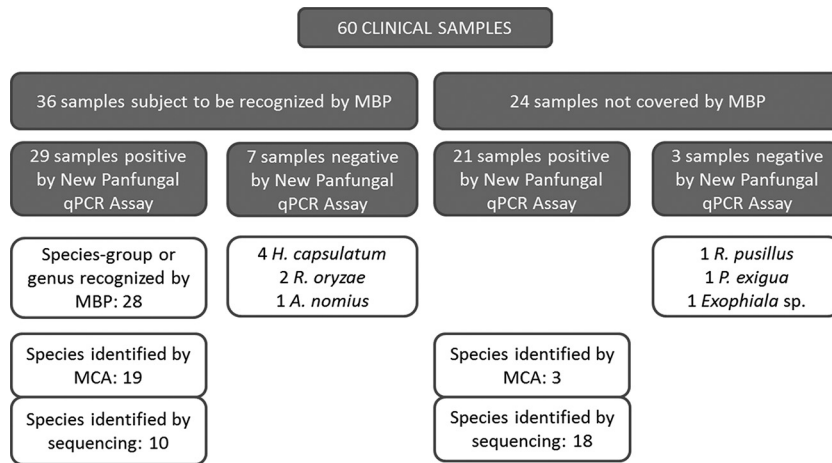
FIG 2 Chart summarizing the results of the validation assay of the new panfungal qPCR assay. Samples were differentiated in two blocks, those that could be recognized by probes and those that were not covered by any probe. Results for each group are detailed in white boxes. MCA, melting curve analysis; MBP, molecular beacon probe; *P. exigua*, *Phoma exigua*; *A. nomius*, *Aspergillus nomius*.

sults within 24 h. When probe detection and melting curve analysis are negative, sequencing the amplicon becomes mandatory, increasing the response time to 120 h. However, this maximum time could be reduced to 48 h in those cases in which the species or genus was identified by molecular beacon probes by performing

additional specific diagnostic tests. The selected design was directed to three groups of fungi (*Aspergillus* spp., Mucorales, and fungi causing endemic mycoses) by the use of five specific probes. Probes were designed using the sequence database belonging to the Mycology Department of the Instituto de Salud Carlos III,
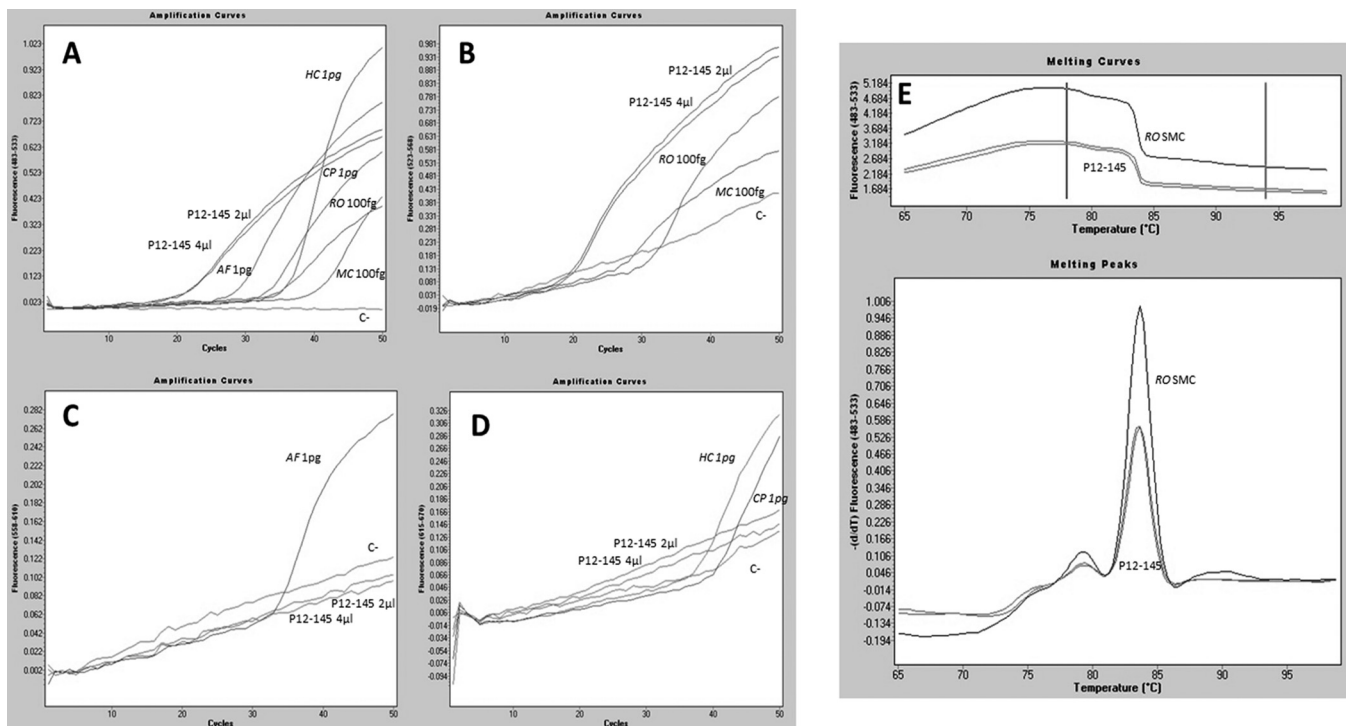

FIG 3 Dependence of fluorescence signal on the number of cycles in the new panfungal qPCR assay for patient P12-145 with proven mucormycosis caused by *R. oryzae*. (A) Fluorescence detection in the FAM fluorescence channel, based on positive signal for the Resolight binding dye indicating panfungal amplification. (B) Fluorescence detection in the HEX fluorescence channel, showing specific detection of *Rhizopus* sp. and *Mucor* sp. DNA and 2 and 4 μl of DNA extracted from sample P12-145. (C) Fluorescence detection in the ROX fluorescence channel. The positive signal corresponded to the *A. fumigatus* positive control. (D) Fluorescence detection of Cy5 dye, showing *H. capsulatum* and *C. posadasii* positive-control amplification. (E) Melting peaks and melting curves detected in the FAM fluorescence channel obtained by melt curve genotyping analysis. *R. oryzae* standard melting curve matched with those melting curves obtained from the P12-145 clinical sample. RO, *R. oryzae*; MC, *M. circinelloides*; AF, *A. fumigatus*; HC, *H. capsulatum*; CP, *C. posadasii*; C−, negative control; SMC, standard melting curve.
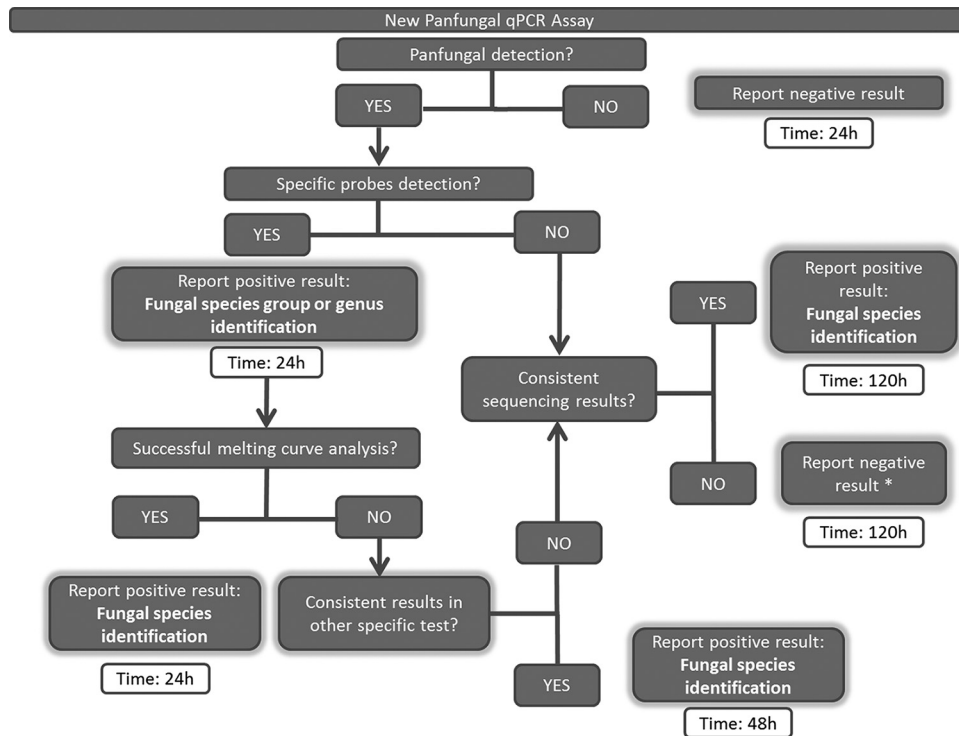
FIG 4 Schematic diagram of procedures to perform and time-to-result analysis for this new panfungal qPCR when there is no suspicion of species involved in the IFI. *, with the exception of those cases in which species or genus was detected by molecular beacon probes, for which samples should be retested or a new sample should be requested.

which contains a large set of ITS sequences representing a wide range of different fungal species. Other groups of fungi were not detected by using specific probes due to the limitations of the real-time PCR equipment. Despite this limitation, the technique is open and flexible to modifications, as new probes could be designed to focus on different groups of fungi depending on the needs of the laboratories, but it must be kept in mind that fluorescent channels available in qPCR equipment are limited.

This new technique has been standardized with 12 clinical strains from 11 fungal species belonging to the collection of the Mycology Department of the Spanish Centre for Microbiology, Instituto de Salud Carlos III. During standardization, the sensitivity and reproducibility of this new panfungal qPCR were analyzed. Despite slight differences observed in detection limits described among different fungal species, the assay was efficient in detecting all fungal species targeted by the molecular beacon probes designed. The technique also exhibited high reproducibility and specificity. Moreover, other species of *Mucor* and *Aspergillus* genera, different from those used for the standardization process, were also detected by the MB-PanMuc and MB-AspDeg probes. Finally, a database with melting curves obtained from different fungal species was constructed. This database included all species used for the standardization process and also species belonging to important fungal pathogens such as *Candida* spp., *Fusarium* spp., and *Scedosporium* spp. that were not detected by specific probes but were included in the specificity test and collected as external melting standards.

The technique was validated by using 60 clinical samples from patients with proven and probable IFI. Samples were mainly biopsy specimens (44/60), followed by respiratory samples (8/60)

and others (7/60). An overall sensitivity of 83.3% was achieved, showing similar sensitivity values in patients with proven and probable IFI. We were able to provide identification to the species level for 77.8% of those cases in which fungal species were covered by molecular beacon probes. In these cases, data from melting curves helped to define the species involved in 67.8% of the cases. Although melting curve analysis failed to give a definite diagnosis in nine of those "probe-positive" cases, the results provided important information that could help to perform additional specific PCR assays or to define an appropriate antifungal therapy for the patient. In addition, in three samples the species were identified by melting curve analysis because they were not targeted by molecular beacon probes. Finally, sequencing was mandatory for 56% of positive samples (28/50) mainly due to IFIs caused by species neither recognized by any probe nor included in the melting curve database constructed. Also, this technique showed some limitations, as certain species (i.e., *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. neoformans*) exhibited similar melting curves (Fig. 1) and had large standard deviations in T_m values (Table 4) that made them indistinguishable. In these cases, complementary techniques (specific PCRs, conventional tests, etc.) should be performed to assess fungal identification. Negative results were found only for biopsy samples; however, these results are biased, as these samples are overrepresented in comparison to respiratory and other samples included in the validation assay. Also, 5 of the 10 negative cases were found in paraffin-embedded biopsy specimens. Extraction of DNA suitable for amplification from these specimens is not optimized (31, 32). To discard the possibility that DNAs from those clinical samples with negative results were degraded, new DNA extraction was performed from five available clinical sam-

ples, but a positive result was obtained for only one sample. Of note, 7 of the negative samples harbored species that, theoretically, were susceptible to be detected by designed molecular beacon probes. However, species identification could not be achieved even by sequencing the amplicon. This could be due to a low fungal burden in the sample, the use of old, long-term-stored samples, or mixed infections, which have been described as main limitations of panfungal qPCR assays (3, 5).

In conclusion, this new panfungal qPCR assay is fast, sensitive, and specific for the detection of several fungal species in clinical samples, avoiding amplicon sequencing in many instances and shortening the time to definite diagnosis by up to 4 or 5 days. Further experiments are warranted in order to enlarge the melting curve database and expand the range of fungal species that can be detected by this technique.

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