Development of a single tube multiplex real-time PCR to detect the most clinically relevant Mucormycetes species

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Abstract

Mucormycetes infections are very difficult to treat and a delay in diagnosis could be fatal for the outcome of the patient. A molecular diagnostic technique based on Real Time PCR was developed for the simultaneous detection of *Rhizopus oryzae*, *Rhizopus microsporus* and the genus *Mucor* spp. in both culture and clinical samples. The methodology used was *Molecular beacon* species-specific probes with an internal control. This multiplex real-time PCR (MRT-PCR) was tested in 22 cultured strains and 12 clinical samples from patients suffering from a proven mucormycosis. Results showed 100% specificity and a detection limit of 1 fg of DNA per microlitre of sample. The sensitivity was 100% for clinical cultured strains and for clinical samples containing species detected by the PCR assay. Other mucormycetes species were not detected in clinical samples. This technique can be useful for clinical diagnosis and further studies are warranted.

Keywords: Mucormycetes, multiplex real time, fungal invasive infection

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Introduction

Mucormycosis is an important emerging fungal infection associated with high morbidity and mortality [1]. Infections caused by fungi belonging to the Mucorales order occur in immuno-compromised patients or those with chronic debilitating conditions such as ketoacidosis or uncontrolled diabetes mellitus, among others. It is a devastating fungal infection that progresses quickly and has a very poor prognosis due to the resistance of Mucormycetes to the newer antifungal agents, such as voriconazole and caspofungin [2].

The incidence of these infections has increased in the last decades as they have been reported from institutions in Europe and the USA [3,4]. The change in the epidemiology has been associated with widespread use of voriconazole or caspofungin [5] for the treatment of invasive aspergillosis, as these fungi are resistant to such antifungal drugs, although it remains unclear whether there is genuine epidemiological association with voriconazole or whether this is the result of changes in immunosuppression strategies [4,6]. The treatment of these mycoses is based on a combination of three types of measures: surgical resection, treatment with high doses of antifungal drugs and adjuvant therapies to control the underlying conditions that promote infection. Of the three measures, surgery is the most crucial and recommended whenever possible [7], given the extremely low rates of response to medical treatment [8]. The most recent therapeutic strategies are based on the role of the LAmB, posaconazole, and the use of adjuvant therapies such as employing deferaxirox [9].

Species identification is essential to choose the most suitable antifungal therapy because the different species of Mucormycetes exhibit different antifungal susceptibility profiles [10–12].

The diagnosis and management of mucormycosis remains a difficult task; indeed, there are no clinical or radiological signs specific to this disease [13]. Classical methods such as histopathology or direct visualization for observation of the

characteristic broad, non-septate and irregularly branched hyphae are not sensitive enough and are unable to identify the species [14]. Regarding cultures, these fungi are difficult to recover from cultures of clinical specimens due to concurrent antifungal therapy or to loss of hyphal viability in tissue homogenization prior to culture [15]. Even though serological tests have been attempted, these are not recommended [16,17]. On the other hand, immunohistochemical reagents that detect *Aspergillus* spp. and Mucormycetes in tissue are commercially available (AbDSerotec), but these methods have their limitations and they are unable to reach the species level [18]. Finally, the development of molecular techniques to detect Mucormycetes species is very recent. Conventional and real-time PCR-based techniques to amplify DNA from microbiological cultures or tissues have been reported [13,19–22], targeting multicopy genes such as 28S rDNA, Cytochrome b or ITS regions. In most cases, sequencing the amplicon or analysing melting curves was necessary to discriminate the different genera. Molecular techniques help improve the identification from cultures and, when the clinical sample is available, the direct diagnosis of mucormycosis using these techniques is the fastest method for appropriate therapy.

In this report, we describe the development of a multiplex real-time PCR technique (MRT-PCR) to detect DNA from the most frequents species involved in invasive mucormycosis.

Material and Methods

Control strains

DNA from different strains was used to standardize the MRT-PCR assay. Isolates were *Rhizopus oryzae* (CNM-CM-3020), *R. microsporus* (CNM-CM-4244) and *Mucor circinelloides* (CNM-CM-2437), all of them belonging to the Mould Collection of the Spanish National Center of Microbiology (CNM-CM). For the purposes of evaluating *Mucor* spp. detection, other species belonging to this genus were included, such as *Mucor circinelloides* (CNM-CM-5170), *Mucor plumbeus* (CNM-CM-5245), *Mucor racemosus* (CNM-CM-3862) and *Mucor velutinosus* (CNM-CM-5510). The specificity of this technique was assessed including other Mucormycetes such as *Cunninghamella bertholletiae* (CNM-CM4907), *Lichtheimia corymbifera* (CNM-CM5039 and CNM-CM5256), *Apophysomyces elegans* (CNM-CM6522), *Rhizomucor pusillus* (CNM-CM6144) and *Actinomucor elegans* (CNM-CM4894), as well as other fungal pathogens: *Aspergillus fumigatus* (CNM-CM-AF237), *Scedosporium apiospermum* (CNM-CM-3169), *Scedosporium prolificans* (CNM-CM-1627), *Fusarium solani* (CNM-CM-3035), *Fusarium oxysporum* (CNM-CM-2914) and *Candida albicans* (ATCC 64551).

DNA extraction

DNA extraction from cultured strains was performed as described previously by Tang *et al.* [23]. DNA extraction from clinical samples was performed using the QiAmp Tissue DNA Mini Kit (Qiagen, Izasa, Madrid, Spain) following the manufacturer's instructions. Fifty microlitres of elution buffer was used for elution. Two microlitres of extracted DNA were used for each PCR reaction. When the biopsies were embedded in paraffin, the paraffin was removed by one lavage with 1.2 mL of xylene followed by two lavages with 1.2 mL of ethanol (96–100%). After incubating the tissue at 37°C in order to evaporate the remains of the ethanol, DNA was extracted in the manner mentioned above.

Primers and probe design

The assay was designed in order to detect the most frequent species in human disease (genus *Mucor* and two species of genus *Rhizopus*) because we had a limitation of the number of fluorescent probes able to be detected by the PCR equipment. Primers and molecular beacon probes were designed on the basis of the nucleotide sequence of the ITS1 ribosomal DNA region from strains belonging to the collection of the Spanish National Center of Microbiology: 15 sequences of *R. oryzae* (submission ID, 1541001), and 10 sequences of *R. microsporus* (Submission ID: 1540471) and the sequence of the ITS2 region for *Mucor* spp belonging to the species *M. circinelloides* (accession numbers, JX094793, JX09794, JX09795, JX09796, JX09797, JX09798, JX09799, JX09800), *M. racemosus*, *M. plumbeus* and *M. velutinosus.* (submission ID, 1541277). Sequences from GenBank were also used for the design of the probe that targeted *Mucor* spp. species (accession numbers, AF412287, AY243947, AY243943, AF412290, AY243945).

The reason for choosing the ITS1 or ITS2 region as target was based on the possibility of designing a suitable probe for each case. The Beacon Designer 5.0 software (Premier Biosoft, Palo Alto, CA, USA) was used to design primers and probes. An internal control was included to ensure that negative results were not due to non-specific inhibition of the PCR assay. The internal control was designed on the basis of a jellyfish-derived sequence [24]. A pair of primers and a specific molecular beacon labelled with Cy5 were designed (Table 1). The primers amplified a 105 bp fragment, which was cloned in a p-GEMt plasmid (Promega, Madrid, Spain) to use as an internal control template. The minimum amount of internal control plasmid (pICJF) to be added to the reaction mix was adjusted in order to prevent the sensitivity of the

TABLE 1. Sequences of primers and probes designed for the multiplex real-time PCR assay

Primers and probes	
Rhizopus oryzae	
Oli Ror1 (f)	5′ TCTGGGGTAAGTGATTGC 3′
Oli Ror2 (r)	5′ GCGAGAACCAAGAGATCC 3′
Ror- MB1	5′ Cyan 500-CGCGATAACCAGGAGTGGCATCGATCAAATCGCG-BHQ1 3′
Rhizopus microsporus	
Oli Rmic1 (f)	5′ CTTCTCAGTATTGTTTGC 3′
Oli Rmic2 (r)	5′ ATGGTATATGGTAAAGGG 3′
Rmic-MB1	5′ HEX-CGCGATCCTCTGGCGATGAAGGTCGTATCGCG-BHQ1 3′
Mucor spp.	
Oli Mucor1 (f)	5′ GTCTTTGAACGCAACTTG 3′
Oli Mucor4 (r)	5′ CCTGATTTCAGATCAAAT 3′
Mucor-MB1	5′ FAM-CGCGATTTCCAATGAGCACGCCTGTTATCGCG-BHQ1 3′
Jellyfish (IC[a])	
Oli1-icjf1 (f)	5′ GCCTGGTGCAAAAATTGCTTATC 3′
Oli2-icjf2 (r)	5′ CTAAGACAAGTGTGTTTATGGTATTG 3′
CIJF-MB	5′ Cy5-CGCGATGCTGTTCTTCCGCCACTTCCAATCGCG-BHQ2 3′

f, forward; r, reverse.
[a]Internal control.

assay being affected. Two fentograms of the internal control plasmid were included in each assay. The primers and probes designed were subjected to a blast search in the GenBank sequence database (http://blast.ncbi.nim.gov/Blast.cgi) and in the database of the Department of Mycology of the Spanish National Center for Microbiology (more than 5000 distinct sequences) to avoid cross-homology with other microorganisms. The primers and probes sequences are shown in Table 1.

Multiplex real-time PCR assay

PCR reactions were performed in the Light Cycler 480 System (Roche Diagnostics, Mannheim, Germany). The kit 2x SensiMix DNA (Quantace, Ecogen, Madrid, Spain) was used according to the manufacturer's instructions. The PCR reaction (20 μL) contained 0.5 μM of each primer of the Mucormycetes species, 0.25 μM of the internal control primers, 3 mM $MgCl_2$, 0.2 μM *R. oryzae* and *R. microsporus* probes, 0.4 μM of *Mucor* spp.probe, 0.1 μM of internal control probe, 2 μL of pICJF and 2 μL of DNA extracted from the sample. The cycling conditions included preincubation at 95°C for 10 min and an amplification programme of 40 cycles as follows: denaturation, 95°C, 15 s; annealing, 50°C, 30 s, extension, 72°C, 15 s; cooling cycle, 40°C, 30 s. Quantification standards were run in conjunction with each set of samples as well as negative controls. Subsequently, a colour compensation experiment was performed to prevent 'crosstalk' between dyes.

The sizes of the amplicons generated were 192 bp for *R. oryzae*, 187 for *R. microsporus* and 263 bp for *Mucor* spp. DNA. PCR products were subjected to electrophoresis in 2% agarose gels (Pronadisa, Madrid, Spain), and amplified fragments were sequenced (ABI Prism 377 DNA sequencer; Applied Biosystems, Madrid, Spain) to confirm the PCR results.

Standardization

Standard curves were constructed with PCR results from five repetitions of different dilutions of *R. oryzae* (CNM-CM-3020), *R. microsporus* (CNM-CM-4244) and *Mucor circinelloides* (CNM-CM-2437) genomic DNA. Dilutions ranged from 20 ng to 2 fg DNA/20 μL of reaction. A line ($y = mx + b$) was constructed by plotting the standard curve of log quantity vs. its corresponding C_T value, a cycle in which fluorescence becomes detectable against background. If this curve exhibited a linear regression coefficient value of >0.980, the standard curve was then used to determine sensitivity, primer efficiencies and reproducibility of the assay. In order to evaluate the specificity, 2 ng of DNA/20 μL from six species of mucormycetes as well as other fungal species and human and mouse genomic DNA were included in the PCR assay (see Control strains).

MRT-PCR assay on cultured clinical strains

The specificity of the technique was assessed using 22 strains belonging to the Department of Mycology of the Spanish National Centre for Microbiology; six of them were *R. oryzae* strains, five *R. microsporus* and 11 *Mucor* spp. (*M. plumbeus* (1), *M. racemosus* (1) and *M. circinelloides* (9)). These strains had been previously identified by molecular methods. Two microlitres of the extracted DNA were used in each MRT-PCR reaction.

MRT-PCR on clinical samples from mucormycosis patients

The utility of MRT-PCR for diagnosis of clinical mucormycosis was evaluated through the use of clinical samples. Twelve biopsies from nine patients with proven mucormycosis were tested. Proven mucormycosis was considered when the fungus was recovered in culture from a specimen or when the typical irregular, broad and non-septate hyphae, were visualized by histopathology. The underlying diseases of patients

and the samples analysed are shown in Table 2. Two microlitres of DNA from each sample were used for the MRT-PCR reaction. When the assay was negative, the DNA from samples was used to amplify the its1 and its2 regions using the universal primers ITS1 and ITS4 and then sequencing to verify the result [25].

Results

In vitro standardization of the MRT-PCR technique

The designed MRT-PCR assay specifically detected *R. oryzae*, *R. microsporus* and the genus *Mucor* ssp. The sensitivity of this technique was 1 fg of DNA per microlitre of sample. The determination coefficient (r^2) of the linear regressions between crossing point values and different dilutions of genomic DNA was 0.98–0.99 ($p < 0.01$). The average coefficients of variation were 2% for *R. oryzae* DNA, 2.2% for *R. microsporus* DNA and 1.08% for *Mucor* spp. DNA. The Ct value for the internal control was 29.62 ± 0.05. The specificity of the MRT-PCR assay was 100%. No positive signal was detected when 2 ng of DNA from other fungi (*Aspergillus fumigatus*, *Scedosporium apiospermum*, *Scedosporium prolificans*, *Fusarium solani*, *Fusarium oxysporum* and *Candida albicans*) and genomic DNA from humans and mice was tested (Promega, Madrid, Spain). In addition, no cross-reaction was observed between these Mucormycetes species. Each species was detected in the corresponding fluorophore channel and other Mucormycetes such as *Cunninghamella bertholletiae*, *Lichtheimia corymbifera*, *Apophysomyces elegans*, *Rhizomucor pusillus* and *Actinomucor elegans* gave a negative result.

Real-time PCR assays for cultured clinical strains

Results were positive in all clinical strains tested. The MRT-PCR assay specifically detected DNA from six clinical strains of *R. oryzae*, five *R. microsporus*, nine *Mucor circinelloides*, one *Mucor racemosus* and one *Mucor plumbeus*. The crossing point values varied according to the amount of DNA obtained for each strain.

MRT-PCR assay on clinical samples from Mucormycosis patients

MRT-PCR was performed on 12 biopsy samples from nine patients. The source of the samples was very heterogeneous, depending on the patient's symptomatology (Table 2). In terms of patients, the MRT-PCR was positive in all cases in which the infection was produced by the species included in the MRT-PCR assay (7/9). The species detected were *R. oryzae* and *Mucor* spp. No patient was infected with *Rhizopus microsporus*. The assay was negative in two cases. The subsequent amplification of ITS regions and sequencing showed that samples that were negative contained *R. pusillus* (Case 1) and *C. bertholletiae* (Case 9). Fig. 1 shows the amplification signals in different channels depending on the species of Mucormycetes detected: (a) *R. oryzae* detection in clinical samples, (b) *Mucor* spp. detection in clinical samples and (c) *R. microsporus* detection in clinical strains. The amount of DNA obtained for each sample appears in Table 2. None of the assays presented inhibition. The internal control was amplified in all cases. Negative controls were never amplified.

Discussion

Molecular techniques have proven to be very useful in the correct identification of fungal species [26]. However, there are few reports that describe molecular diagnostic methods based on real-time PCR for Mucormycetes species. Some authors described a method to detect Mucormycetes species based on the analysis of the melting curves, but MRT-PCR approaches have never been described before [19,20].

TABLE 2. MRT-PCR results for clinical samples from patients with proven Mucormycosis

Case	Underlying disease	Fungal infection	Sample (biopsy)	PCR result	Amount DNA ng/μl
1	ND	Disseminated	Skin	Negative[a]	–
			Lung	Negative[a]	–
2	ND	Gastrointestinal	Stomach	*Rhizopus oryzae*	83×10^{-5}
			Mediastine		32×10^{-5}
3	Diabetes	Sinonasal	Nose	*Rhizopus oryzae*	13×10^{-2}
4	Autotransplant	Disseminated	Necropsy	*Mucor* spp.	38×10^{-5}
			Necropsy		31×10^{-5}
5	SOTR	Skin/Subcutaneous	Skin	*Rhizopus oryzae*	52×10^{-4}
6	Polytrauma	Subcutaneous	Parotid gland	*Rhizopus oryzae*	61×10^{-7}
7	Renal failure	Lung	Lung	*Rhizopus oryzae*	15×10^{-5}
8	SOTR	Rhinocerebral	ND	*Rhizopus oryzae*	15×10^{-7}
9	OD	Lung	Lung	Negative[b]	–

SOTR, solid organ transplant recipient; OD, oncohaematological disease; ND, no data.
[a]*Rhizomucor pusillus* by using primers its1 and its4 and then sequencing.
[b]*Cunninghamella. bertholletiae* by using primers its1 and its4 and then sequencing [28].

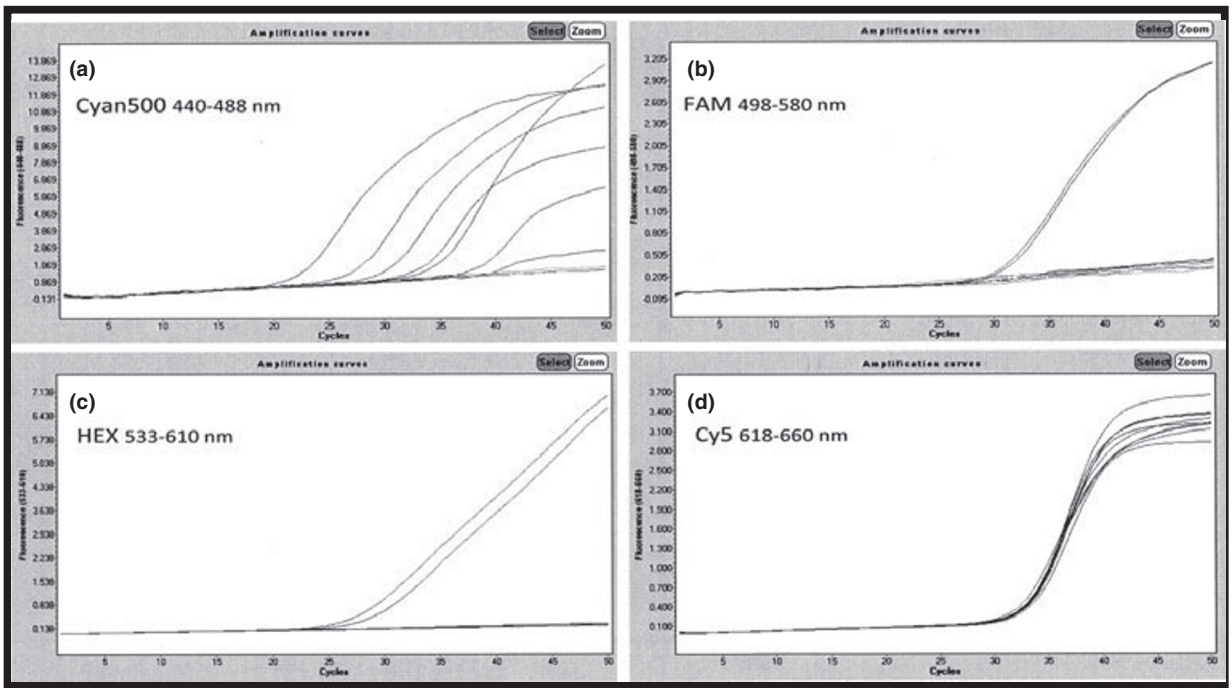

FIG. 1. The dependence of fluorescence signal on the number of cycles in multiplex real-time PCR. (a) The signal of Cyan 500 dye with *Rhizopus oryzae*-specific hybridization probe (clinical samples). (b) The signal of FAM dye with *Mucor* spp.-specific hybridization probe (clinical samples). (c) The signal of HEX dye with *Rhizopus microsporus*-specific hybridization probe (clinical strains). (d) The signal of Cy5 dye with pICJF-specific hybridization probe (clinical samples).

Different species of Mucormycetes exhibited varying antifungal susceptibility, hence the importance of correct identification [10]. Amphotericin B is the agent of choice to treat mucormycosis, although some strains of *Rhizopus* spp. and *Cunninghamella* spp. have high MIC values to this drug. Azole drugs had limited *in vitro* activity. In a recent study voriconazole was not active; itraconazole showed a wide range of MICs and only posaconazole showed good activity for all species, except for *Cunninghamella bertholletiae*. Finally, terbinafine showed good activity, except for *R. oryzae* and *M. circinelloides* [11].

In this context, species identification becomes essential for the proper choice of antifungal. The technique developed in this work is a single-tube MRT-PCR able to specifically detect the most common species causing mucormycosis from cultures and clinical samples. Designed probes and primers targeted the ITS regions of rDNA. An internal control was included in the assay to detect PCR inhibition. The assay was specific (100%), had a good sensitivity and reproducibility, and the results were obtained within 2–3 h. The MRT-PCR developed was first tested on 22 clinical strains belonging to *Rhizopus* and *Mucor* genera. All strains yielded positive results while other control species included in the assay were negative. Subsequently, the technique was validated using 12 biopsy samples from nine patients with proven mucormycosis. The MRT-PCR detected 100% of infections caused by *Rhizopus* spp. or *Mucor* spp. Only samples from patients one and nine were negative. The DNA from these samples was amplified and sequenced using universal ITS1 and ITS4 primers. The sequence confirmed that other species were involved in these cases.

To date there have been several publications which explain multiple amplification reactions based on conventional PCR and subsequent product sequencing stages to attain identification [19,20,27,28]. Some of these works were based on nested PCR that is not recommended for the diagnostic test as these assays were considered prone to producing false-positive results [29]. The advantages of our technique compared with others already published is that in a single tube and with a single amplification it can detect the most frequent Mucormycetes species causing the infection without post-amplification manipulation. Furthermore, it includes an internal control which ensures that the amplification reaction has not been inhibited. However, this technique has the limitation of the real time PCR equipments used, because the number of detection channels is not large and therefore only the most frequent species of Mucormycetes can be included in the study. In the future we hope to include new species as technological advances so allow.

As the more frequent species in human disease belong to the *Mucor* and *Rhizopus* genera, a molecular method able to specifically detect those species in a quick and sensitive way would enable earlier diagnosis. As quantitative assays, they may prove useful in monitoring a patient's general outcome and response to antifungal therapy.

In conclusion, the multiplex PCR assay developed seems to be a promising tool to complement the diagnosis techniques that are currently routinely used in microbiology laboratories. Molecular diagnosis of invasive mucormycosis is feasible and studies are needed using a larger sample number.

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Transparency Declaration

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