

Molecular Identification of *Mucor* and *Lichtheimia* Species in Pure Cultures of *Zygomycetes*

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Abstract

Background: The *Mucorales* are an important opportunistic fungi that can cause mucormycosis in immunocompromised patients. The fast and precise diagnosis of mucormycosis is very important because, if the diagnosis is not made early enough, dissemination often occurs. It is now well established that molecular methods such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are feasible and reliable tools for the early and accurate diagnosis of mucormycosis agents.

Objectives: The present study was conducted to evaluate the validity of PCR-RFLP for the identification of *Mucorales* and some important *Mucor* and *Lichtheimia* species in pure cultures of *Zygomycetes*.

Materials and Methods: Specific sense and anti-sense primers were used to amplify the *Mucorales*, *Mucor*, and *Lichtheimia* DNA. The PCR products were digested by *AflIII*, *XmnI*, and *AclI* restriction enzymes, and the resultant restriction pattern was analyzed.

Results: On the basis of the molecular and morphological data, we identified *Mucor plumbeus* (10.83%), *M. circinelloides* (9.17%), *Lichtheimia corymbifera* (9.17%), *M. racemosus* (5.83%), *M. ramosissimus* (3.33%), and *L. blakesleeana* (0.83%).

Conclusions: It seems that PCR-RFLP is a suitable technique for the identification of *Mucorales* at the species level.

Keywords: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism, *Mucorales*, *Mucor*, *Lichtheimia* (*Absidia*)

1. Background

Based on the results of molecular phylogenetic analysis, the *Mucorales*, the core group of the traditional Zygomycota, have been reclassified into the subphylum Mucoromycotina of the new division, *Glomeromycota* (1).

These fungi are ubiquitous saprophytes that are scattered widely in nature, food, soil, and air (2). The *Mucorales* are regarded as important animal and human pathogens, and they are responsible for mucormycosis (formerly known as zygomycosis) (3, 4), the third most common invasive fungal infection (5-7). The *Mucorales* are increasingly recognized as opportunistic pathogens in immunocompromised or immunosuppressed patients (8, 9).

Mucormycosis is a very aggressive invasive fungal disease. It is a serious condition that affects a variety of patient groups. Mucormycosis can be caused by any of the six families of *Mucorales*, although the members of the *Mucoraceae* including *Rhizopus*, *Mucor*, *Absidia* (*Lichtheimia*), and *Rhizomucor* are more frequently isolated in human infections (5, 7, 10, 11).

Members of the genus *Rhizopus* are the most common isolates recovered in a clinical setting, with *Rhizopus arrhizus* occurring the most frequently. Members of the

genus *Mucor* come second to *Rhizopus* with respect to frequency, while *Cunninghamella*, *Apophysomyces*, *Lichtheimia*, *Saksenaia*, *Rhizomucor*, *Cokeromyces*, and *Syncephalastrum* each constitute a significantly lower percentage of clinical isolates (7, 12, 13).

Due to the infection's rapidly progressive course and unexpectedness, many mucormycosis cases are only diagnosed postmortem. The infection therefore constitutes a huge diagnostic and therapeutic challenge (9). The number of cases of mucormycosis has increased over the last few decades, making the fast and accurate diagnosis of these infections imperative. Unfortunately for patients who are affected by mucormycosis, no routine laboratory tests are available to diagnose this disease. Clinical diagnosis instead relies on histopathology and the isolation of etiologic fungi from infected tissues (2, 14). The identification of *Mucorales* is primarily based on standard mycological methods. The *Mucorales* are characterized by anamorph structures. The mycelium is typically non-septate (coenocytic) or irregularly septate. Sporangiospores are produced in multi-sporangia. The sporangia are characterized by the inclusion of a variously shaped columella (15).

The growth of the *Mucorales* in media is rapid, with

mycelia elements expanding to cover the entire plate in only a few (one to seven) days. However, culture-based identification is often difficult and time-consuming, and it relies entirely on the experience of the technician or expertise mycologist. Conventional phenotypic methods usually identify isolates to only the genus level, and sometimes only as *Mucorales*. If the diagnosis is not made early enough, dissemination often occurs (8, 16, 17).

Based on the above, the development and validation of a new detection system, perhaps involving polymerase chain reaction (PCR) methods, for the fast and accurate diagnosis of mucormycosis is necessary. PCR-restriction fragment length polymorphism (RFLP) is a rapid and highly reliable method for the identification and differentiation of important common *Mucorales* species (16).

2. Objectives

This study aimed to identify the *Mucor* and *Lichtheimia* (formerly *Absidia*) species among other genera of *Mucorales* isolated in culture media using the PCR-RFLP method.

3. Materials and Methods

3.1. Isolation of Fungi

Of the course of a year, 500 samples were taken from different city districts, parks, hospitals, greengrocers, food stores, laboratories, public restrooms, mosques, and cattle houses. The samples were cultured on sabouraud dextrose agar (SDA; Merck KGaA, Darmstadt, Germany) and potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) supplemented with chloramphenicol. A diversity of different types of filamentous fungi and yeasts were identified in the media. Finally, 120 pure cultures belonging to the *Mucorales* were gained after the purification of the colonies. Primary identification of the *Mucorales* was performed on the basis of the macroscopic and microscopic features of the colonies.

In the next step, a mixture of two genus-specific sense primers and one degenerate anti-sense primer from the 18S rRNA gene region were selected to determine the two genera of *Lichtheimia* and *Mucor* within the *Mucorales*.

3.2. DNA Extraction From Pure Fungal Cultures

All of the pure colonies obtained were sub-cultured on 2% SDA and PDA and then incubated for one to seven days at 27°C in stable conditions. The genomic DNA was extracted and purified from each colony using the phenol-chloroform method as follows. Briefly, hyphae (without sporangia) from fresh 48 hours cultures on SDA or PDA were suspended in a 200 μ L lysis buffer (100 mM Tris-HCl,

10 mM EDTA (pH = 8), 2% Triton X-100, 1% SDS, 100 mM NaCl) (Merck KGaA, Darmstadt, Germany) in 2 ml Eppendorf tubes and grinded well. Then, the suspension (without the hyphal elements) of each tube was transferred to a new Eppendorf tube. Next, 200 μ L Phenol-Chloroform (1:1) was added and vortexed (or shaken by hand for 5 minutes) rigorously with 200 μ L of glass beads (0.5 mm in diameter) to release the DNA. After centrifugation for 5 minutes at 5,000 rpm, the supernatant was mixed with an equal volume of 2-propanol and 0.1 volume of 3 M sodium acetate (pH = 5.2), vortexed, and incubated at -20°C for 10 min. It was then centrifuged for 12 min at 12,000 rpm. All of the solution was gently removed and then 100 μ L of 70% ethanol was gently added to the tube and centrifuged for 5 minutes at 5000 rpm. The ethanol was then removed from the tube, dried, and resuspended in 50 μ L of distilled deionized water, and it was kept at -20°C as the purified DNA until use.

3.3. PCR

Selected primers targeted an 830 bp sequence in the 18S fungal ribosomal gene, which excludes the amplification of human DNA and other filamentous fungi. The specific sense primers were *MucL1*: 5' TGATCTACGTGACATATTCT 3' and *AbsL1*: 5' TGA TCTACACGGCATCAAAT 3' (Bioneer, Daejeon, South Korea), which corresponded to the sequences of *Mucor* sp. and *Absidia* (*Lichtheimia*), respectively. A degenerate anti-sense primer (*MR1*: 5' AGTAGTTTGCTTCGGTCAA3') was applied for the *Mucorales*. The sense primers annealed to the region of the template starting at position 75, while the antisense primer annealed to the region at position 901 (18).

Each amplification reaction included 12.5 μ L of premix (containing 2.5 U Taq DNA polymerase, PCR buffer, 1.5 mM MgCl₂, and 200 μ M dNTP) (Ampliqon, Odense, Denmark), 2 μ L (about 10 ng) of template DNA, 0.5 μ L of each primer (3 \times 0.5 = 1.5 μ L), and 9 μ L of distilled deionized water in a final volume of 25 μ L. Amplification was performed on a 2700 thermocycler (Applied Biosystems, Singapore) as follows. One cycle of 1 minute at 94°C (primary denaturation), 30 cycles of 1 minute at 94°C (denaturation), 1 minute at 60°C (annealing), 1 minute at 72°C (extension), and finally one cycle of 5 minute at 72°C. Negative controls (no DNA template) were included in each run to detect the presence of any DNA contamination in the reagents and reaction mixtures.

3.4. Restriction Enzymes

The PCR products of 120 *Mucorales* were individually digested with selected restriction enzymes including *AflIII*, *XmnI* (Thermo Fisher Scientific, Vilnius, Lithuania), and *AclI* (New England BioLabs, USA), which were specified to the

Mucor sp.: *Mucor circinelloides*, *M. racemosus*, *M. ramosissimus*, *M. plumbeus*, *L. corymbifera*, and *L. blakesleeana*.

3.5. RFLP

Eighteen s amplified fragments of the 120 *Mucorales* were separately digested by the three abovementioned enzymes. The amplicons were digested for 1 hour at 37°C in a total volume of 25 µL (containing 2 µL of the enzyme, 2.5 µL of related buffer, 10 µL of PCR product, and 10.5 µL of distilled water). The digested amplification products were subjected to electrophoresis, and the sizes of the restriction fragments were determined by comparison with a 100 bp ladder standard DNA molecular weight marker (Fermentas, Vilnius, Lithuania). The restriction site, specificity, and fragment size of each enzyme are detailed in Table 1.

Table 1. The Restriction Site, Specificity, and Fragment Size of Each Enzyme

Enzyme (Restriction Site)	Specificity	Fragment Size, bp
<i>AflIII</i> (5' CTTAAG 3')	<i>Mucor</i> sp.	750 + 87
<i>XmnI</i> (5' GAATAGCTTC 3' or 5' AGCTTCGGT 3')	<i>Mucor circinelloides</i> , <i>M. racemosus</i> , <i>M. ramosissimus</i> , <i>M. plumbeus</i>	613 + 224
<i>AclI</i> (5' AACGTT 3')	<i>Lichtheimia corymbifera</i> , <i>L. blakesleeana</i>	518 + 306

3.6. Electrophoresis

Agarose gel in a TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) at 100 V for 45 - 120 minutes in a 1%, 1.5%, and 2% gel was used for the electrophoresis of the extracted DNA, PCR products, and RFLP products, respectively.

3.7. Characteristic Features of the *Mucor* and *Lichtheimia* Species

The *Mucor* sp. typically exhibits rapid growth, producing globose sporangia on sporangiophores that are either solitary or branched. The sporangia contain the entire columella and spores that are mucous bound. The sporangial wall collapses irregularly, if at all. The sporangia may also be deliquescent (dissolving). Rhizoids and stolons are absent. These features distinguish the *Mucor* spp. from the other producers of globose sporangia (8). The genus *Lichtheimia* is defined by its pyriform, apophysate sporangia (15). The characteristic features of some *Mucor* and *Lichtheimia* species are summarized in Table 2 (8, 15, 19-23).

4. Results

The genomic DNA belonging to the *Mucorales* was successfully amplified with the selected primers and a product of approximately 830 base pair (bp) was amplified for all *Mucorales*. The PCR products were electrophoresed in 1.5% agarose gels in the presence of ethidium bromide and then visualized under UV light (Figures 1 and 2). The PCR amplicons were separately digested by *AflIII*, *XmnI*, and *AclI* enzymes. The obtained sizes of the RFLP products were exactly comparable with the sizes detailed in Table 1.

Two fragments (750 + 78) were visualized following the *AflIII* restriction. The specific restriction pattern of *M. circinelloides*, *M. racemosus*, *M. ramosissimus*, and *M. plumbeus* was established using *XmnI* (613 + 224). Additionally, two fragments (518 + 306) were visualized following digestion with *AclI*, which represented the specific pattern for *Lichtheimia corymbifera* and *L. blakesleeana* (Figures 3 and 4).

The discrimination of these species was conducted based on the macroscopic and microscopic features as well as the growth ability in different temperatures (Figure 4). Finally, *Mucor* sp. and *Lichtheimia* sp. represented approximately 39.17% of all the pure colonies. Thirty-five colonies (29.17%) were identified as *Mucor* sp., namely *M. circinelloides* (9.17%), *M. racemosus* (5.83%), *M. plumbeus* (10.83%), and *M. ramosissimus* (3.33%). Twelve colonies (10%) were identified as *Lichtheimia*, which belonged to *L. corymbifera* (9.17%) and *L. blakesleeana* (0.83%).

5. Discussion

The identification of *Zygomycetes* is mainly based on macroscopic and microscopic characteristics, which is a difficult and time-consuming process that sometimes needs the expertise of a reference laboratory (14). Additionally, the precise identification of the *Mucorales* down to a species level may hold great importance for further research on antifungal effectiveness (18, 24, 25). Molecular techniques have showed enormous potential for rapidly and accurately identifying the ecological agents of mucormycosis, which helps in conducting epidemiologic investigations. Molecular detection assays for the *Mucorales* are, however, not yet widely available (18, 26).

Different regions of the rRNA operon have most frequently been the targets for the detection of *Zygomycetes*, with previously reported PCRs for zygomycosis. Several prior reports have described the utilization of universal fungal primers from the 18S, 28S, or ITS rRNA gene regions for PCR amplification followed by the sequencing or hybridization of the product to specific probes (2, 13, 27, 28).

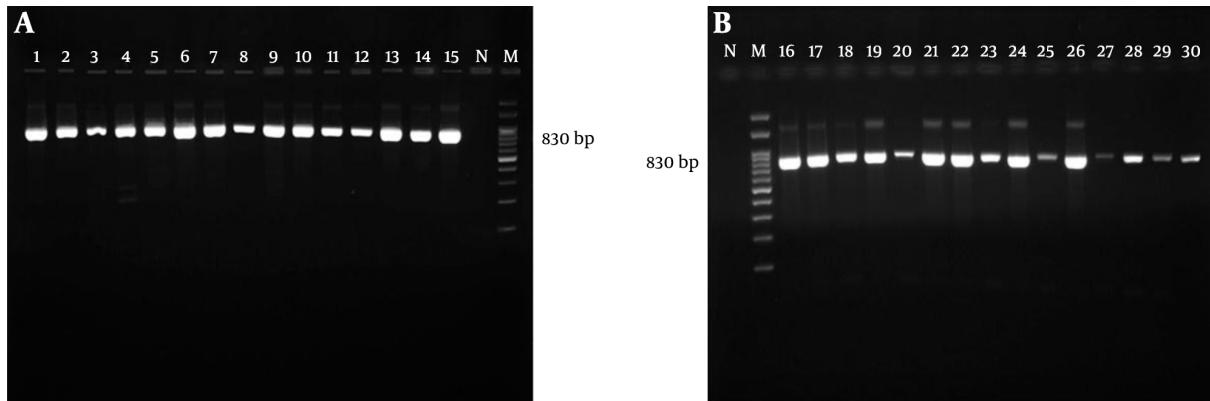
Table 2. Characteristic Features of the Some *Mucor* and *Lichtheimia* Species

Organism name	Colony Morphology	Sporangium Morphology, μm	Columella Morphology, μm	Sporangiospore Morphology, μm	Rhizoids and Apophysis	Sporangiophore Morphology	Other
<i>Mucor ramosissimus</i>	Rapidly growing low colonies; gray to buff	Globose, 25 - 80	Round to flattened, 20 - 37 by 17 - 30; collars may be seen; smaller sporangia lack columella	Oval to round, smoothwalled, brownish; 3.3 - 5.5 by 3.5 - 8	Absent	Sympodially branched; may have racquet shaped swellings	Optimal growth at 24°C, poor growth at 37°C
<i>Mucor circinelloides</i>	Floccose with rapid growth; pale gray to yellowish, brown at 37°C	Globose, up to 60	Spherical, up to 50 in diameter; collars may be present	Smooth walled and oval; 4.4 - 7	Absent	Sympodially branched and circinate	Growth from 5°C to 37°C
<i>Mucor plumbeus</i>	Grey to light olive-green	Globose up to 80, Hyaline, dark brown to brownish grey with age, with spinulose walls	Pyriiform, ovoid with a truncate base, up to 25 - 50	Globose, sometimes more or less ellipsoidal or irregularly shaped, 7 - 8	Absent	With slightly encrusted walls, branching	Optimal growth at 5 - 20°C
<i>Mucor racemosus</i>	Low to medium-high colonies; light to medium brown	Globose, light brown, encrusted walls, up to 80	Ellipsoidal to pyriform, up to 40 long	Oval to subspherical, smooth walled; 5 - 8	Absent	Branched	Optimal growth at 25°C, poor or no growth over 32°C
<i>Lichtheimia corymbifera</i>	Floccose; first white, turning brown to greenish brown with age	Pyriform 20 - 80	Usually with an apical projection, Columella: dome shaped	Single-celled, hyaline, subglobose to broadly ellipsoidal	Present apophysis: flask shaped	Erect, simple or slightly branched, typically rising along the stolon but not opposite the rhizoids, apically with a well-developed, funnel shaped or swollen apophysis	Capable of growth at 48°C to 52°C
<i>Lichtheimia blakesleeana</i>	Woolly, white, and grey-brown to olive green with age	Pyriform 20 - 80	Usually with a apical projection, Columella: dome shaped	Single-celled, hyaline, subglobose or more rarely, broadly ellipsoidal	Present apophysis: flask shaped	Apically with a well-developed, funnel shaped or swollen apophysis	No growth at 48°C

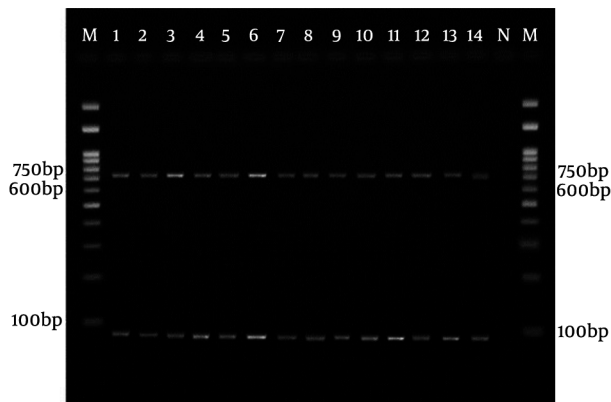
PCR-RFLP is a reliable and easy to perform technique that can be used in epidemiological and research studies. PCR-RFLP-based methods target the 18S ribosomal gene of *Zygomycetes* on DNA extracted from human specimens and may therefore provide clinicians with a rapid and definitive diagnosis of mucormycosis (29). In the present study, a PCR-RFLP method based on the 18S ribosomal gene of *Zygomycetes*, which had been previously developed by Machouart et al. was used to identify the *Mucor* and *Aspidia* species from pure cultures. The identification of this region by PCR amplification with selective primers has proved to be reliable for the identification of *Zygomycetes* (18).

Bialek et al. (30) developed a PCR-based method targeting the 18S rRNA gene for the identification of mucormycosis and aspergillosis agents in paraffin wax embedded tis-

sue. Piancastelli et al. (31) identified *L. corymbifera* among other fungi with PCR-RFLP using the ITS region as a target sequence and the *AclI* restriction enzyme. In our study, *AflII* was used for *Mucor* identification to the genus level and, after that, the *XmnI* restriction enzyme was applied to distinguish *M. circinelloides*, *M. racemosus*, *M. ramosissimus*, and *M. plumbeus*. The *AclI* restriction enzyme was used to identify *L. corymbifera* and *L. blakesleeana*. Digestion with *AflII*, *XmnI*, and *AclI* generated two fragments of 750 + 87, 613 + 224, and 518+306 bp, respectively. The restriction site and fragment sizes can be seen in Table 1. *XmnI* does not cut the amplicons obtained from *M. hiemalis* or *M. indicus*. Therefore, following RFLP, the differentiation of the four above-mentioned species was performed based on comparing the macroscopic, microscopic, and other features. These features are summarized in Table 2.

Figure 1. Agarose Gel Electrophoresis of 18sS rRNA PCR Products of Different *Mucorales*

All samples yielded a single band of approximately 830 bp in lanes 1 to 30. Lanes N, negative control; M, 100 bp molecular size marker.

Figure 2. Agarose Gel Electrophoresis of 18sS rRNA PCR Products of Different *Mucorales* After Restriction Digestion With *AflIII*

Lanes 1 to 13, *Mucor* sp.; N, negative control; M, 100 bp molecular size marker.

Figure 3. Agarose Gel Electrophoresis of 18sS rRNA PCR Products of Different *Mucorales* After Restriction Digestion With *XmnI* and *AclI*

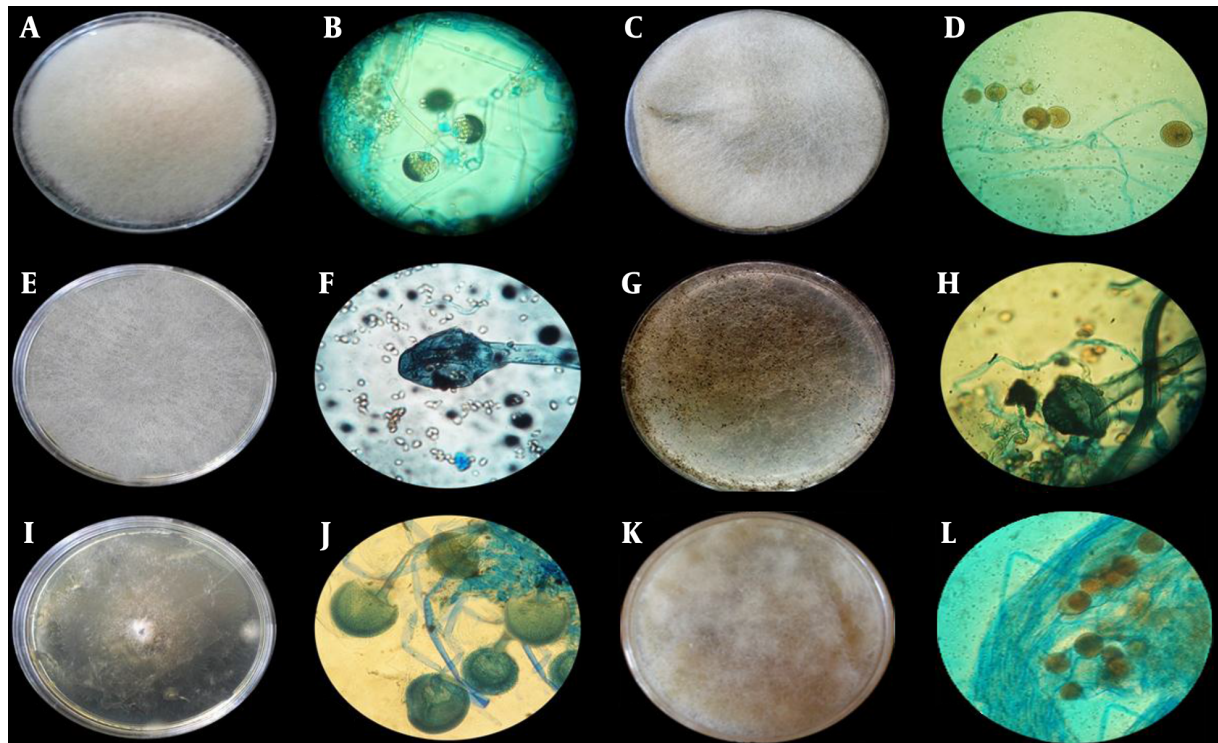
Lanes 12, 11, 10, 9, 8, 2 and 13, *M. circinelloides*, *M. racemosus*, *M. ramosissimus* or *M. plumbeus*; Lanes 3, 1, 7, 6, 5, 4, and 14, *L. corymbifera* or *L. blakesleeana*; N, negative control; M, 100 bp molecular size marker.

Iwen et al. (19) identified *M. circinelloides* as a cause of primary *zygomycosis* using a sequence analysis of the ITS region as well as phenotyping methods. The *Mucor* species are considered to be a distant third behind the *Rhizopus* species and *Absidia corymbifera* in terms of causing *zygomycosis*. Only five species are suspected of causing human disease. These include the thermotolerant species *M. racemosus*, which either does not grow or else grows poorly at 37°C. The presence of fungal species has been considered to be an environmental microbiological indicator, and some of the fungi have been found to cause fungal infection (26).

A considerable number of mucormycosis cases have been associated with *M. circinelloides*, which appears to be the most common cause of the disease. There have been a

few mucormycosis cases associated with *M. ramosissimus*. However, no mycosis cases associated with *M. plumbeus* have yet been reported (32). In this study, the genera of *Mucor* and *Lichtheimia* were represented in 29.17% and 10% of the 120 pure *Mucorales* cultures, respectively. Alvarez et al. (33) studied 190 isolates morphologically identified as *Zygomycetes* using sequencing of the ITS region of the rDNA, which revealed that *M. circinelloides*, *L. corymbifera*, and *M. indicus* represented approximately 9.5%, 5.3%, and 2.6% of these isolates, respectively.

Among the *Absidia* species, the most important species associated with mucormycosis is *A. corymbifera*. Based on physiological, phylogenetic, and morphological data, it

Figure 4. Macroscopic and Microscopic Appearance of Identified *Mucorales*

A, Colony of *M. plumbeus*; B, Microscopic morphology of *M. plumbeus*; globos sporangia with spinolus walls and pyriform columella; C, colony of *M. circinelloides*; D, microscopic morphology of *M. circinelloides* with globos sporangia and spherical columella; E, colony of *L. corymbifera*; F, microscopic morphology of *L. corymbifera* with pyriform sporangia and flask like apophysis; G, colony of *L. Blakesleenana*; H, microscopic morphology of *L. Blakesleenana* with pyriform sporangia and flask like apophysis; I, colony of *M. ramosissimus*; J, microscopic morphology of *M. ramosissimus* with globos sporangia and round columella; K, colony of *M. racemosus*; L, microscopic morphology of *M. racemosus* with globos, light brown and encrusted walls sporangia, and ellipsoidal columella.

was proposed that three *Absidia* species, namely *A. corymbifera*, *A. blakesleeana*, and *A. hyalospora*, should be reclassified as a separate family, the *Lichteimiaceae* fam. nov., and the three species renamed as *L. corymbifera*, *L. blakesleeana*, and *L. hylospora*. *L. blakesleeana* was subsequently reduced to a synonym of *L. hyalospora* (34, 35). In Dannaoui et al. (24) study, almost all of the PCR results for *M. circinelloides* were negative. However, in the present study, PCR amplification of all the pure cultures using specific primers was carried out successfully, and the obtained bands were fully sharp.

Finally, our findings revealed that molecular methods can be used for the rapid detection and differentiation of species that are responsible for infection, and they can hence help in conducting epidemiologic investigations. In contrast to other methods, a PCR-based approach has the potential to be time efficient, highly specific, and endowed with a good sensitivity (36).

In summary, the present study maintains that the diagnosis of *Zygomycetes* to a species level based on macroscopic and microscopic features is very difficult. At the

same time, there are several limitations to the method used in our study. Therefore, it seems that more research is needed to modify the present molecular approaches. In a future study, we intend to design a PCR-RFLP method that needs to lower specific primers and enzymes in order to identify the genera and species belonging to *Zygomycetes*.

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