



Molecular Identification of Causative Agents in 25 Cases with Mucormycosis in Iran

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Abstract

Background: Mucormycosis is a life-threatening infection due to the members of the Mucorales order with increasing incidence during the last decades. *Rhizopus* spp. are the most common causes of disease; however, this infection could be developed by various other species.

Objectives: This study presented the clinical features and predisposing factors of several patients with mucormycosis along with the causative agents using sequence analysis.

Methods: Clinical specimens of 25 cases with mucormycosis were included in this study. Direct examination and culture were performed for all specimens and then isolated fungi were identified based on their morphology and sequence analysis of ribosomal DNA.

Results: The patients were comprised of 17 (68%) females and 8 (32%) males with the mean age of 47.16 ± 17.4 years. Rhino-cerebral mucormycosis was the most common clinical form (24 cases) followed by pulmonary mucormycosis (one case). Diabetes mellitus was the most common predisposing factor ($n = 17$, 68%). The culture was positive in 15 specimens and the isolated fungi were morphologically identified as *Rhizopus* spp., subsequently, all of the isolates were identified as *Rhizopus oryzae* at the species level using the molecular method.

Conclusions: Diabetes and *R. oryzae* played the most important roles as the predisposing factor and etiologic agent of mucormycosis, respectively.

Keywords: Invasive Fungal Infections, Iran, Mucorales, *Rhizopus oryzae*, Zygomycosis

1. Background

Mucormycosis is an opportunistic fungal infection caused by members of the Mucorales order. The infection is less common than candidiasis and aspergillosis; however, the incidence of mucormycosis has been increased steadily during the last decades (1, 2). Some of the most important predisposing conditions for mucormycosis are prolonged neutropenia, corticosteroid and deferoxamine therapy, diabetic ketoacidosis, hematologic malignancies, aplastic anemia, myelodysplastic syndrome, solid organ or hematopoietic stem cell transplantation, penetrating trauma, and burns (3, 4). Although mucormycosis in developed countries is mostly reported in patients with underlying diseases such as hematological malignancies,

chemotherapy, and bone marrow transplantation, uncontrolled diabetes mellitus is the main predisposing factor for mucormycosis in developing countries (5, 6). In high-risk patients, 5% - 12% of the invasive fungal infections are usually caused by Mucorales; however, the higher rates (20% - 44%) have also been reported (7, 8).

Based on the anatomical location, mucormycosis falls into six clinical forms, including rhino-cerebral (48%), pulmonary (24%), cutaneous (19%), gastrointestinal (7%), disseminated (3%), and uncommon or rare forms (6%) such as endocarditis, osteomyelitis, and peritonitis (3, 7, 9). *Rhizopus* species have usually been recovered from mucormycosis specimens whereas other Mucorales, including *Mucor* spp., *Cunninghamella* spp., *Apophysomyces* spp.,

Saksena spp., *Rhizomucor* spp., *Syncephalastrum* spp., and *Cokeromyces* spp. are less common etiologic agents of infection (4, 10).

Laboratory diagnosis is based on microscopic examination and culture of clinical specimens. Polymerase chain reaction (PCR) and PCR-based sequencing have been used for laboratory diagnosis of the infection and species identification of the causative agents (4, 11). Although direct microscopy of the involved tissue, as well as culture and microscopic observation of reproductive structures, are the most common methods in the clinical laboratory, application of molecular methods can improve the management of patients with mucormycosis (12).

Here, the clinical and laboratory data of 25 cases with mucormycosis was reported whose clinical specimens have been referred to a Medical Mycology Laboratory in Tehran.

2. Objectives

In order to provide accurate microbial epidemiology data, sequence-based identification of the causative fungi was also performed.

3. Methods

3.1. Specimen Collection and Processing

A set of 25 clinical specimens or previously isolated fungi from patients with mucormycosis were included in this study (Table 1). Of them, 15 cases were collected from 2009 to 2013 and 10 cases were obtained from January 2014 to October 2016. These specimens were collected in Medical Mycology Laboratory, Tehran University of Medical Sciences, Tehran, Iran. Demographic data and clinical histories, including underlying diseases, biopsy site, antifungal therapies, and the outcome of the disease were collected from the laboratory and hospital records. This study was approved by the Ethics Committee of Tehran University of Medical Sciences.

Microscopic examination of these specimens was performed by KOH (10% - 20% potassium hydroxide) wet mount. Furthermore, a part of all the specimens was cultured on Sabouraud dextrose agar medium (SDA, Himedia, India) and incubated at 30°C for initial three days and then at room temperature (23 - 26°C).

3.2. Morphological and Molecular Identification

Slide culture was prepared and morphological characteristics of the diagnostic structures (sporangiohores, columella, sporangium, sporangiospore, and rhyzoid) were imaged and analyzed using an optical microscope

(Olympus BX41, Olympus Corporation, Japan) and OLYSIA Bio Report software.

For molecular identification, all isolates were sub-cultured on SDA medium and their DNAs were extracted and purified as described previously (13). The entire internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2) was amplified by using universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') in PCR conditions described previously (14). The purity of PCR-amplified products was checked by electrophoresis on 1.5% agarose gel and all PCR-amplicons were sent for Sanger sequencing (Bioneer company, South Korea). Speciation of isolates was done by comparing the sequences with GenBank data using basic local alignment search tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and all sequences were deposited in GenBank under the accession numbers of MF850346 to MF850360.

4. Results

In direct examination of all the specimens, broad non-septate hyphal elements were observed (Figure 1). Females (n = 17, 68%) were more affected than males (n = 8, 32%). The age of the patients ranged from 10 to 69 years (mean = 47.16 ± 17.4 years). All of the patients were immunocompromised and diabetes mellitus was the most common predisposing factor (n = 17, 68%), followed by solid organ transplant and hematological disorder/malignancy such as leukemia, each of them contained two patients. Rhino-cerebral infection (n = 24) was the most common type of disease, also one case of pulmonary mucormycosis was recorded in this study (Table 1). Thirteen patients out of 22 patients (59.09%) were expired (according to available data. The data of some cases were missing) (Table 1).

The culture was positive for 15 specimens and the remaining specimens yielded no fungal growth even after numerous repeated cultures. The following morphologic characteristics were obtained in slide cultures by using OLYSIA Bio Report software: (i) single or often paired sporangiohores, mostly unbranched, brownish and between 400 μm to 2 mm in length, (ii) sporangiums mostly spherical to conical, brownish-gray and between 50 - 250 μm in diameter and (iii) spherical to conical columellas and branched rhizoid with 100 - 250 μm in length. The isolated fungi were morphologically identified as *Rhizopus* spp. and further identification was not feasible at the species level (Figure 1). However, all the isolates were identified at the species level as *R. oryzae* using the molecular method (Table 1).

Table 1. Demographic and clinical data of 25 patients with mucormycosis and morpho-molecular identification results of etiologic agents

No.	Age/Sex	Province of Residence	Type of Infection	Biopsy Site	Underlying Disease	Direct Examination	Culture	Treatment	Outcome	Morphological Identification	Molecular Identification	Accession Number
1	F/57	Alborz	RC	Paranasal sinus	Diabetes	Positive	Negative	AmB	Expired	NG	NP	NP
2	F/49	Markazi	RC	Paranasal sinus/eye	Diabetes	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850346
3	M/63	Alborz	RC	Nasal cavity	Tuberculosis	Positive	Positive	NA	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850347
4	M/24	Tehran	RC	Paranasal sinus	ALL/thalassemia major/diabetes	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850348
5	F/21	Lorestan	RC	Paranasal sinus	AML-M3	Positive	Positive	NA	Cured	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850349
6	F/33	Tehran	RC	Paranasal sinus	ALL	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850350
7	F/26	Tehran	RC	Paranasal sinus/eye	DKA	Positive	Negative	AmB	Expired	NG	NP	NP
8	F/48	Isfahan	RC	Nasal cavity	Diabetes	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850352
9	F/10	Alborz	RC	Jaw	Diabetes	Positive	Positive	AmB	Cured	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850353
10	M/28	Tehran	RC	Nasal cavity	Addiction	Positive	Positive	NA	Cured	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850354
11	F/69	Tehran	RC	Paranasal sinus	Wegner's syndrome	Positive	Negative	NA	Expired	NG	NP	NP
12	M/24	Tehran	RC	Paranasal sinus/nasal cavity	Aplastic anemia	Positive	Positive	AmB/PSC	Cured	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850356
13	M/57	Tehran	RC	Paranasal sinus	Diabetes	Positive	Negative	AmB	Cured	NG	NP	NP
14	F/42	Tehran	RC	Paranasal sinus	PNH	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850358
15	F/52	Tehran	RC	Paranasal sinus	Diabetes	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850359
16	F/69	Tehran	RC	Paranasal sinus	Diabetes	Positive	Negative	AmB	Cured	NG	NP	NP
17	M/40	Qom	RC	Paranasal sinus	Diabetes	Positive	Positive	AmB	Cured	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850355
18	F/63	Alborz	RC	Paranasal sinus	Diabetes	Positive	Negative	NA	NA	NG	NP	NP
19	F/64	West Azarbaijan	RC	Paranasal sinus	Metastatic colon cancer	Positive	Negative	NA	Expired	NG	NP	NP
20	F/54	Alborz	P	BAL	Diabetes/liver transplant	Positive	Positive	NA	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850351
21	M/67	Tehran	RC	Paranasal sinus/nasal cavity	CLL/diabetes	Positive	Positive	AmB	Expired	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850357
22	M/43	Alborz	RC	Nasal cavity	Diabetes	Positive	Positive	AmB	Cured	<i>Rhizopus</i> spp.	<i>R. oryzae</i>	MF850360
23	F/40	Khuzestan	RC	Nasal cavity	Diabetes/kidney transplant	Positive	Negative	NA	NA	NG	NP	NP
24	M/67	Tehran	RC	Paranasal sinus	Diabetes	Positive	Negative	NA	NA	NG	NP	NP
25	F/69	East Azerbaijan	RC	Nasopharynx	Diabetes	Positive	Negative	AmB	Cured	NG	NP	NP

Abbreviations: ALL, acute lymphoblastic leukemia; AmB, amphotericin B; AML, acute myeloblastic leukemia; BAL, bronchoalveolar lavage; CLL, chronic lymphoblastic leukemia; DKA, diabetic ketoacidosis; F, female; M, male; NA, not available; NG, no growth; NP, not performed; P, pulmonary; PNH, paroxysmal nocturnal hemoglobinuria; PSC, posaconazole; RC, rhinocerebral.

5. Discussion

Mucormycosis is an opportunistic fungal infection that was first reported in 1885 (4). Over the past two decades, the incidence of mucormycosis has dramatically increased, as a result of an increase in the population at risk (6). Despite antifungal therapy and aggressive surgical interventions, the mortality rate associated with mucormycosis is > 47% emphasizing the importance of this disease (15). In the present study, we analyzed the clinical data of 25 Iranian cases with mucormycosis and the

causative agents were identified through morphological and molecular procedures.

In our study, the mean age of the patients was 47 years similar to some previous reports (range, 40 - 52 years) (16-19). In contrast, Dai et al. (9) and Saegeman et al. (7) have reported the mean ages of 58.8 and 60 years, respectively. Although mucormycosis has been reported to be more common in males compared with females in most of the published series (1, 3, 4, 7, 9, 16, 20), in the present study, females (68%) were more affected than males (32%), which is in accordance with reports by Komur et al. (21) from Turkey

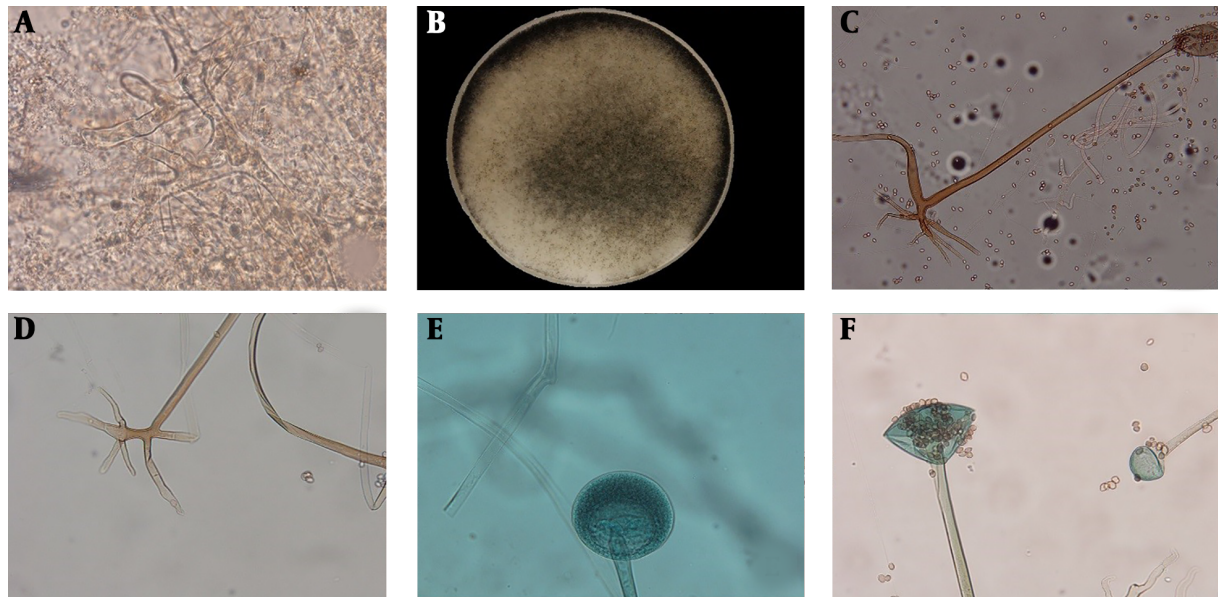


Figure 1. Broad aseptate hyphal elements with right branching in direct examination of a clinical specimen (A), macroscopic view of grown colony on Sabouraud dextrose agar medium (B), the microscopic features of sporangiophore (C), rhizoids (D), and immature- (E) and mature- (F) sporangium in slide cultures of *Rhizopus oryzae* ($\times 400$)

and AlAkhrass et al. (22) from the United States of America. These differences could be due to variations in study populations.

Rhinocerebral mucormycosis is the most common clinical form of this infection (4, 6, 17, 21, 23), which is in agreement with our results. In our study, similar to a report by Roden et al. (17) from the United States of America, rhinocerebral mucormycosis with or without orbital involvement was the major manifestation in diabetic patients. These findings are inconsistent with the results of Zaki et al. (3) from Egypt, which demonstrated pulmonary mucormycosis as the major manifestation in diabetic patients. In this report, diabetes mellitus (68%) was the common risk factor for mucormycosis, which supports other studies (3, 4, 20, 24), as expected in tropical and developing countries (3, 6). However, in some other studies, hematologic malignancy has been reported as the most common predisposing factor for mucormycosis (21, 25).

Recovery of mucoralean fungi from clinical specimens has been found problematic because of negative culture results, which could be due to the tissue processing prior to culture (26, 27) as well as initiation of therapy prior to specimen collection, which result in a negative culture. In the present study, the positive culture was obtained in 60% of the cases, which is similar to the data reported in a review of 929 mucormycosis cases (1940 - 2003) with 50% positive cultures (17). However, Zaki et al. (3), reported a recovery rate of 100%. These discrepancies could be due to

the difference in the procedure of specimen processing.

According to morphological characteristics, all isolates were identified as *Rhizopus* spp. and the results of molecular identification were 100% confirmed at the genus level, similar to Alvarez et al. (28). Therefore, conventional morphological methods, which are available in all Medical Mycology Laboratories could be trusted at the genus level at least for the *Rhizopus* as most common genus. However, these procedures are time-consuming and need high levels of expertise, especially in the case of uncommon species. Also, accurate identification may not be achieved for all genera as Yang et al. (15) reported a lower concordance rate (58.3%) between conventional and molecular identifications. Similarly, Kontoyiannis et al. (18) showed a 20% error rate for conventional identification in comparison to PCR-sequencing results. Therefore, simultaneous application of both morphologic and molecular procedures could be of great importance to provide accurate results.

Members of the genus *Rhizopus* are the principal causes of mucormycosis (1, 4, 7, 22). Although 10 of 25 specimens yielded no colonies in this study, all 15 grown isolates were identified as *R. oryzae*, which implies a prevalence of at least 60% for this species as the most common agent in this study. Similar results could be found in studies by Bala et al. and Alvarez et al. (4, 28). However, others (3, 29, 30) reported species other than *R. oryzae* as the prevailing etiology of mucormycosis. These differences emphasize the

need for precise identification in all cases of mucormycosis.

Finally, the present study used sequence-based method for precise identification of the isolates, though, the results could be beneficial for a better understanding of the microbial etiology of mucormycosis. However, the number of the patients were limited and positive culture was not obtained for some cases, which are some shortcomings of this study.

5.1. Conclusions

It could be concluded that the spectrum of fungal etiology of mucormycosis could differ based on the study populations and geographic locations. Regarding full agreement between the results of morphologic and molecular methods in our study, conventional procedures could be trusted at genus level at least for *Rhizopus* species; however, application of molecular methods is recommended due to their high identification power.

Footnotes

Authors' Contribution: Mahsa Naeimi performed all the experiments. Parivash Kordbacheh designed and supervised the study. Seyed Jamale Hashemi, Mehraban Falahati and Farideh Zaini were the scientific and practical consultants and also provided some of the laboratory materials. Hossein Mirhendi was the consultant for molecular identification and provided some of the isolates. Mahin Safara, and Mohsen Gerami Shoar assisted in morphological identification and image analysis. Shahram Mahmoudi was in charge of the analysis of the results of molecular identification. The manuscript was drafted by Mahsa Naeimi and Shahram Mahmoudi and the final version was prepared by Parivash Kordbacheh and Hossein Mirhendi.

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