Molecular Diagnosis & Therapy (2022) 26:511–525
https://doi.org/10.1007/s40291-022-00595-1

ORIGINAL RESEARCH ARTICLE

Optimization of a Quantitative PCR Methodology for Detection of *Aspergillus* spp. and *Rhizopus arrhizus*

Alexandre Mendonça[1] · Joana Carvalho-Pereira[1] · Ricardo Franco-Duarte[1] · Paula Sampaio[1]

Accepted: 2 May 2022 / Published online: 16 June 2022

Abstract

Introduction Multiplex quantitative polymerase chain reaction (qPCR) methods for the detection of *Aspergillus* spp. based only on SYBR Green and melting curve analysis of PCR products are difficult to develop because most targets are located within ITS regions. The aim of this study was to adapt our previously developed methodology based on a multiplex PCR assay coupled with GeneScan analysis to provide a qPCR method.

Methods A SYBR Green-based real-time PCR assay was optimized to detect *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *R. arrhizus* in a multiplex assay and applied to cultured fungi and spiked plasma.

Results Different melting temperatures allowed identification of all five pathogens and discrimination between them, even in samples with low amounts of fungal gDNA (from 1.3 to 33.0 pg/µL), which has been reported previously as problematic. No false-positive results were obtained for non-target species, including bacteria and human DNA. This method allowed detection of fungal pathogens in human plasma spiked with fungal DNA and in coinfections of *A. niger*/*R. arrhizus*.

Discussion This work provides evidence for the use of a qPCR multiplex method based on SYBR Green and melting curve analysis of PCR products for the detection of *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *R. arrhizus*. The proposed method is simpler and less expensive than available kits based on fluorescent probes and can be used for aiding diagnosis of the most relevant invasive filamentous fungi, particularly in low-income health care institutions.

Key Points

The present work contributes an optimized method to detect *Aspergillus* spp. and *Rhizopus arrhizus*.

Using a quantitative PCR methodology, the detection of fungal pathogens can be performed in a simpler and less expensive way.

Our method shows the ability to deliver fast (~1.5 h) and accurate identification of the most frequent filamentous fungi involved in systemic infections in one single step, showing great potential for aiding diagnosis of these infections.

1 Introduction

Aspergillus spp. are found in nature, being present in the soil as saprophytes that sporulate with abundance, releasing conidia into the environment. Conidia inhalation is the primary route of human infection and, in immunocompromised patients, it can lead to invasive infections, linked with high rates of mortality and morbidity [1, 2]. The main etiologic agent of invasive aspergillosis is *Aspergillus fumigatus*, which is associated with more than 300,000 cases per year [3, 4] and a mortality rate of 30–70% [3, 5, 6]. However, other *Aspergillus* species are emerging as successful pathogens that can also cause severe invasive infections, such as *A. niger*, *A. flavus*, and *A. terreus* [7]. Isavuconazole and voriconazole are the preferred agents for first-line treatment

✉ Ricardo Franco-Duarte
ricardofilipeduarte@bio.uminho.pt

Alexandre Mendonça
alexandremendonca.1997@gmail.com

Joana Carvalho-Pereira
Joanaicpereira@gmail.com

Paula Sampaio
psampaio@bio.uminho.pt

[1] CBMA (Centre of Molecular and Environmental Biology), Department of Biology, University of Minho, Braga, Portugal

of pulmonary invasive aspergillosis, whereas liposomal amphotericin B is moderately supported [8]. However, a fast, accurate, and reliable diagnosis is crucial for an appropriate therapeutic plan, leading to less time in intensive care units and higher survival rates. This is also critical for the hospital itself, since it was shown that invasive aspergillosis hospitalizations in United States topped US$600 million [9].

Infections caused by fungi of the order Mucorales are also considered as emerging infections, associated with high mortality rates [3, 10]. Mucormycosis is the third most common fungal infection, after the ones caused by *Candida* and *Aspergillus*. Mucormycosis can be caused by fungal pathogens of different genera, including *Rhizopus, Apophysomyces* and *Lichtheimia, Rhizomucor*, *Mucor*, and *Cunninghamella*. Though *Rhizopus* is predominant worldwide, *Apophysomyces* and *Lichtheimia* species are rising in predominance in Asia and Europe, respectively [11]. *Rhizopus arrhizus*, formerly called *R. oryzae*, is linked to over 10,000 cases per year [3, 10, 12]. Similarly to what happens in invasive aspergillosis, mucormycosis also mainly affects immunocompromised patients, such as patients with hematological malignancy, solid organ transplants, corticosteroid therapy, and even patients with diabetes mellitus [10, 12, 13]. Rhinocerebral mucormycosis is considered the most common type, accounting for 33–55% of the infections [12]; however, disseminated mucormycosis is the most severe condition, being commonly associated with patients with a hematological condition in 23–62% of the cases [14–16]. Mortality rates for mucormycosis range from 40 to 80% with varying rates depending on underlying conditions and sites of infection [11]. Improved survival is related to earlier diagnosis and application of early treatment approaches involving aggressive surgical debridement followed by antifungal therapy. Treatment with liposomal amphotericin B is highly recommended while intravenous isavuconazole and intravenous or delayed-release posaconazole tablet are recommended with moderate strength [17]. However, depending on the geographical location, not all recommended treatments are available and the clinical status of the patient must also be considered (see recommendation on the [17] review).

The proper way to diagnose invasive aspergillosis and mucormycosis is by achieving a *proven* diagnosis, through cultures, direct microscopy, and histopathology, using specimens from sterile sites [18, 19]. Although these traditional methodologies fall short in terms of turn-around time and sensitivity, they continue to be employed for comparison and confirmation [20]. These techniques still require a trained mycologist to differentiate fungal species, especially with the similar microscopic morphology of fungal structures. Regarding invasive aspergillosis diagnosis, direct microscopy, preferably using optical brighteners, histopathology, and culture, are strongly recommended [8]. Still, the majority of *Aspergillus* spp. are rarely isolated from CSF and blood cultures, even in disseminated infections, with sensitivity values ranging from 1 to 5% [21, 22]. On the other hand, bronchoalveolar fluid (BAL) cultures present sensitivity values of 47.4%, but BAL are not samples from a sterile site, which introduces the variable of contamination [23]. Traditional cultures, when applied to diagnose mucormycosis, are commonly linked to blood, BAL, and CSF samples; however, the overall sensitivity is < 40% and the results are falsely negative in over 50% of mucormycosis cases [24–26].

In order to overcome the limitations associated with traditional methodologies, serum and BAL galactomannan (GM) measures are recommended as markers for the diagnosis of invasive aspergillosis. ELISA Platelia *Aspergillus* assay™ (Bio-Rad, USA), FDA approved, is the most frequently used kit in the clinical context to specifically detect GM. This assay was optimized for serum and showed sensitivity values ranging from 44 to 100% and specificity capacity ranging from 78.6 to 100% [27, 28]. Regarding mucormycosis, Fungitell Assay is not indicated, since *Rhizopus* and other Mucorales species are known to produce very low levels of β-(1,3)-D-glucan [29] and there are no other commercially available antigen assays to aid the diagnosis [26].

Polymerase chain reaction (PCR)-based methodologies, generally, are directly applied to clinical samples from sterile and non-sterile sites, to detect fungal DNA. These techniques detect the genetic material of the pathogen, in singleplex or multiplex reactions using specific primers, and the PCR products are analyzed through several techniques such as sequencing [30–32], fluorescence in situ hybridization (FISH) [33], restriction fragment length polymorphism (RFLP) [20, 34], capillary electrophoresis [35], melting curve analysis (MCA) [30, 36] or with the direct use of probes [31, 37, 38]. However, in clinical settings the preferred methodology is quantitative real-time PCR (qPCR), since the reaction occurs in an entirely closed system avoiding contamination, and with low hands-on time. There are several commercially available qPCR kits that can be used in clinical laboratories to aid diagnosis of invasive aspergillosis and mucormycosis. Table 1 presents the most frequently used kits. Implementation of PCR-based methods in consensus definitions guidelines for diagnosing invasive fungal

diseases (IFD) has been difficult due to limited standardization, however, the ISHAM Fungal PCR Initiative Working Group (www.fpcri.eu) has been working on these standardizations and the 2017 ESCMID-ECMM-ERS guideline indicates that PCR should be considered in conjunction with other diagnostic tests [8].

Most commercial kits are probe-based amplification qPCR methods that require different fluorescence combinations when multiplexed and a differentiated qPCR instrument, making this technique expensive. The main objective of this work was to adapt a previously developed GeneScan-based multiplex PCR method [35] for the detection of systemic fungal infections to a multiplex qPCR method based on SYBR Green and melting curve analysis of PCR products. The formerly developed methodology presented a panel for filamentous fungi that was able to specifically identify and distinguish *Aspergillus* spp. (*A. fumigatus*, *A. niger*, *A. flavus*, and *A. terreus*) and *R. arrhizus* [35].

Table 1 List of commercially available quantitative PCR-based assays for detection of pathogenic fungi

Product	Assay method	Detected species	Specimens	Sensitivity/specificity	References
MycAssay™ Aspergillus (Microgen Bioproducts Ltd., Camberley, UK)	qPCR method (probes – molecular beacons)	18 *Aspergillus* spp.	BAL Serum	80–100%/82.4–98.6%	[54–56]
Fungiplex® Aspergillus and Fungiplex® Aspergillus azole-R (Bruker Corporation, Billerica, US)	qPCR method (probes)	*A. fumigatus* *A. terreus* *A. niger* *A. flavus* Azole resistance markers (TR34 and TR46)	WB Serum Plasma BAL	60%/91.2%	[57, 58]
Aspergillus spp. ELITe MGB® (ELITech Group, Puteaux, France)	qPCR method (probes)	*A. niger* *A. terreus* *A. flavus* *A. nidulans* *A. versicolor* *A. glaucus*	BAL BA	90–100%/91.2%	[59, 60]
AsperGenius® (PathoNostics)	qPCR method (probes)	*A. fumigatus* *A. terreus*	BAL Serum Plasma Biopsy CSF	65.5–88.9%/77.8–93.3%	[54, 55, 61–64]
MycoReal Aspergillus (Ingenetix)	qPCR method (MCA analysis)	*A. fumigatus* *A. flavus* *A. niger* *A. terreus* *A. nidulans*	BAL WB CSF Tissues	NA	[56, 65, 66]
AspID (Olm Diagnostics)	qPCR method (probes)	*A. terreus*	BAL	94.1%/76.5%	[67–69]
MucorGenius® (PathoNostics)	qPCR method (probes)	*Rhizopus* spp. *Mucor* spp. *Lichtheimia* spp. *Cunninghamella* spp. *Rhizomucor* spp.	BAL Biopsy Serum	75–90%/97.9%	[70–74]
MycoGENIE® *Aspergillus* and *Mucorales* spp. and MycoGENIE® *A. fumigatus* TR34 and L98H (Ademtech, Pessac, France)	qPCR method (probes)	*Aspergillus* spp. Mucorales spp. *A. fumigatus* with azole resistance markers	Serum BAL Biopsy	71–100%/84.6–100%	[58, 67, 68]

BA bronchial aspirate, *BAL* bronchoalveolar fluid, *CSF* cerebrospinal fluid, *NA* not available, *qPCR* quantitative polymerase chain reaction, *WB* whole blood

However, this methodology was based on two steps after DNA extraction from clinical samples, the multiplex PCR reaction and a capillary electrophoresis for the separation of PCR products and size determination by GeneScan analysis. An extra step for species identification in the clinical setting is considered a drawback. Thus, in this study, the previously describe methodology was changed from a two-step technique after DNA extraction to a one-step method. The adapted qPCR method was designed as a simple and cost-effective technique. The developed qPCR multiplex method was able to deliver fast and accurate results in a more cost-effective manner and can be used in low-income hospitals, or in situations where the qPCR instrument is not suitable for multiplex reactions based on probes with different fluorescence wavelengths.

2 Materials and Methods

2.1 Primers Selection

All primers used throughout the work were designed by Carvalho-Pereira and co-workers [35], to identify pathogenic fungi through the methodology of multiplex PCR coupled with GeneScan analysis. The sequences of the used primers are shown in Table 2, according to the specific identification panel.

2.2 Strains Tested

DNA from several clinical fungal isolates, previously identified, was used to optimize and assess *locus*-specific amplification and representative melting temperature of each species for the qPCR method (Supplementary Data 1, see electronic supplementary material [ESM]). The specificity of the qPCR method was evaluated using DNA from *Penicillium* spp., *Mucor velutinas*, *A. versicolor*, *Scopulariopsis brevicaulis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Candida albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis, C. auris*, *Escherichia coli*, and *Staphylococcus aureus*. These primers were already tested with 121 previously identified clinical and environmental fungal isolates [35]. All strains are conserved at the Collection of the Department of Biology (CDB), University of Minho, Braga, Portugal.

Table 2 Primer sequences selected. These primers are included in the Portuguese Patent n° PT 115216 [35]

Filamentous fungi panel	
Species	Primer sequence (5′ to 3′)
Aspergillus fumigatus	**F-**gccctcttccgttattcctt **R-**gcgcattgatagctacctcaggc
Aspergillus niger	**F-**ccctccttccaaacaaacaa **R-**tccagatcggctacacagaa
Aspergillus flavus	**F-**gggatcgacactcggactt **R-**ctggtaagagcttgtgggtg
Aspergillus terreus	**F-**gcggatgcaaggtgtaattt **R-**tactgcgcgttagttgaagc
Rhizopus arrhizus	**F-**agaagcaaaatcatcgtcgaaag **R-**cgtaggtccagcgtaaacttg

F refers to forward primer; R refers to reverse primer

2.3 Extraction of Genomic DNA from Filamentous Fungi

Before the genomic DNA extraction, filamentous fungi strains were grown at 30 °C for 4 days on YPD agar medium (agar 2%, glucose 2%, bactopeptone 1%, and yeast extract 1%). DNA was extracted from filamentous fungi according to the protocol described by Carvalho-Pereira et al. [35]. Shortly after, approximately 500 µL of fungal biomass was frozen in liquid nitrogen and then macerated to promote a mechanical disruption of cell walls. Chloroform : isoamyl alcohol (24 : 1) was used to isolate DNA in the aqueous phase, and sodium acetate (3 M) was used to promote DNA precipitation. The pellet was dried overnight and resuspended in 50 µL of warm sterile water. Samples were incubated in thermoblock at 65 °C for 45 min and posteriorly homogenized. The genomic DNA was purified using JetQuick® Genomic DNA Purification Kits (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. All manipulations were performed in a category 2 laminar flow cabinet to prevent contamination.

2.4 qPCR Amplification Conditions—Singleplex and Multiplex

Singleplex qPCR reactions were performed using several fungal strains, in order to assess locus-specific amplification, and the representative melting temperature of each species. Multiplex qPCR reactions were performed to optimize the methodology, to assess parameters such as sensibility and reproducibility and to evaluate the effectiveness of the method in mimicked samples. Different concentrations of magnesium chloride ($MgCl_2$ 1 mM, 1.5 mM, and 2 mM), annealing temperatures (from 60 to 65 °C) and primer concentrations (from 0.13 to 0.23 µM) were tested. To avoid non-specific binding of primers and non-specific products, two PCR enhancers, betaine (from 0.8 to 1 M) and nonionic detergent Nonidet P-40 (NP-40, 0.8%), were also tested in the PCR reaction.

Real-time PCR reactions were carried out with the CFX96™ Real-time PCR Detection Systems (Bio-Rad) with the PCR program consisting of a pre-incubation step

at 95 °C for 3 min, then 40 cycles were performed which included the following steps: 95 °C for 15 seconds, annealing at 60 °C (for singleplex reactions) and 61 °C (for multiplex reactions) for 30 seconds, and extension at 72 °C for 30 seconds. Melting curve analysis consisted of a heat preservation step at 65 °C for 5 seconds, then a step corresponding to the temperature ramp from 65 to 95 °C, with a heating rate of 0.05 °C/s.

In singleplex qPCR, the baseline and threshold values used were automatically defined by the Bio-Rad CFX software. However, in multiplex qPCR the baseline and threshold values used were manually defined according to protocols for detecting SARS-CoV-2 [39, 40]. Baseline was defined between cycles 3–15 to identify a constant and linear component of the background levels and to normalize the amplification curves [39]. The threshold was adjusted to be equal to the exponential phase of the negative control (no template control: water) (in case of a threshold cycle (Ct) value > 30) [40]. All results were analyzed in the software Bio-Rad CFX Manager.

2.5 Standard Curves

Serial 10-fold dilutions of fungal gDNA in water were performed specifically to be 200 ng/µL, 20 ng/µL, 2 ng/µL, 200 pg/µL, and 20 pg/µL, and the PCR was performed. The standard curves were used to determine the efficiency, the correlation coefficient and coefficient of variation (CV) of the qPCR multiplex assay. These values were calculated based on the results of five replicates.

2.6 Identification of Mixed Fungal DNA

To detect different species in a single reaction, we combined *R. arrhizus* and *A. niger* DNA. For this analysis, 0.5 µL of each gDNA was used at 2 ng/µL in a reaction volume of 15 µL corresponding to 66 pg/µL in the final reaction. The PCR multiplex reaction was performed as described in Sect. 2.4.

2.7 Total DNA Extraction from Human Plasma Spiked with Fungal DNA

Blood samples (approximately 10 mL) were collected from healthy donors in K3-EDTA tubes. Samples were centrifuged at 3000 rpm for 10 min and plasma was divided into 500 µL aliquots. Plasma aliquots were spiked with 400 ng of fungal DNA, and total DNA extraction was performed according to the Triton/Heat/Phenol (THP) protocol described by Xue et al. [41]. Extraction DNA was then resuspended in 25 µL of warm sterile water. The efficiency of the extraction was determined by comparing qPCR results with the DNA before and after plasma extraction [42]. To assess the concentration of fungal DNA present in the spiked samples, a qPCR standard curve of amplification was generated using a range of known fungal DNA concentrations. From this curve, the fungal DNA concentration was extrapolated. All manipulations were performed in a category 2 laminar flow cabinet to avoid contamination.

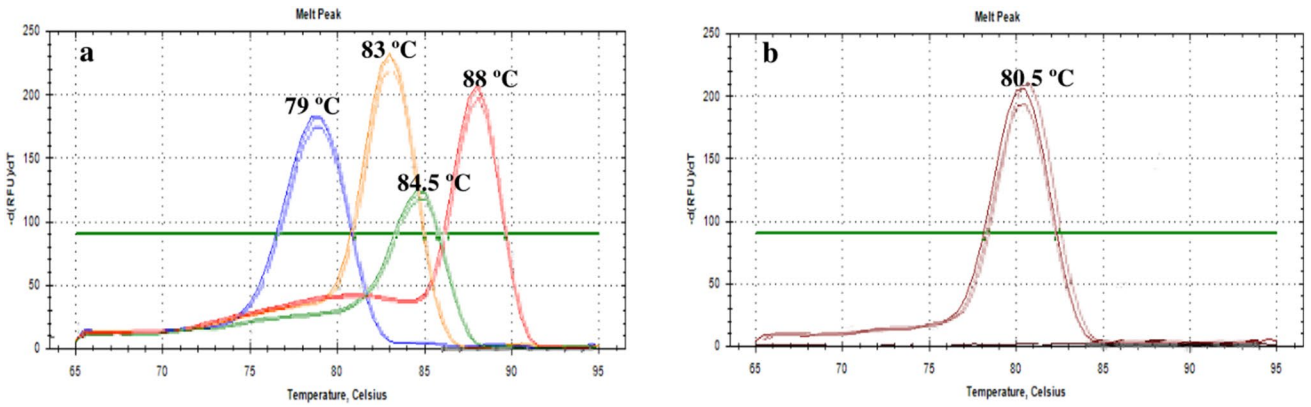


Fig. 1 Representative melting curve analysis profiles obtained by singleplex with 20 ng of gDNA. **a** Aspergillus species—*A. fumigatus*, 79 °C (blue); *A. flavus*, 83 °C (orange); *A. niger*, 84.5 °C (green); *A. terreus*, 88 °C (red). **b** *Rhizopus arrhizus* 80.5 °C (brown) ($n = 3$)

3 Results and Discussion

3.1 Melting Curve Analysis for Reference Strains—Singleplex

Primers for amplification of each species were first tested in singleplex. The qPCR conditions for this initial analysis were the same as those used by Carvalho-Pereira and colleagues [35] and the representative melting temperatures (Tm) for each *Aspergillus* species (Fig. 1a) and *Rhizopus arrhizus* (Fig. 1b) were determined.

The Tm values obtained were clearly distinct for the four *Aspergillus* species: *A. fumigatus* 79 °C (± 0.5 °C); *A. flavus* 83 °C (± 0.5 °C); *A. niger* 84.5 °C (± 0.5 °C); and *A. terreus* 88 °C (± 0.5 °C), and for *R. arrhizus* 80.5 °C (± 0.5 °C), indicating that all five species could be distinguished in a multiplex qPCR reaction. The specificity of each primer pair was evaluated with DNA from the target species, but also with DNA from non-target filamentous fungi (*Penicillium* spp., *Mucor velutinas*, *Aspergillus versicolor*, *Scopulariopsis brevicaulis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes,* and *Microsporum canis*), yeast species (*C. albicans*, *C. glabrata, C. tropicalis, C. krusei, C. parapsilosis,* and *C. auris*) and bacteria (*Escherichia coli*, *Staphylococcus aureus*). No cross-amplification was observed (Supplementary Data 2, see ESM).

3.2 Multiplex qPCR

Even though there were no cross-amplifications in singleplex reactions, interactions between primers in multiplex reactions can lead to non-target amplifications. This is a known problem when primers are designed within the same region, as is the case with most of the methods developed that favor amplification at the internal transcribed spacer (ITS) regions. In this study, because primers were designed for specific regions outside the ITS regions, multiplex amplification was expected to be successful. However, a non-specific melting peak at 84.5 °C was observed in the multiplex analysis when DNA from *A. fumigatus* was used, suggesting that primers might bind to different regions or with each other, resulting in primer-dimers. There were no non-specific melting peaks when using DNA from the remaining species (Fig. 2).

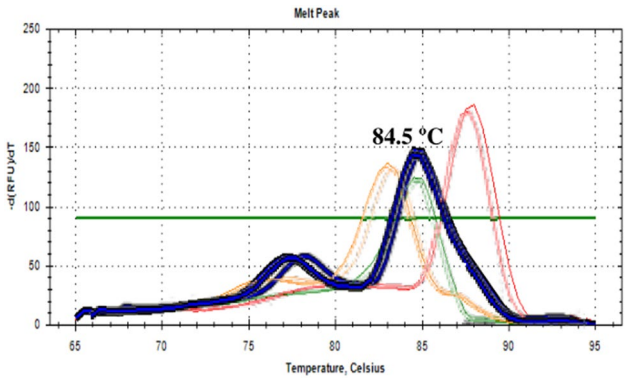


Fig. 2 Representative melting curve analysis profiles obtained by multiplex with 20 ng of gDNA. *A. flavus* (orange); *A. niger* (green); *A. terreus* (red); *A. fumigatus* (blue). It can be observed by the analysis of *A. fumigatus* melting peak that the non-specific binding (84.5 °C) is hampering the specific one (79 °C) ($n = 3$)

To overcome this limitation, two PCR reaction enhancers were tested. These enhancers are known to increase the yield of the desired PCR product and decrease the formation of non-specific products. Betaine helps the amplification of templates that form secondary structures, especially GC-rich templates [43–45]. The nonionic detergent Nonidet P-40 (NP-40) is also known to enhance DNA amplification by reducing the formation of non-specific products [44, 45]. Betaine (Fig. 3b), NP-40 (Fig. 3c), and betaine + NP-40 (Fig. 3d) were tested for amplification with DNA from *A. fumigatus* and compared with the condition without PCR enhancers (Fig. 3a). The use of betaine allowed correct amplification of the target sequence of *A. fumigatus* in multiplex, as the non-specific melting peak was no longer observed (Fig. 3b). NP-40 also increased the yield of the specific melting peak, but the non-specific peak was still observed (Fig. 3c). The combined use of betaine and NP-40 seems to disfavor the amplification of the target sequence at the conditions used in this study (Fig. 3d). Thus, betaine was the selected enhancer. However, the expected Tm for *A. fumigatus* was 79 °C, but with betaine the melting peak was 78 °C. It is known that the intrinsic mechanism of action of betaine lowers DNA melting temperatures [46]. Therefore, it was essential to redetermine the Tm of each species in the multiplex reaction with betaine and to compare them with the singleplex reaction with betaine (Fig. 4). Table 3 shows the Tm values obtained for each species after introduction of betaine into the qPCR reaction. When comparing the melting temperatures observed for each species considering the singleplex and multiplex reactions, no statistically significant differences were observed. To balance and improve the amplification profiles, the multiplex qPCR conditions were further optimized. The results showed that for the multiplex qPCR reaction, 1.5 mM $MgCl_2$, and annealing temperature of 61 °C, and 1 M betaine were the best conditions (Fig. 5).

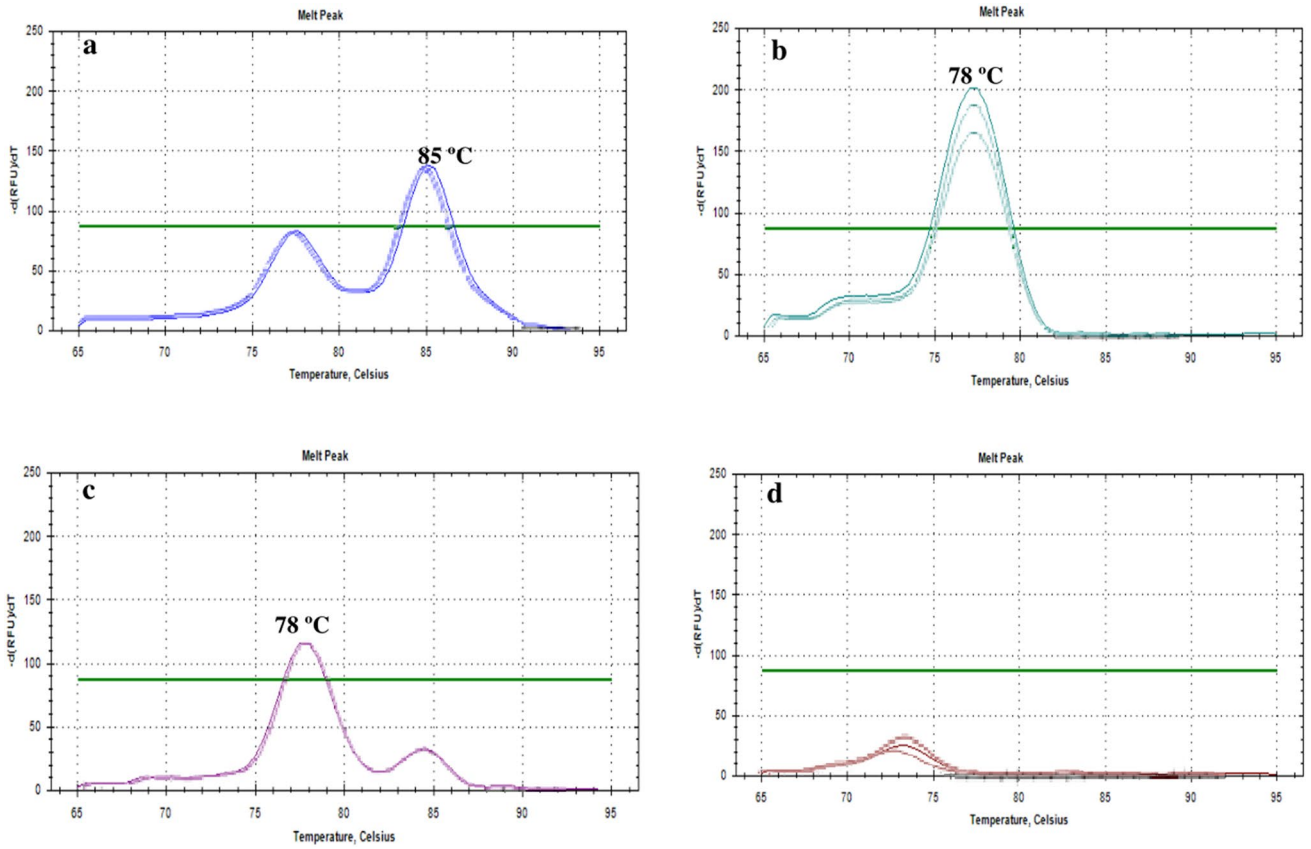


Fig. 3 Representative melting curve analysis profiles obtained by multiplex with 20 ng of gDNA of *A. fumigatus*, when using: **a** no introduction of PCR enhancers; **b** betaine 0.8 M; **c** NP-40 0.8%; **d** betaine 0.8 M + NP-40 0.8% ($n = 3$)

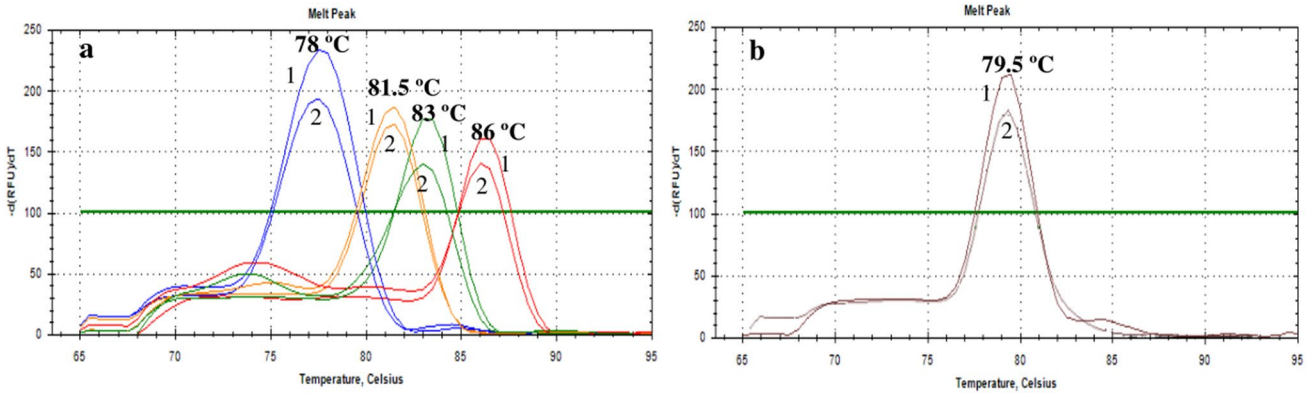


Fig. 4 Representative melting curve analysis profiles obtained by: **a** 1—singleplex and 2— multiplex amplifications with 20 ng of gDNA: *A. fumigatus*, 78 °C (blue); *A. flavus*, 81.5 °C (orange); *A. niger*, 83 °C (green); *A. terreus*, 86 °C (red). Each melting temperature (Tm) obtained in the singleplex reaction corresponded to the same Tm in the multiplex. **b** 1—singleplex and 2—multiplex with 20 ng of gDNA: *R. arrhizus*, singleplex 79.5 °C (brown)

Table 3 Melting temperature values obtained for each species, according to the addition of betaine to the qPCR reaction

Filamentous fungi panel		
Species	Melting temperature value (°C)	
	Singleplex qPCR	Multiplex qPCR
Aspergillus fumigatus	78.0 (± 0.5)	78.0 (± 0.5)
Aspergillus niger	83.0 (± 0.5)	83.0 (± 0.5)
Aspergillus flavus	81.5 (± 0.5)	81.5 (± 0.5)
Aspergillus terreus	86.0 (± 0.5)	86.0 (± 0.5)
Rhizopus arrhizus	79.5 (± 0.5)	79.5 (± 0.5)

qPCR quantitative polymerase chain reaction

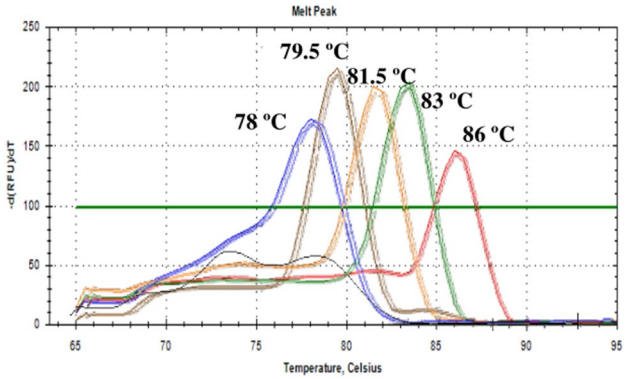


Fig. 5 Representative melting curve analysis profiles obtained by multiplex with 2 ng of gDNA, with optimized conditions of MgCl2 (1.5 mM), betaine (1 M) annealing temperature (61 °C), and primers concentration. *A. fumigatus,* 78 °C (blue); *A. flavus,* 81.5 °C (orange); *A. niger,* 83 °C (green); *A. terreus,* 86 °C (red); *R. arrhizus,* 79.5 °C (brown); negative control (black) ($n = 3$)

Regarding primers concentration, primers for *A. fumigatus* and *A. terreus* at 0.13 µM, primers for *A. flavus* and *R. arrhizus* at 0.16 µM, and primers for *A. niger* at 0.23 µM were found to improve the amplification curves and to balance the melting curve profiles and limit the interactions in the negative control. In addition, increasing betaine concentration from 0.8 to 1 M did not change the Tm values of the amplification product in all species tested. All target strains were correctly identified, including two clinical isolates from Braga Hospital, identified as *A. fumigatus* and *R. arrhizus*. Each singleplex and multiplex qPCR reaction was performed in a final volume of 15 µL, and the representative mixtures are listed in Table 4. In our previous study [35], PCR fragments were detected by capillary electrophoresis followed by GeneScan analysis after 50 PCR cycles. However, 50 cycles are not recommended for qPCR because they increase the probability of non-specific amplifications [47]. Therefore, PCR cycles were increased to 45 to raise the sensitivity of the method in detecting small amounts of gDNA (Supplementary Data 3, see ESM).

3.3 Quantitative Performance and Limit of Detection

To validate the assay, standard curves of 10-fold dilutions of gDNA of each species were analyzed (Supplementary Data 4, see ESM). The correlation coefficients of the standard curves ranged from 0.99 to 0.97 with low variability between replicates (< 2.5% CV). The efficiencies of the primers ranged from 91 to 103% using samples diluted in water, which is within the recommended values [48]. Specifically, amplification efficiencies were 96.3% for *A. fumigatus*, 92.0% for *A. flavus*, 103.3% for *A. niger*, 98.7% for

Table 4 Singleplex and multiplex qPCR reactions

qPCR Mix	Singleplex qPCR	Multiplex qPCR
Reaction volume	15 µL	15 µL
NZYSpeedy qPCR Green Master Mix (NZYTech)	7.5 µL	7.5 µL
Primers concentration	0.2 µM of each primer	0.13 µM of each *A. fumigatus* and *A. terreus* primers; 0.16 µM of each *A. flavus* and *R. arrhizus* primers; 0.23 µM of each *A. niger* primers
Fungal DNA	1 µL, corresponding to 20 ng	1 µL, corresponding to 20 ng and 2 ng
Betaine (5 M)		3 µL
Magnesium chloride (25 mM)		0.9 µL
Water	5.9 µL	0.1 µL

qPCR quantitative polymerase chain reaction

A. terreus, and 91.4% for *R. arrhizus* (Supplementary Data 4, see ESM).

Invasive fungal infections are associated with low levels of gDNA from the pathogen in clinical samples. Therefore, it was important to determine the limit of detection (LOD) of the multiplex reaction, which is defined by the absolute highest Cq value that reaches the threshold, generating a positive result, and detects amplification of the target sequence (with 95% confidence) (Fig. 6). The results show that all target species present a clear and distinct Tm peak in the melting curve analysis when 13.0, 1.3, and 0.13 ng/µL of gDNA were used (Fig. 6 dilutions 1, 2, and 3). When 0.013 ng/µL of gDNA was used, *A. terreus* was not detected despite the appearance of a melting peak with the expected Tm but below the threshold (Fig. 6e, dilution 4). In addition, *A. niger* and *A. flavus* were not detected when 0.0013 ng/µL of gDNA was used (Fig. 6c, d, dilution 5). *A. fumigatus* and *R. arrhizus* were detected at 0.0013 ng/µL gDNA (Fig. 6a, b, dilution 5). None of the species were detected at 0.00013 ng/µL gDNA (Supplementary Data 5, see ESM). Thus, the results show that LOD for *A. terreus* was 0.13 ng/µL, for *A. niger* and *A. flavus* the value was 0.013 ng/µL, and for *A. fumigatus* and *R. arrhizus* it was 0.0013 ng/µL.

3.4 Identification of Mixed Fungal DNA

The ability of the qPCR method to detect different species in a single reaction was also evaluated. Since some studies have reported coinfections between aspergillosis and mucormycosis, particularly between *R. arrhizus* and *A. niger* [49–51], this combination was tested (Fig. 7). For this analysis, 66 pg/µL of each gDNA were used in a reaction volume of 15 µL, which is within the previously defined LOD values. The qPCR reaction resulted in two Tm peaks with the expected values (79.5 °C for *R. arrhizus* and 83.0 °C for *A. niger*). This result indicates that even using a low concentration of gDNA, it was possible to identify the two species in mixed infections.

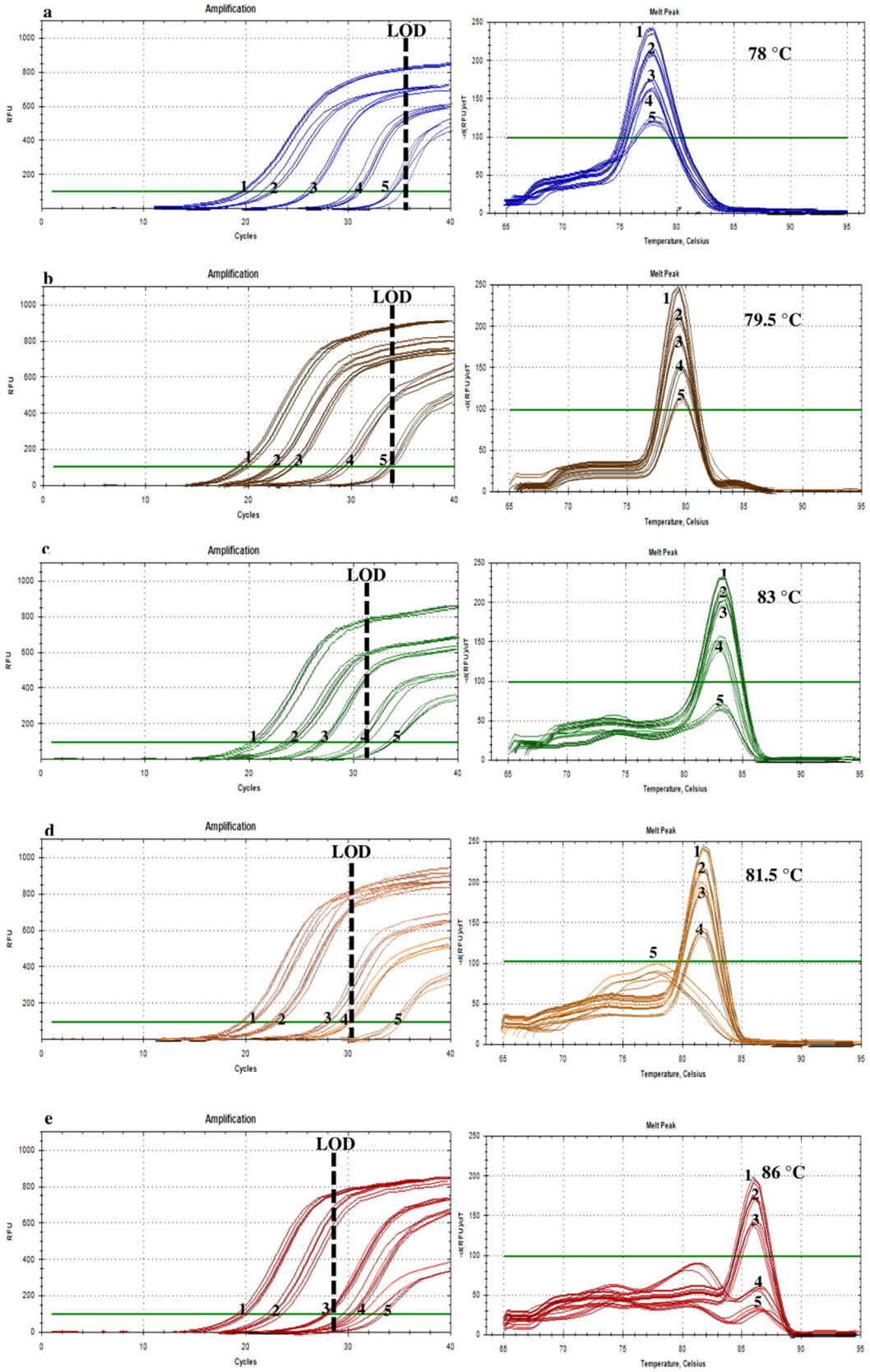
3.5 Human Plasma Spiked with Fungal DNA

The ability of the method to detect fungal DNA in clinical blood samples was mimicked by spiking DNA in plasma from healthy donors. For this purpose, plasma aliquots were spiked with fungal DNA. The extraction efficiency was $< 15\% \pm 3.48$, which is relatively low but within the values presented by commercially available recommended kits (QIAmp DNA Midi Kit, [41]). After extraction, to assess the concentration of fungal DNA present in the spiked samples, a qPCR standard curve of amplification was generated using a range of known fungal DNA concentrations (Fig. 8). From this curve, the fungal DNA concentration was extrapolated, which ranged from 0.4 to 1.7 ng/µL. These values were lower than expected, around 16 ng/µL (400 ng/25 µL, see Sect. 2.7), but still within the detection limit of this multiplex qPCR. Therefore, the multiplex qPCR reactions were performed and the amplifications were compared with the gDNA isolated from each fungal species, as positive control (Fig. 9). Additional non-template controls were also tested using DNA extracted from non-spiked plasma and water (Fig. 9). Amplification of this DNA from the spiked samples with primers for the ITS region was performed as control (Supplementary Data 6, see ESM). The results showed that the developed qPCR method was able to detect fungal DNA in biological samples with *A. fumigatus* (26.0 pg/µL, Cq = 26.96), *A. niger* (40.0 pg/µL, Cq = 26.45), *A. terreus* (66.0 pg/µL, Cq = 25.79), *R. arrhizus* (100.0 pg/µL Cq = 25.24), and *A. flavus* DNA (113.0 pg/µL, Cq = 25.03), without showing cross-amplification with human DNA and maintaining the representative Tm values for each species.

Curiously, a valid amplification was obtained with *A. terreus* even at 66 pg/µL, which was not consistent with the previously defined LOD for this species (130 pg/µL). Because there was a 10-fold dilution between the last gDNA concentration that provided a true amplification (2 ng/µL) and the first concentration that delivered a negative result (0.2 ng/µL), amplifications were performed with 1 ng/µL and 500 pg/µL of gDNA of *A. terreus* (Fig. 10). The results showed correct *A. terreus* amplifications (Tm of 86 °C) with both DNA concentrations. So, the LOD for *A. terreus* was considered to be 0.033 ng/µL.

4 Conclusion

Identification of fungal pathogens by traditional methods is time consuming and hinders patient treatment, especially because of the low sensitivity and specificity of these methods. For several years, the adoption of molecular methods for the diagnosis of invasive infections was limited, in part because of the lack of standardization of these methods. However, technological advances and genetic knowledge have allowed the development of molecular methods that are robust enough to be validated and verified, and to be


a
Amplification
LOD
RFU
Cycles
Melt Peak
78 °C
-d(RFU)/dT
Temperature, Celsius
b
Amplification
LOD
RFU
Cycles
Melt Peak
79.5 °C
-d(RFU)/dT
Temperature, Celsius
c
Amplification
LOD
RFU
Cycles
Melt Peak
83 °C
-d(RFU)/dT
Temperature, Celsius
d
Amplification
LOD
RFU
Cycles
Melt Peak
81.5 °C
-d(RFU)/dT
Temperature, Celsius
e
Amplification
LOD
RFU
Cycles
Melt Peak
86 °C
-d(RFU)/dT
Temperature, Celsius

◂**Fig. 6** Representative amplification curve profiles and melting curve analysis profiles obtained by multiplex with: (1) 13 ng/µL; (2) 1.3 ng/µL; (3) 0.13 ng/µL; (4) 0.0013 ng/µL; (5) 0.00013 ng/µL of gDNA. *A. fumigatus,* 78 °C (blue); *R. arrhizus,* 79.5 °C (brown); *A. niger,* 83 °C (green); *A. flavus,* 81.5 °C (orange); *A. terreus,* 86 °C (red) ($n = 5$)

commercially available. Molecular techniques, particularly qPCR methods, have allowed a significant reduction in turn-around time, resulting in more efficient, specific, and fast diagnosis. This is critical for a timely delineation therapeutic plan that increases survival, but also leads to a reduction in the number of patients admitted to the intensive care unit, which generates approximately $30,000 per patient for the hospital [52].The correct way to improve the diagnosis of invasive fungal infections seems to be the interpretation of the results of more than one method, preferably a serological assay and a PCR-based method [53].

The Filamentous Fungi Panel optimized for multiplex qPCR had the advantage of being able to detect and clearly distinguish the four most prevalent pathogenic species involved in invasive aspergillosis with no cross-amplification with human DNA. Additionally, this qPCR method was still able to distinguish *Aspergillus* species from *R. arrhizus* in a single reaction, which is a problem in several multiplex PCR-based methods [48]. Most of the commercially available options target the polygenic ITS while our markers target specific monogenic regions. This has the advantage of limiting cross-amplification, but has a direct impact on the LOD. While with monogenic markers LOD is in the nanograms range, lower LOD can be achieved with lower polygenic markers, ranging from 1000 to 100 femtograms [50–64]. The SYBR-Green assay was chosen to provide rapid and accurate results in a cost-effective manner, providing a tool to help in the diagnosis of filamentous fungi infections, especially for low-income health facilities in developing countries. However, apart from the cost-effective advantage of SYBR Green, probe-based techniques provide a higher sensitivity and reproducibility, and for this reason most commercially available and FDA-approved qPCR methods are probe-based techniques. However, it is important to emphasize that adapting this panel to probe-based detection would not only increase the associated costs, but also the need for a qPCR platform capable of detecting at least five different wavelengths at the same time. Nevertheless, our qPCR method was able to rapidly (approximately 1 h and 30 min) and accurately identify the most common filamentous fungi involved in systemic infections in a single step after DNA extraction from the samples, showing great potential to

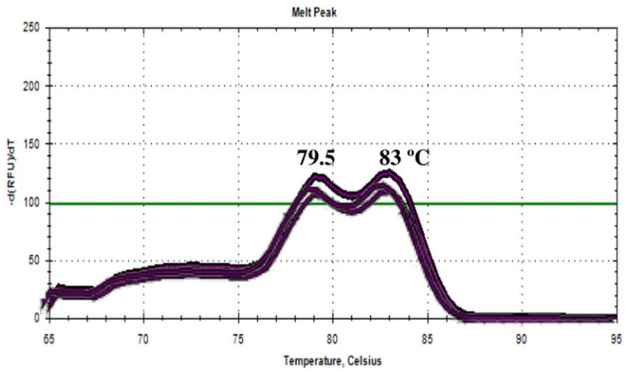


Fig. 7 Representative melting curve analysis profile obtained by multiplex with 66 pg/µL gDNA of *R. arrhizus* (79.5 °C) and 66 pg/µL gDNA of *A. niger* (83 °C) ($n = 3$)

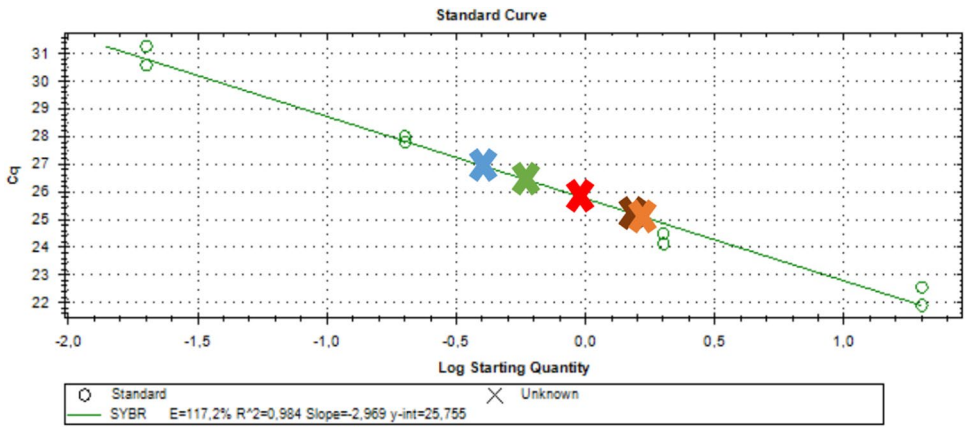
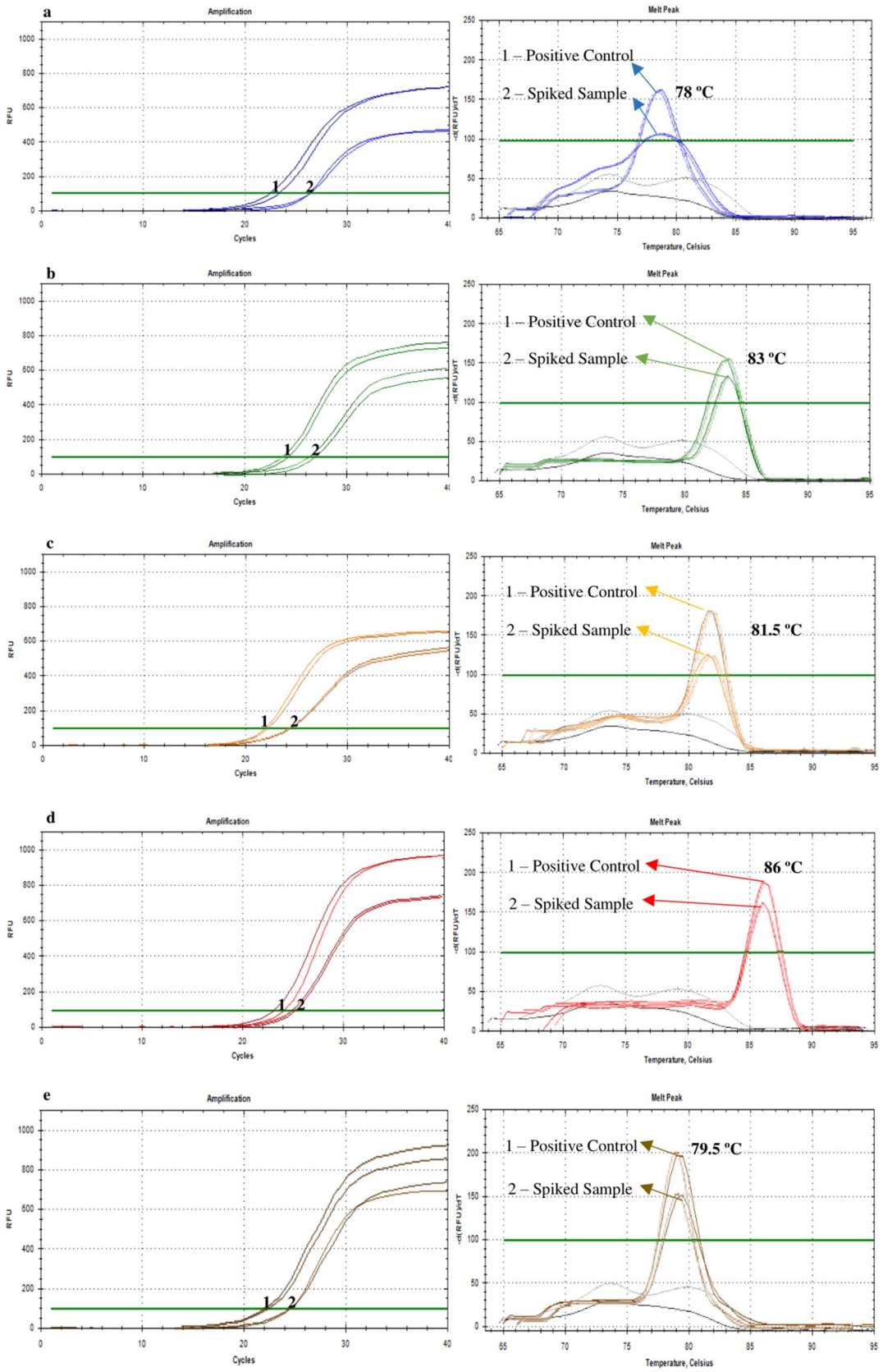


Fig. 8 The log of each known concentration in the dilution series (*x* axis) is plotted against the quantification cycle (Cq) value for that concentration (*y* axis). Duplicate dilution series corresponding to 20, 2, 0.2 and 0.02 ng/µL of gDNA. *A. fumigatus* DNA yield of 0.4 ng (blue); *A. niger* DNA yield of 0.6 ng (green); *A. terreus* DNA yield of 1.0 ng (red); *R. arrhizus* DNA yield of 1.5 ng (brown); *A. flavus* DNA yield of 1.7 ng (orange)


a
Amplification
RFU
Cycles
Melt Peak
-d(RFU)/dT
Temperature, Celsius
1 – Positive Control
2 – Spiked Sample
78 ºC
b
Amplification
RFU
Cycles
Melt Peak
-d(RFU)/dT
Temperature, Celsius
1 – Positive Control
2 – Spiked Sample
83 ºC
c
Amplification
RFU
Cycles
Melt Peak
-d(RFU)/dT
Temperature, Celsius
1 – Positive Control
2 – Spiked Sample
81.5 ºC
d
Amplification
RFU
Cycles
Melt Peak
-d(RFU)/dT
Temperature, Celsius
1 – Positive Control
2 – Spiked Sample
86 ºC
e
Amplification
RFU
Cycles
Melt Peak
-d(RFU)/dT
Temperature, Celsius
1 – Positive Control
2 – Spiked Sample
79.5 ºC

◂**Fig. 9** Representative melting curve analysis profiles obtained by multiplex with: (1) positive control—1.3 ng/µL of gDNA; (2) human plasma spiked with fungal gDNA. *A. fumigatus,* 78 °C (blue); *A. niger*, 83 °C (green); *A. flavus*, 81.5 °C (orange); *A. terreus*, 86 °C (red); *R. arrhizus*, 79.5 °C (brown); negative control—human DNA (grey); non-template control—water (black) ($n = 2$)

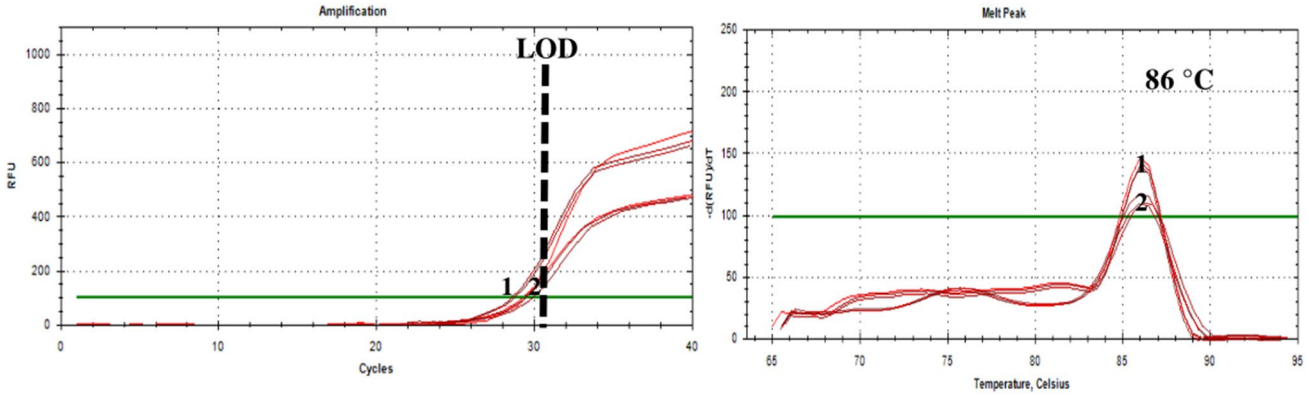


Fig. 10 Representative amplification curve profiles and melting curve analysis profiles obtained by multiplex with: (1) 0.06 ng/µL; (2) 0.03 ng/µL, gDNA of *A. terreus* (86 °C) ($n = 3$)

aid in the diagnosis of these infections. With the samples used, this method proved to be suitable for identification of the fungal pathogens. However, the proposed methodology should be further validated with clinical samples from *proven* infection, such as blood samples and biopsies, and samples from BAL and CSF, this being a limitation of this study. Since clinical samples usually contain a lower fungal burden, future work should validate the detection limits in clinical settings.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40291-022-00595-1.

Declarations

Conflict of interest All authors report no conflicts of interest relevant to this article.

Data and code sharing Not applicable.

Funding Nothing to declare.

Ethics approval and consent Nothing to declare.

Author contributions All authors contributed to the study conception and design. Experiments were performed by AM and JCP, data was analyzed by RFD and PS. All authors wrote and revised the manuscript.

References

1. Challa S. Pathogenesis and pathology of invasive aspergillosis. Curr Fungal Infect Rep. 2018;12:23–32.
2. Dagenais TRT, Keller NP. Pathogenesis of *Aspergillus fumigatus* in invasive aspergillosis. Clin Microbiol Rev. 2009;22:447–65.
3. Firacative C. Invasive fungal disease in humans: are we aware of the real impact? Mem Inst Oswaldo Cruz. 2020;115:1–9.
4. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases—estimate precision. J Fungi. 2017;3:5.
5. Mühlemann K, Wenger C, Zenhäusern R, Täuber MG. Risk factors for invasive aspergillosis in neutropenic patients with hematologic malignancies. Leukemia. 2005;19:545–50.
6. Cooper B, Dubberke ER, Morris Engemann A, Freifeld AG, Greene JN, Ito JI, et al. Prevention and treatment of cancer-related infections, version 2.2016 clinical practice guidelines in oncology NCCN categories of evidence and consensus. JNCCN J Natl Compr Cancer Netw. 2016;14:882–913.
7. Gregg KS, Kauffman CA. Invasive aspergillosis: epidemiology, clinical aspects, and treatment. Semin Respir Crit Care Med. 2015;36:662–72.
8. Ullmann AJ, Aguado JM, Arikan-Akdagli S, Denning DW, Groll AH, Lagrou K, et al. Diagnosis and management of *Aspergillus* diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. Clin Microbiol Infect. 2018;24:e1-38.
9. Zilberberg MD, Nathanson BH, Harrington R, Spalding JR, Shorr AF. Epidemiology and outcomes of hospitalizations with invasive aspergillosis in the United States, 2009–2013. Clin Infect Dis. 2018;67:727–35.
10. Petrikkos G, Skiada A, Lortholary O, Roilides E, Walsh TJ, Kontoyiannis DP. Epidemiology and clinical manifestations of mucormycosis. Clin Infect Dis. 2012;54:S23–34.
11. Prakash H, Chakrabarti A. Global epidemiology of mucormycosis. J Fungi. 2019;5:26.
12. Maran G. Mucormycosis: an emerging disease? Clin Infect Dis. 2006;12:7–23.

13. Chander J, Kaur M, Singla N, Punia RPS, Singhal SK, Attri AK, et al. Mucormycosis: battle with the deadly enemy over a five-year period in India. J Fungi. 2018;4:46.
14. Kubota N, Miyazawa K, Shoji N, Sumi M, Nakajima A, Kimura Y, et al. A massive intraventricular thrombosis by disseminated mucormycosis in a patient with myelodysplastic syndrome during deferoxamine therapy. Haematologica. 2003;88:EIM13.
15. Kontoyiannis DP, Wessel VC, Bodey GP, Rolston KVI. Zygomycosis in the 1990s in a tertiary-care cancer center. Clin Infect Dis. 2000;30:851–6.
16. Nosari A, Oreste P, Montillo M, Carrafiello G, Draisci M, Muti G, et al. Mucormycosis in hematologic malignancies: an emerging fungal infection. Haematologica. 2000;85:1068–71.
17. Cornely OA, Alastruey-Izquierdo A, Arenz D, Chen SCA, Dannaoui E, Hochhegger B, et al. Global guideline for the diagnosis and management of mucormycosis: an initiative of the European Confederation of Medical Mycology in cooperation with the Mycoses Study Group Education and Research Consortium. Lancet Infect Dis. 2019;19:405–21.
18. Peter Donnelly J, Chen SC, Kauffman CA, Steinbach WJ, Baddley JW, Verweij PE, et al. Revision and update of the consensus definitions of invasive fungal disease from the European organization for research and treatment of cancer and the mycoses study group education and research consortium. Clin Infect Dis. 2020;71:1367–76.
19. Mendonça A, Santos H, Franco-Duarte R, Sampaio P. Fungal infections diagnosis—past, present and future. Res Microbiol. 2021. https://doi.org/10.1016/j.resmic.2021.103915.
20. Franco-Duarte R, Černáková L, Kadam S, Kaushik KS, Salehi B, Bevilacqua A, et al. Advances in chemical and biological methods to identify microorganisms—from past to present. Microorganisms. 2019;7:130.
21. Arvanitis M, Anagnostou T, Fuchs BB, Caliendo AM, Mylonakis E. Molecular and nonmolecular diagnostic methods for invasive fungal infections. Clin Microbiol Rev. 2014;27:490–526.
22. Badiee P, Alborzi A, Karimi M, Pourabbas B, Haddadi P, Mardaneh J, et al. Diagnostic potential of nested PCR, galactomannan EIA, and beta-D-glucan for invasive aspergillosis in pediatric patients. J Infect Dev Ctries. 2012;6:352–7.
23. Guegan H, Robert-Gangneux F, Camus C, Belaz S, Marchand T, Baldeyrou M, et al. Improving the diagnosis of invasive aspergillosis by the detection of *Aspergillus* in broncho-alveolar lavage fluid: comparison of non-culture-based assays. J Infect. 2018;76:196–205.
24. Lackner M, Caramalho R, Lass-flörl C. Laboratory diagnosis of mucormycosis: current status and future perspectives. Future Microbiol. 2014;9:683–95.
25. Walsh TJ, Gamaletsou MN, McGinnis MR, Hayden RT, Kontoyiannis DP. Early clinical and laboratory diagnosis of invasive pulmonary, extrapulmonary, and disseminated mucormycosis (zygomycosis). Clin Infect Dis. 2012;54:55–60.
26. Skiada A, Pavleas I, Drogari-Apiranthitou M. Epidemiology and diagnosis of mucormycosis: an update. J Fungi. 2020;6:1–20.
27. Dichtl K, Seybold U, Ormanns S, Horns H. Evaluation of a novel *Aspergillus* antigen enzyme-linked immunosorbent assay. J Clin Microbiol. 2019;57:e00136–19.
28. Visited on-line: october 28, 2021. Bio-Rad. https://www.bio-rad.com/SearchResults?search_api_fulltext=elisa+platelia+aspergillus.
29. Visited on-line: november 16, 2021. Fungitell. https://www.fungitell.com/.
30. Gebhart C. Molecular microbiology: diagnostic principles and practice, 2nd edn. Lab Med. 2014. p. 489–500.
31. Zeller I, Schabereiter-Gurtner C, Mihalits V, Selitsch B, Barousch W, Hirschl AM, et al. Detection of fungal pathogens by a new broad range real-time PCR assay targeting the fungal ITS2 region. J Med Microbiol. 2017;66:1383–92.
32. Oliveira MME, Franco-Duarte R, Romeo O, Pais C, Criseo G, Sampaio P, et al. Evaluation of T3B fingerprinting for identification of clinical and environmental *Sporothrix* species. FEMS Microbiol Lett. 2015;362:1–7.
33. Da Silva RM, Da Silva Neto JR, Santos CS, Frickmann H, Poppert S, Cruz KS, et al. Evaluation of fluorescence in situ hybridisation (FISH) for the detection of fungi directly from blood cultures and cerebrospinal fluid from patients with suspected invasive mycoses. Ann Clin Microbiol Antimicrob. 2015;14:3.
34. Jafari Z, Motamedi M, Jalalizand N, Shokoohi GR, Charsizadeh A, Mirhendi H. A comparison between CHROMagar, PCR-RFLP and PCR-FSP for identification of Candida species. Curr Med Mycol. 2017;3:10–5.
35. Carvalho-Pereira J, Fernandes F, Araújo R, Springer J, Loeffler J, Buitrago MJ, et al. Multiplex PCR based strategy for detection of fungal pathogen DNA in patients with suspected invasive fungal infections. J Fungi. 2020;6:308.
36. Wen X, Chen Q, Yin H, Wu S, Wang X. Rapid identification of clinical common invasive fungi via a multi-channel real-time fluorescent polymerase chain reaction melting curve analysis. Med (United States). 2020;99:e19194.
37. Camp I, Manhart G, Schabereiter-Gurtner C, Spettel K, Selitsch B, Willinger B. Clinical evaluation of an in-house panfungal real-time PCR assay for the detection of fungal pathogens. Infection. 2020;48:345–55.
38. Valero C, De La Cruz-Villar L, Zaragoza Ó, Buitrago MJ. New panfungal real-time PCR assay for diagnosis of invasive fungal infections. J Clin Microbiol. 2016;54:2910–8.
39. Nolan T, Huggett J, Sanchez E. Good practice guide for the application of quantitative PCR (qPCR). Natl Meas Syst. 2013. p. 43–4.
40. Genomoics B. Real-time fluorescent RT-PCR kit for detecting SARS-CoV-2. 2020. p. 19–21.
41. Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clin Chim Acta. 2009;404:100–4.
42. Lion T. Human fungal pathogen identification: methods and protocols. Berlin: Springer; 2017.
43. Ralser M, Querfurth R, Warnatz HJ, Lehrach H, Yaspo ML, Krobitsch S. An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun. 2006;347:747–51.
44. Zhang Z, Kermekchiev MB, Barnes WM. Direct DNA amplification from crude clinical samples using a PCR enhancer cocktail and novel mutants of Taq. J Mol Diagn. 2010;12:152–61.
45. Visited on-line: november 19, 2021. Promega, PCR Amplification. Promega. https://worldwide.promega.com/resources/guides/nucleic-acid-analysis/pcr-amplification/.
46. Visited on-line: november 19, 2021. Ampliqon, PCR Enzymes & Reagens. Ampliqon. https://ampliqon.com/download.ashx?sku=A351104.
47. Visited on-line: november 19, 2021. Bio-Rad, PCR Troubleshooting. Bio-Rad. https://www.bio-rad.com/en-pt/applications-technologies/pcr-troubleshooting?ID=LUSO3HC4S#gel2.
48. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55:611–22.
49. Aqsa A, Droubi S, Glaser A. *Aspergillus* and *Rhizopus* fungal coinfection in a patient with multiple myeloma. Cureus. 2020;12:e8050.
50. Seo H, Kim JY, Son HJ, Jung J, Kim MJ, Chong YP, et al. Diagnostic performance of real-time polymerase chain reaction assay on blood for invasive aspergillosis and mucormycosis. Mycoses. 2021;50:0–2.

51. Bajpai T. A case of coinfection of *Aspergillus*, *Mucor* & *Rhizopus* case report in renal transplant patient from central India. J Clin Microbiol. 2016;3:32–5.
52. Arnold HM, Micek ST, Shorr AF, Zilberberg MD, Labelle AJ, Kothari S, et al. Hospital resource utilization and costs of inappropriate treatment of candidemia. Pharmacotherapy. 2010;30:361–8.
53. Arvanitis M, Anagnostou T, Mylonakis E. Galactomannan and PCR based screening for invasive aspergillosis among high-risk hematology patients: a diagnostic meta-analysis. Clin Infect Dis. 2015;61:1263–72.
54. Wickes BL, Wiederhold NP. Molecular diagnostics in medical mycology. Nat Commun. 2018;9:5135.
55. Denis J, Forouzanfar F, Herbrecht R, Toussaint E, Kessler R, Sabou M, et al. Evaluation of two commercial real-time PCR Kits for *Aspergillus* DNA detection in bronchoalveolar lavage fluid in patients with invasive pulmonary aspergillosis. J Mol Diagn. 2018;20:298–306.
56. Rath PM, Steinmann J. Overview of commercially available PCR assays for the detection of *Aspergillus* spp. DNA in patient samples. Front Microbiol. 2018;9:740.
57. Visited on-line: march 17, 2021. Bruker. www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/molecular-diagnostics/fungiplex-aspergillus.html.
58. Scharmann U, Kirchhoff L, Hain A, Buer J, Koldehoff M, Steinmann J, et al. Evaluation of three commercial PCR assays for the detection of azole-resistant *Aspergillus fumigatus* from respiratory samples of immunocompromised patients. J Fungi. 2021;7:1–10.
59. Visited on-line: march 22, 2021. ElitechGroup. elitechgroup.com/product/aspergillus-elite-mgb-kit.
60. Grancini A, Orlandi A, Lunghi G, Consonni D, Pozzi C, Rossetti V, et al. Evaluation of real time PCR *Aspergillus* spp. in bronchoalveolar lavage samples. New Microbiol. 2018;41:67–70.
61. Chong GLM, Van De Sande WWJ, Dingemans GJH, Gaajetaan GR, Vonk AG, Hayette MP, et al. Validation of a new *Aspergillus* real-time PCR assay for direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. J Clin Microbiol. 2015;53:868–74.
62. Chong GM, van der Beek MT, von dem Borne PA, Boelens J, Steel E, Kampinga GA, et al. PCR-based detection of *Aspergillus fumigatus Cyp51A* mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay® in 201 patients with haematological disease suspected for invasive aspergillosis. J Antimicrob Chemother. 2016;71:3528–35.
63. Visited on-line: march 20, 2021. Pathonostics. www.pathonostics.com/product/aspergenius.
64. White PL, Posso RB, Barnes RA. Analytical and clinical evaluation of the pathonostics aspergenius assay for detection of invasive aspergillosis and resistance to azole antifungal drugs directly from plasma samples. J Clin Microbiol. 2017;55:2356–66.
65. Visited on-line: march 30. Ingenetix. https://www.ingenetix.com/produkt/human/Aspergillus/.
66. Kidd SE, Chen SCA, Meyer W, Halliday CL. A new age in molecular diagnostics for invasive fungal disease: are we ready? Di. Front Microbiol. 2020;10:1–20.
67. Dannaoui E, Gabriel F, Gaboyard M, Lagardere G, Audebert L, Quesne G, et al. Molecular diagnosis of invasive aspergillosis and detection of azole resistance by a newly commercialized PCR kit. J Clin Microbiol. 2017;55:3210–8.
68. Jenks JD, Spiess B, Buchheidt D, Hoenigl M. Methods for detection of *Aspergillus fumigatus* resistance in clinical samples. Curr Fungal Infect Rep. 2019;13:129–36.
69. Prattes J, Hoenigl M, Zinke SEM, Heldt S, Eigl S, Johnson GL, et al. Evaluation of the new AspID polymerase chain reaction assay for detection of *Aspergillus* species: a pilot study. Mycoses. 2018;61:355–9.
70. Millon L, Scherer E, Rocchi S, Bellanger AP. Molecular strategies to diagnose mucormycosis. J Fungi. 2019;5:1–8.
71. Lackner N, Posch W, Lass-Flörl C. Microbiological and molecular diagnosis of mucormycosis: From old to new. Microorganisms. 2021;9:1518.
72. Mercier T, Reynders M, Beuselinck K, Guldentops E, Maertens J, Lagrou K. Serial detection of circulating mucorales DNA in invasive mucormycosis: a retrospective multicenter evaluation. J Fungi. 2019;5:1–10.
73. Guegan H, Iriart X, Bougnoux ME, Berry A, Robert-Gangneux F, Gangneux JP. Evaluation of MucorGenius® mucorales PCR assay for the diagnosis of pulmonary mucormycosis. J Infect. 2020;81:311–7.
74. Visited on-line: march 23, 2021. Pathonostics. https://www.pathonostics.com/product/mucorgenius.