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Identification of Mucorales From Clinical Specimens: A 4-Year Experience in a Single Institution

Mina Yang, M.D.[1,*], Jang Ho Lee, M.T.[2,*], Young-Kwon Kim, M.T.[3], Chang-Seok Ki, M.D.[1], Hee Jae Huh, M.D.[1], and Nam Yong Lee, M.D.[1]

Department of Laboratory Medicine and Genetics[1], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; Department of Clinical Laboratory Science[2], Semyung University, Jecheon; Department of Biomedical Laboratory Science[3], Konyang University, Daejeon, Korea

Mucormycosis, a fatal opportunistic infection in immunocompromised hosts, is caused by fungi belonging to the order Mucorales. Early diagnosis based on exact identification and multidisciplinary treatments is critical. However, identification of Mucorales fungi is difficult and often delayed, resulting in poor prognosis. This study aimed to compare the results of phenotypic and molecular identification of 12 Mucorales isolates collected from 4-yr-accumulated data. All isolates were identified on the basis of phenotypic characteristics such as growth rate, colony morphology, and reproductive structures. PCR and direct sequencing were performed to target internal transcribed spacer (ITS) and/or D1/D2 regions. Target DNA sequencing identified five *Lichtheimia* isolates, two *Rhizopus microsporus* isolates, two *Rhizomucor pusillus* isolates, one *Cunninghamella bertholletiae* isolate, one *Mucor fragilis* isolate, and one *Syncephalastrum racemosum* isolate. Five of the 12 (41.7%) isolates were incorrectly identified on the basis of phenotypic identification. DNA sequencing showed that of these five isolates, two were *Lichtheimia* isolates, one was *Mucor* isolate, one was *Rhizomucor* isolate, and one was *Rhizopus* microspores. All the isolates were identified at the species level by ITS and/or D1/D2 analyses. Phenotypic differentiation and identification of Mucorales is difficult because different Mucorales share similar morphology. Our results indicate that the molecular methods employed in this study are valuable for identifying Mucorales.

Key Words: Mucorales, Mucormycosis, Mycological typing

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Corresponding author: Nam Yong Lee
Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Gangnam-gu, Seoul 06351, Korea
Tel: +82-2-3410-2706
Fax: +82-2-3410-2719
E-mail: micro.lee@samsung.com

Co-corresponding author: Hee Jae Huh
Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Gangnam-gu, Seoul 06351, Korea
Tel: +82-2-3410-1836
Fax: +82-2-3410-2719
E-mail: pmhhj@gmail.com

*These authors contributed equally to this work.

Mucormycosis (previously described as zygomycosis) is an emerging opportunistic infection caused by fungi belonging to the order Mucorales [1]. Mucormycosis, which was once considered a rare disease, is now one of the most common invasive mold infections in patients with hematologic malignancies, second only to aspergillosis [2], and in immunocompetent hosts [3]. Mucorales fungi can be easily differentiated from *Aspergillus* fungi on culture. However, sometimes it is difficult to differentiate them on the basis of the results of imaging studies and histopathological analysis. Accurate identification is critical for treatment because these fungi cause two different infections. Amphotericin B (AMB) and azole are the best drugs for treating mucormycosis and aspergillosis, respectively. Overall mortality rate associated with mucormycosis is >47% [3, 4]. Therefore, rapid and accurate diagnosis and treatment of mucormycosis are essential for positive prognosis [5]. Unfortunately, identifica-

tion of Mucorales fungi by conventional methods is demanding. In this study, we comparatively identified Mucorales fungi by using phenotypic and molecular identification methods.

We analyzed 12 Mucorales isolates that were collected from Samsung Medical Center, Seoul, Korea, during 2010-2014. Fungal isolates that were repeatedly isolated from a single patient or from patients with suspected invasive fungal infections and that were morphologically identified as Mucorales were also included in the study. Contaminants growing on the edge of culture plates away from inoculation sites were excluded. Clinical specimens were inoculated on Sabouraud dextrose agar and were cultured at 30°C for initial three days and at room temperature (23-26°C) thereafter. Isolates were identified by using phenotypic characteristics such as growth rate, colony morphology, and reproductive structures [6]. Scotch tape method and lactophenol cotton blue stain were used for slide preparation. DNA was extracted by using MagNa Pure LC DNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) or I-genomic BYF DNA Extraction Mini Kit for fungi (iNtRON Inc., Seongnam, Korea), according to the manufacturer's recommendations. Briefly, 5-10 mg of mycelium was mixed with phosphate-buffered saline and vortexed. The sample was mixed with proteinase K, incubated at 95°C for 10 min, and cooled. DNA was isolated by using magnetic bead technology according to the manufacturer's instructions. The isolated DNA was amplified in a thermal cycler (Model 9700; Applied Biosystems, Foster City, CA, USA), and amplified products were sequenced by using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Internal transcribed spacer (ITS) was amplified by using primer sets ITS-1/ITS-4 and ITS-5/ITS-4, and D1/D2 region of the 28S subunit of ribosomal DNA was amplified by using

Table 1. Clinical information of patients and comparison of morphological and molecular identification methods

Patient No.	Age/sex	Specimen	Microscopic morphological ID	Molecular identification			Underlying disease	Diagnosis	Antifungal agent/ outcome
				Sequencing ID	ITS	D1/D2			
					Accession No. (identity)	Accession No. (identity)			
1	56/M	Endotracheal	*Cunninghamella bertholletiae*	*C. bertholletiae*	ND	FJ345351.1 540/540 (100%)	Kidney transplant	Pneumonia	Expired
2*		Sputum	*Lichtheimia* sp.	*L. corymbifera*	EU330179.1 538/538 (100%)	FJ719444.1 462/471 (98%)			
3	52/M	Blood	*Lichtheimia* sp.	*L. corymbifera*	HQ285610.1 702/702 (100%)	ND	DM, liver cirrhosis	ARDS	Expired
4	69/M	Sputum	*Rhizomucor* sp.	*L. ramosa*	JN315007.1 466/468 (99%)	JN315038.1 550/550 (100%)	Lung cancer	Lung cancer	Expired
5	69/M	Urine	*Rhizomucor* sp.	*L. corymbifera*	HQ285610.1 702/702 (100%)	ND	Diffuse large B cell lymphoma	Diffuse cerebral dysfunction	Expired
6	53/M	Stool	R/O *Rhizomucor* sp.	*Mucor fragilis*	JF299225.1 408/408 (100%)	ND	Liver cirrhosis	Liver transplant work up	
7	61/M	Sputum	R/O *Rhizomucor* sp.	*R. pusillus*	JN315022.1 517/517 (100%)	ND	Alcoholism	R/O fungal ball	
8	46/M	Endotracheal	*Rhizopus* sp.	*Rhizopus microsporus*	AB381937.1 678/679 (99.9%)	AB363776.1 671/679 (98.8%)	Liver cirrhosis	Pneumonia	Expired
9	68/M	Nasal	Unidentified Mucorales	*Rhizopus microsporus*	AY243961.1 623/623 (100.0%)	AB250181.1 618/618 (100.0%)	DM	Invasive mucormycosis	Amphotericin B/Expired
10	26/M	Endotracheal	R/O *Mucor* sp.	*R. pusillus*	AB369914.1 610/610 (100%)	AF113475.1 566/566 (100%)	B-ALL	Pneumonia	Expired
11	56/M	Stool	*Syncephalastrum racemosum*	*S. racemosum*	HM999978.1 164/166 (99%)	HM849721.1 422/432 (98%)	HCC	Liver transplant work up	
12	56/M	Endotracheal	R/O L *corymbifera* complex	*L. corymbifera*	FJ719398.1 575/575 (100%)	ND	Myelodysplastic syndrome	Pneumonia	Expired

*Clinical information is missing during the procedure of data handling and it was not traceable.
Abbreviations: R/O, rule out; ND, not done; ARDS, acute respiratory distress syndrome; DM, diabetes mellitus; HCC, hepatocellular carcinoma.

primer set NL-1/NL-4, as proposed in CLSI guidelines [7]. Sequences obtained were used to perform BLAST search in the GenBank database available at the NCBI website (http://www.ncbi.nlm.nih.gov). Outputs were sorted on the basis of percentage identity and were considered significant at ≥98% identity and ≥90% query coverage. Isolate were considered as misidentified if their phenotypic identification was different from their molecular identification at the genus level.

Five isolates were identified as *Lichtheimia* spp., (formerly *Absidia* spp.; four *Lichtheimia corymbifera* isolates and one *Lichtheimia ramosa* isolate) on the basis of DNA sequencing analysis. Further, two *Rhizopus microsporus* isolates, two *Rhizomucor pusillus* isolates, one Cunninghamella bertholletiae isolate, one *Mucor fragilis* isolate, and one *Syncephalastrum racemosum* isolate were identified. Results of phenotypic and molecular identification are listed in Table 1. Discrepancy between phenotypic and molecular identification at the genus level was observed for four isolates. Of these, three isolates initially identified as *Rhizomucor* on the basis of phenotypic identification were reclassified as *Lichtheimia* (*L. ramosa* and *L. corymbifera*) and *Mucor* (*M. fragilis*) on the basis of molecular identification. One isolate initially identified as *Mucor* was re-classified as *Rhizomucor* (*R. pusillus*) on the basis of molecular identification. One unidentified Mucorales isolate was classified as *Rhizopus microspores* on the basis of molecular identification. One patient (no. 9) was diagnosed as having invasive fungal infection on the basis of histopathological analysis. Concordance rate between phenotypic and molecular identification was 58.3% (7/12). Further, ITS and D1/D2 analyses provided more effective results.

Kontoyiannis *et al*. [8] identified 20 Mucorales isolates, with 20% error rate for phenotypic identification compared with molecular identification. In contrast, Alvarez *et al*. [9] reported 100% concordance rate between phenotypic and molecular identification at the genus level. A study by Jang *et al*. [10] in Korea compared phenotypic and molecular methods for identifying mold; however, only 9% of all isolates tested were Mucorales (four isolates). Some species such as *Cunninghamella* and *Syncephalastrum* can be identified easily on the basis of their morphology. *Rhizopus* spp. can be easily identified on the basis of the presence of rhizoids and striated or grooved sporangiospores. However, identification of *Rhizomucor*, *Lichtheimia*, or *Mucor* spp. based on their morphological characteristics is difficult. In the present study, isolates 4, 5, and 6 were morphologically identified as *Rhizomucor* because they had round sporangia compared with pear-shaped sporangia of *Lichtheimia*. Isolate 9 could not be classified as Mucorales because its rhizoids and sporangia were not observed. Because no rhizoids were observed in isolate 10, it was classified as *Mucor*. Absence of rhizoids is the key to differentiate *Mucor* spp. from other Mucorales fungi. However, this is not always applicable because of some exceptions such as that observed above. This explains the low concordance rate between phenotypic and molecular identification. Therefore, molecular identification of Mucorales fungi is more reliable than phenotypic identification [11-13]. ITS and D1/D2 analyses performed in this study provided comparable performances. However, as DNA targets, ITS and D1/D2 regions were not superior to each other. Because ITS shows increased level of demarcation in some species while D1/D2 region shows increased level of demarcation in other species, they are considered complementary.

Mucorales fungi show variable susceptibility to antifungal drugs. *Rhizopus* spp. are less susceptible to itraconazole, posaconazole, and AMB than *Lichtheimia* spp. and less susceptible to AMB than *Mucor* spp. [1]. Of 4,011 fungal isolates collected in this institute during the study period, 23.5% belonged to *Aspergillus* spp. and 0.7% belonged to Mucorales. Mucorales fungi cause a small portion of fungal infections. However, antifungal susceptibility testing of Mucorales fungi has provided limited data. Prompt treatment of mucormycosis is important because it is associated with high mortality rate, as evidenced by Chamilos *et al*. [5] who showed that delaying AMB-based treatment for >6 days after the onset of mucormycosis symptoms increased mortality rate by two fold (compared with previous treatments) and decreased survival rate to <20% at 12 weeks after diagnosis. Although the isolates identified in this study were repeatedly recovered from immunocompromised patients, these patients did not have systemic mucormycosis. Therefore, the possibility of contamination cannot be excluded, especially for urine or stool specimens.

In conclusion, phenotypic differentiation and identification of Mucorales are difficult because different Mucorales share similar morphology. Molecular identification methods were effective for identifying Mucorales fungi. In addition, this study is noteworthy because it identified all medically important Mucorales fungi reported in Korea by using phenotypic and molecular identification methods.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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