Molecular identification of *Mucorales* in human tissues: contribution of PCR electrospray-ionization mass spectrometry

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Abstract

Molecular methods are crucial for mucormycosis diagnosis because cultures are frequently negative, even if microscopy suggests the presence of hyphae in tissues. We assessed PCR/electrospray-ionization mass spectrometry (PCR/ESI-MS) for *Mucorales* identification in 19 unfixed tissue samples from 13 patients with proven or probable mucormycosis and compared the results with culture, quantitative real-time PCR, 16S–23S rRNA gene internal transcribed spacer region (ITS PCR) and 18S PCR sequencing. Concordance with culture identification to both genus and species levels was higher for PCR/ESI-MS than for the other techniques. Thus, PCR/ESI-MS is suitable for *Mucorales* identification, within 6 hours, for tissue samples for which microscopy results suggest the presence of hyphae.

Keywords: Culture, electrospray-ionization mass spectrometry, *Mucorales* identification, mucormycosis, PCR, species level

Introduction

Mucormycosis is an invasive fungal disease affecting severely immunocompromised patients, patients with uncontrolled diabetes and patients with severe trauma contaminated with soil [1]. The rapid diagnosis of mucormycosis is essential because mortality is high but can be decreased by rapid treatment initiation, including surgery, when possible [2]. Microbiologic diagnosis is based principally on microscopy and the culture of a fresh specimen, and on histologic analysis of formalin-fixed paraffin-embedded biopsy specimens. Culture results are often negative [1], but when positive, the isolate can be used for species identification and antifungal susceptibility testing [3]. Various nucleic acid–based identification methods for *Mucorales* identification have been developed, including quantitative real-time PCR (qPCR) for a portion of the 18S ribosomal RNA gene (18S rRNA gene) [4], PCR amplification and sequencing of 18S–28S rRNA gene internal transcribed spacer region (ITS PCR) [5] and sequencing of a region of the 18S rRNA gene (18S PCR) [6–8].

PCR coupled with electrospray-ionization mass spectrometry (PCR/ESI-MS) is a promising method [9,10] never before evaluated specifically for *Mucorales* detection and identification on tissues. PCR/ESI-MS involves 16 single-plex PCR assays on standardized DNA extracts, with broad-range primers targeting nuclear or mitochondrial genes; rapid determination of the molecular weight and base composition of the amplicon by electrospray-ionization mass spectrometry; and comparison with a specific database generated by the manufacturer. We compared the efficacy of PCR/ESI-MS with three other published molecular methods [4–6] for identification purposes on unfixed tissue biopsy specimens containing *Mucorales* hyphae visible upon direct or pathologic examination.

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Materials and methods

We collected 19 tissue biopsy specimens from 13 patients with proven mucormycosis according to the European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria [11]. Twelve specimens (all culture positive) from seven patients were obtained from patients included in the AMBIZYGO trial (NCT00467883; Lanternier *et al*., personal communication) and were frozen at −80°C (Table 1). Seven consecutive biopsy specimens from six patients with *Mucorales hyphae* visible on microscopy were collected prospectively over a 2-year period at Saint-Louis Hospital, Paris, France (Table 1). Each specimen was split in two. One part was placed on Sabouraud dextrose agar. For culture-positive specimens, one colony was subcultured and sent to the French National Reference Center for Invasive Mycoses and Antifungals, where species identification was performed by a polyphasic approach, as described elsewhere [12,13]. The other part of the sample was frozen at −80°C for molecular studies. It was then thawed, and 1 g of tissue was cut off with a sterile scalpel and used for DNA extraction on the PLEX-ID platform (Abbott Molecular). DNA extracts were subjected to PCR/ESI-MS, qPCR assays, and ITS PCR and 18S rRNA gene sequencing (Table 2).

PCR/ESI-MS analysis was performed with the Fungal Spectrum assay, targeting part of the 18S rRNA gene and the mitochondrial cytochrome *b* gene [9]. PCR was performed after DNA extraction in six wells for broad fungal identification and in 10 wells for specific identification. Amplicon mass was analysed with a PLEX-ID analyser (Abbott Molecular). Base composition was determined and compared with the PLEX-ID v2.0 Fungal Spectrum database. Results were considered validated if Q-score (confidence) was $\geq$0.85 and level (quantity) was $\geq$3. The three qPCR assays for *Mucorales* identification were performed as described elsewhere [4]. qPCR was a reliable method for fungal load determination, providing a quantification cycle (Cq) value. Species-level identification was intrinsically impossible in these qPCR assays. The transcribed spacer region (ITS) was amplified with ITS1–ITS2, ITS5–ITS2 and ITS3–ITS4 primers, together with part of the 18S rRNA gene (18S PCR) [6], and the amplicons were then subjected to sequencing. The species with the best score in the Mycobank database was considered [14].

We used Cohen's kappa coefficient to assess the consistency between the four methods and culture identification [15,16]. Sensitivity/specificity calculations were not possible because only microscopy-positive biopsy specimens were studied.

Results

Mucorales identification with PCR/ESI-MS, qPCR, ITS PCR and 18S PCR assays was compared with polyphasic culture

TABLE 1. Molecular identification of *Mucorales* in 19 unfixed human tissue samples from 13 patients with mucormycosis[a]

Patient no.	Sample no.	Culture identification	Biopsy site	PCR/ESI-MS identification	Positive qPCR assays (Cq)[b]	ITS PCR identification	18S PCR identification
1	1	*Saksenaea vasiformis*	Bone	*Apophysomyces elegans*	Amplification failure	*Saksenaea* spp.	*Apophysomyces elegans*
2	2	*Lichtheimia corymbifera*	Lung	*Lichtheimia corymbifera*	*Lichtheimia* spp. (29.2)	*Lichtheimia ramosa*	*Lichtheimia ramosa*
3	3	*Lichtheimia corymbifera*	Kidney	*Lichtheimia corymbifera*	*Lichtheimia* spp. (34.6)	*Lichtheimia corymbifera*	Amplification failure
	4		Skin (left arm)	*Lichtheimia corymbifera*	*Lichtheimia* spp. (35.6)	Amplification failure	Amplification failure
	5		Skin (left forearm)	*Lichtheimia corymbifera*	*Lichtheimia* spp. (39)	Amplification failure	Amplification failure
	6		Skin (left thigh)	*Lichtheimia corymbifera*	*Lichtheimia* spp. (38.4)	Amplification failure	Amplification failure
4	7	*Lichtheimia ramosa*	Kidney	*Lichtheimia corymbifera*	*Lichtheimia* spp. (30.3)	*Lichtheimia corymbifera*	*Lichtheimia corymbifera*
5	8	*Rhizopus arrhizus*	Mandibular bone	*Rhizopus arrhizus*	*Rhizopus* spp./*Mucor* spp. (33)	Amplification failure	Amplification failure
	9		Gum (left)	*Rhizopus arrhizus*	*Rhizopus* spp./*Mucor* spp. (15.7)	*Rhizopus arrhizus*	*Rhizopus* spp.
	10		Gum (right)	*Rhizopus arrhizus*	*Rhizopus* spp./*Mucor* spp. (31)	*Rhizopus arrhizus*	*Rhizopus* spp.
6	11	*Cunninghamella* spp.	Skin (thigh)	*Amplification failure*	Amplification failure	Amplification failure	Amplification failure
7	12	*Rhizopus arrhizus*	Sinus	*Rhizopus arrhizus*	*Rhizopus* spp./*Mucor* spp. (32.7)	*Rhizopus arrhizus*	*Rhizopus* spp.
8	13	*Lichtheimia ramosa*	Skin (thigh)	*Lichtheimia ramosa*	*Lichtheimia* spp. (34.6)	*Lichtheimia ramosa*	*Lichtheimia* spp.
9	14	*Mucor circinelloides*	Skin (hand)	*Mucor circinelloides*	*Rhizopus* spp./*Mucor* spp. (42)	Amplification failure	Amplification failure
10	15	*Rhizopus arrhizus*	Sinus	*Rhizopus arrhizus*	*Rhizopus* spp./*Mucor* spp. (26.1)	*Rhizopus arrhizus*	*Rhizopus* spp.
11	16	Negative	Liver	*Lichtheimia corymbifera*	*Lichtheimia* spp. (34.3)	*Lichtheimia corymbifera*	*Lichtheimia corymbifera*
	17	Negative	Liver	*Lichtheimia corymbifera*	*Lichtheimia* spp. (32)	*Lichtheimia corymbifera*	*Lichtheimia corymbifera*
12	18	Negative	Lung	*Lichtheimia corymbifera*	*Lichtheimia* spp. (37.5)	Amplification failure	Amplification failure
13	19	Negative	Skin (face)	*Rhizopus microsporus*	*Rhizopus* spp./*Mucor* spp. (36.4)	Amplification failure	Amplification failure

18S PCR, 18S rRNA gene PCR identification; ITS PCR, 16S–23S rRNA gene internal transcribed spacer PCR; PCR/ESI-MS, PCR/electrospray-ionization mass spectrometry; qPCR, quantitative real-time PCR.
[a]Microscopic examination demonstrated nonseptate hyphae suggestive of *Mucor*ales in all samples except samples11 (negative) and sample 12 (not available). Samples 1 to 12 were from patients enrolled in the AMBIZYGO trial. Samples 13 to 21 were from patients managed at Saint-Louis Hospital, Paris, France.
[b]Quantification cycle: the higher the value, the lower the fungal DNA content.

TABLE 2. Characteristics of identification assays and Cohen kappa statistics measured in agreement with culture identification in the validation study

Characteristic	PCR/ESI-MS	qPCR	ITS PCR	18S PCR
Master Mix	PLEX-ID kit (Abbott Biosciences)	LightCycler 480 Probes Master (Roche)	10× buffer for Taq Gold (Applied Biosystems)	10× buffer for Taq Gold (Applied Biosystems)
No. of assays	16	3	3	1
PCR volume	75 µL	20 µL	50 µL	50 µL
DNA volume	30 µL	9 µL	9 µL	9 µL
$MgCl_2$ concentration	NA	NA	2 mM	2.5 mM
dNTP concentration	NA	NA	200 µM	100 µM
Primer concentration	NA	1 µM	0.2 µM	1 µM
PCR program	• 10 min at 95°C • (30 s at 95°C, 30 s at 48°C, 30 s at 72°C) 8 cycles • (15 s at 95°C, 20 s at 56°C, 20 s at 72°C) 37 cycles • 2 min at 72°C	• 10 min 95°C • (15 s at 95°C and 1 min at 60°C) 50 cycles	• 7 min at 95°C • (30 s at 95°C, 30 s at 55°C, 1 min at 72°C) 40 cycles • 10 min at 72°C	• 5 min at 95°C • (30 s at 95°C, 30 s at 50°C, 1 min at 72°C) 40 cycles • 5 min at 72°C
Platform	MasterCycler Pro S (Eppendorf)	LightCycler 480 (Roche)	GeneAmp PCR System 9700 Thermocycler (Applied Biosystems)	GeneAmp PCR System 9700 Thermocycler (Applied Biosystems)
Kappa statistics				
Genus-level identification	0.80	0.76	0.49	0.37
Species-level identification	0.72	/[a]	0.14	No identification[b]

18S PCR, 18S rRNA gene PCR identification; ITS PCR, 18S–28S rRNA gene internal transcribed spacer PCR; PCR/ESI-MS, PCR/electrospray-ionization mass spectrometry; qPCR, quantitative real-time PCR.
NA: not available
[a]Species-level identification was intrinsically impossible with qPCR.
[b]No identification at the species level was possible using 18S PCR.

identification for 15 culture-positive biopsy specimens: genus-level identification was achieved for 13/15, 13/15, 9/15 and 7/12 specimens, respectively; species-level identification was achieved for 12/15, 0/15, 6/15 and 0/15 specimens, respectively (Table 1). Species-level identification by PCR/ESI-MS was possible in specimens with low fungal DNA loads (Cq > 39). In contrast, detection by ITS PCR and 18S PCR was unsuccessful for Cq > 34.6 and Cq > 34.3, respectively (Table 1). The kappa coefficient with respect to culture methods was higher for PCR/ESI-MS than for the other methods for identification to the genus (0.80) and species (0.72) levels (Table 2). Discrepancies between PCR/ESI-MS and culture were observed for the following: (a) *Saksenaea vasiformis* (sample 1), misidentified as *Apophysomyces elegans;* (b) *Lichtheimia ramosa* (sample 7), misidentified as *Lichtheimia corymbifera* but well identified in sample 13; and (c) *Cunninghamella* spp. (sample 11), neither detected nor identified (Table 1).

In the four culture-negative biopsy specimens, species-level identification by PCR/ESI-MS was possible; these identifications were consistent with qPCR findings for identification to the genus level (Table 1) and with ITS PCR and 18S PCR findings for identifications to the species level in two of four samples.

Discussion

The microbiologic diagnosis of mucormycosis is subject to several limitations, including an inability to obtain a positive culture, an absence of reference standard molecular techniques and confusion with other mold infections, necessitating expert laboratory intervention for accurate diagnosis. This study made use of 19 biopsy specimens from 13 patients—a large number given the low but increasing incidence of mucormycosis in France [17]. Polyphasic identification of the *Mucorales* isolates [12,13] was performed for all culture-positive cases, in contrast to previous studies [5–8,18]. We also focused on unfixed tissues, the key to optimizing molecular detection and identification [19].

PCR/ESI-MS identified *Mucorales* to species level more effectively than the other molecular methods tested, providing results within 6 hours. The limitations of PCR/ESI-MS for identifying rare species, such as *Cunninghamella* spp. or *Saksenaea vasiformis,* could be overcome by improving the PCR/ESI-MS database.

The identification of *Mucorales* species can directly affect epidemiologic studies and treatment strategies, given the differences in antifungal drug susceptibility between species [3]. PCR/ESI-MS is suitable for rapid identification but is currently

costly (US$150 to $200 per test) and has only limited availability.

Transparency declaration

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

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