Original Article

Molecular epidemiology of zygomycosis and their related factors in tertiary referral centers in southern Iran

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Abstract

Introduction: For the best management of the zygomycosis in immunocompromised patients, the present study aims to detect and identify the etiologic agents by DNA sequencing method and their related factors in clinical samples of patients.

Methodology: Clinical samples from 1,058 patients admitted in 11 university hospitals in Shiraz, Southern Iran were collected between July 2015 and July 2018. All samples (bronchoalveolar lavage, sputum, blood, tissue) were examined by routine microscopic and culture tests for zygomycetes. The etiologic agents were identified by the molecular method and sequencing.

Results: Direct microscopic examinations or pathology smear, culture, and PCR were positive in 61 (5.8%), 15 (1.4%), and 103 (9.7%) patients, respectively. According to EORTC/MSG criteria, the rates of proven, probable, and possible zygomycosis were 59.2% (61/103), 14.6% (15/103), and 26.2% (27/103 patients), respectively. The most prevalent etiologic agents according to sequencing were *Rhizopus oryzae* (44 cases), *Rhizopus microsporus* (31 cases), *Rhizopus stolonifer* (15 cases). Twenty-two patients (21.4%) with positive PCR died. There were significant relations between zygomycosis and the underlying disease ($p = 0.043$) and prior antifungal therapy ($p = 0.023$). White blood cell count was in the normal range in 14.1% of patients, and the means of erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) were 65 mm/hour and 57 mg/L, respectively.

Conclusions: Molecular methods and sequencing may have considered as suitable tools to diagnose zygomycosis. Identification of the etiologic agents may be considered as the future antifungal therapy and management of the respective patients.

Introduction

Zygomycosis is caused by Mucorales or Entomophthorales fungi. These orders are environmental fungi with rapid progress and can cause life-threatening infections in immunocompromised patients with underlying diseases like diabetes mellitus, neutropenia, bone marrow transplants, and hematological malignancies [1–2]. Interestingly, there are reports of patients with this infection without immunocompromising conditions [3,4]. Spores of zygomycetes are ubiquitous and inhalation of spores is the predominant transmission route, but inoculation of spore at the trauma site and use of contaminated food are the other transmission ways. The most prevalent members of these orders with variable antifungal susceptibility patterns include *Mucor*, *Lichtheimia*, *Rhizopus*, and *Rhizomucor*. The spectrum of disease associated with this infection is wide with high mortality rate. Rhinocerebral zygomycosis presents with sinusitis and orbital cellulitis or proptosis. Common spectrum of disease associated with

zygomycetes are include infection of cutaneous, gastrointestinal tract, lung, sinus, brain and angio-invasion. Disseminated infections were reported in immunocompetent patients (2%) [5,6]. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are markers in response to nonspecific inflammation conditions in the human body. In healthy individuals, blood cells settle slowly, but during the infection, quick settling occurred. Monitoring of CRP and ESR in the blood of the patients can exhibit the severe condition of many diseases. The CRP and ESR values along with other clinical signs and symptoms can help to the diagnosis of the infections. For the diagnosis of zygomycosis, histopathology would be the most efficient but this method cannot identify the etiologic agents as species. Sufficient information about etiologic agents in each region by the molecular method and sequencing could be used for the best management and treatment of infected patients. The aim of the present study was the identification of zygomycosis by DNA sequencing in clinical samples of immunocompromised

patients and evaluation of related factors such as CRP, White blood cells (WBC) count, ESR, and previous antifungal therapy for the best management of them.

Methodology

Ethical Statement

The ethics committee of the national institute for medical research development (Nimad) and clinical Microbiology Research center, Shiraz University of Medical Sciences approved this study (EC-958784), which was carried out following the Declaration of Helsinki. Sampling was done as a part of the diagnosis process. Written informed consent was obtained from participants. For participants under 16 years old, all parents were informed about the nature of the study and the written consent obtained from a parent or guardian.

Study Patients and Sample Collection

The study population consisted of the patients with suspected invasive fungal infections, based on clinical and radiological features, and no response to antibacterial between July 2015 and July 2018. Samples (sputum, tissue, bronchoalveolar lavage, and blood) were collected from patients in sterile conditions. They received by the Professor Alborzi Clinical Microbiology Research Center, Shiraz, southern Iran, in the biosafety cabinets from different hospitals (Namazi, Shahid Faghihi, Amir, Rajaee, Dena, Kowsar, Ali Asghar, Abu-Ali Sina, Amir al-Momenin Burn Injury, Hafez and Ordibehesht hospital). Demographic data including gender, age, background disease, clinical sign and symptom, CRP (nephelometry), ESR (Westergren tube), WBC count (Sysmex k21), prior antifungal therapy and the results of pathology smear (gomori methenamine silver and periodic Acid-Schiff staining) were collected from patients' records. The patients were categorized (proven/probable/possible), according to EORTC/MSG criteria and clinical features [7].

Conventional Mycological Methods

Lab Procedures were handled under the sterile condition in biological safety cabinet class 1 to avoid personal and environmental contamination. Samples (except blood) were examined by microscopic examination using 10% potassium hydroxide for the detection of fungal elements. Also, they were cultured on Sabouraud dextrose agar (Merck, Darmstadt, Germany) and incubated for 14 days at room temperature. Isolated species were identified by the lactophenol cotton blue smear and molecular method.

Blood samples were cultured in BACTEC medium (Becton-Dickinson, Sparks, MD, USA).

Molecular identification

Semi-nested PCR for the diagnosis of mucormycosis was performed on all clinical samples and isolated fungi from the culture media. Tissue samples were lysed by mixing with 100 µL of sterile distilled water, 100 µL of lysis buffer, 20 µL of proteinase K and 20 µL of carrier RNA and incubated overnight at 56°C. Blood samples from the patients were obtained and serum was separated. DNA was extracted using a DNA extraction kit (Invisorb Spin DNA Extraction Kit, Berlin, Germany), as recommended by the manufacturer. Semi-nested PCR was used with two sets of primers for the first and second rounds: ZM1 (5′-ATT ACC ATG AGC AAA TCA GA-3′), and ZM2 (5′-TCCGTC AAT TCC TTT AAG TTT C-3′); and ZM1, and ZM3 (5′- CAA TCC AAT TTC ACC TCT AG-3′), respectively according to Rickert *et al.* [8]. Also, for identification of some isolated fungi from culture media, ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′), and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used [9]. All PCRs were run in a thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) and products were evaluated by electrophoresis on 2% agarose gels and analyzed by sequencing. Final sequences were aligned with reference sequences available at the home library in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and MycoBank (http://www.mycobank.org/). Fungal identifications were made based on maximum identities $\geq 99\%$ and query coverage $\geq 98\%$ and then submitted to GenBank.

Statistical analysis

Data were analyzed using SPSS software (IBM SPSS statistics 23, IBM Corp., Armonk, NY, USA). Chi-squared test (χ^2) was used to investigate the relationship between the prevalence of infection, gender, age, prior antifungal therapy, and underlying conditions. *p*-value < 0.05 was considered statistically significant.

Results

One thousand fifty-eight (1,058) patients were entered into this study. Of them, 483 (45.7%) and 575 (54.3%) were female and male, respectively. The mean age was 18 years (> 1 to 89 years, Std. Deviation 21.98). During the study, and before zygomycosis identified, 541 patients (51.1%) used antifungal agents for prophylaxis or treatment of other subspecies fungal

infections. The most prevalent examined samples were the blood followed by sputum, bronchoalveolar lavage, and tissue (Table 1). The most predisposing factors were hematologic disorders (myelocytic leukemia, acute lymphocytic leukemia, and other hematopoietic disorders) followed by diabetes, transplantation, and immunodeficiency.

Direct microscopic examination and pathology smear results were positive in 61 patients (non-septated hyphae) and culture was positive in bronchoalveolar lavage and wound of 15 patients (non-sterile specimens). None of the blood cultures was positive. PCR was positive in 103/1,058 patients (9.7%). In this study, a positive result of pathology smear was considered a proven infection. All positive culture results were from unsterile samples. According to EORTC/MSG criteria for invasive fungal diseases, all

Table 1. The clinical characteristic features of study population admitted to university hospitals and evaluated for zygomycosis in Shiraz, southern Iran.

Characteristics	n (%)
Gender	
Female	483 (45.7%)
Male	575 (54.3%)
Age	
> 1 year	404 (38.2%)
1-9	138 (13%)
10-19	35 (3.3%)
20-29	49 (4.6%)
30-39	60 (5.7%)
40-49	40 (3.8%)
50-59	170 (16.1%)
≥ 60	162 (15.3%)
Ward	
Internal Medicine	188 (17.8%)
ICU	199 (18.8%)
Pediatric	160 (15.1%)
Endocrine	201(19%)
Hematology	228 (21.6%)
Transplant	38 (3.6%)
Immunology	19 (1.8%)
Specimens	
Blood	809
Respiratory secretion	214
Tissue[†]	25
Cerebrospinal fluid	21
Others[‡]	76
Zygomycetes PCR	
Positive	103 (9.7%)
Negative	955 (90.3%)
Outcome	
Died	97 (9.2%)
Lived	961 (90.8%)

[*]Some patients have more than one specimen.; [†]Tissues include: Sinuses, oral lesion, liver, bone marrow and spleen; [‡]Others include: urine, wound discharge, body fluids.

Table 2. Demographic data of 103 PCR positive patients with zygomycosis in Shiraz, southern Iran.

Characteristics	n (%)
Gender	
Female	45 (43.7%)
Male	58 (56.3%)
Age	
Mean	18 years
Minimum	1 year
Maximum	81 years
Ward	
ICU	12 (11.7%)
Pediatric hematology	39 (37.9%)
Adult hematology	20 (19.4%)
Endocrine	22 (21.4%)
Transplant	8 (7.8%)
Immunology	2 (1.9%)
Specimens[*]	
Blood	81
Respiratory secretion	110
Tissue	11
Others[†]	56
Clinical Forms of zygormycosis	
Pulmonary infection	55 (53.4%)
Rhino-cerebral infection	37 (35.9%)
Liver abscess	3 (2.9%)
Eyes infection	5 (4.8%)
Skin lesion infection	2 (1.9%)
CNS	1(0.97%)
Culture result	
Positive	15 (14.6%)
Negative	88 (85.4%)
Outcome	
Died	22 (21.4%)
Lived	81(78.6%)

[*]Some patients have more than one specimen; [†]Others include: Cerebrospinal fluid, eye sample, body fluids and wound discharge.

patients were with the risk of infections and clinical criteria, and the rates of proven, probable and possible mucormycosis were 59.2% (61/103 patients), 14.6% (15/103) and 26.2% (27/103 patients), respectively. Detection of nucleic acid is not included in the EORTC/MSG criteria. The demographic characteristics of PCR positive patients were: median age 18 years (range 1–81 years), and female to male ratio was 45/58 (Table 2). The frequencies of risk factors in these patients were hematologic disorders 57.3% (59/103), diabetes 21.4% (22/103), patients admitted to ICU wards 11.7% (12/103), solid organ transplant patients 7.8% (8/103) and other immunocompromised patients 1.9% (2/103). Antifungal agents were used in 57/103 (55.3%) of patients before entering the study. Out of 26 patients with a history of using voriconazole monotherapy, 3 patients and from 13 patients with caspofungin monotherapy 2 patients had *Mucor* PCR

Table 3. The rates of antifungals used in patients before entered in this study.

Antifungal agents	Total patients	PCR positive patients
Amphotericin B	123 (11.6%)	14 (13.6%)
Fluconazole	72 (6.8%)	3 (2.9%)
Caspofungin	13(1.2%)	2 (1.9%)
Voriconazole	26 (2.5%)	3 (2.9%)
Itraconazole	4 (0.4%)	0 (0%)
Combination*	303 (28.6%)	35 (34%)
Not used	517 (48.9%)	46 (44.7%)
Total	**1,058 (100%)**	**103 (100%)**

*Combination therapy: use more than one antifungal like amphotericin and caspofungin or amphotericin, caspofungin and voriconazole.

positive results (Table 3). There were significant differences in terms of the underlying disease ($p = 0.043$) and antifungal therapy ($p = 0.023$), and no significant gender differences in PCR positive result ($p = 0.841$). Given the wide age range, we could not calculate the significance of the difference between ages. In PCR positive patients, the range of WBC count was between 100-135,000 (mean 10,860 cells/microliter of blood), the WBC count was in normal range (4,000-10,000) in 14.6% (15/103) of patients, and 40.1% (42/103) and 44.7% (46/103) of patients were with lower and higher WBC count than normal range, respectively. The ESR range was 3-142 mm/hour with mean 65 mm/hour (39% < 20 and 61% ≥ 20 mm/hour). The range of CRP was 2-150 mg/L, mean 57 mg/L. 73.6% of patients had CRP > 6 mg/L (cut-off value in our university).

According to the culture medium, *Rhizopus* and *Mucor* species were isolated from the specimens and identification by genus was difficult. The results from molecular sequencing methods correlated well with automated microbiological identification systems for common clinical isolates. The etiologic agents according to sequencing were *Rhizopus oryzae* (44 cases), *R. microsporus* (31 cases), *R. stolonifer* (15 cases), *Saksenaea vasiformis* (3 cases), *Basidiobolus*

ranarum (3 cases), *Apophysomyces elegans* (3 cases), *Sporodiniella umbellata* (2 cases) and *Lichtheimia corymbifera* (2 cases) (Table 4). After the diagnosis of the infection, amphotericin B and/or posaconazole prescribed for patients. In this study, 22/103 (21.4%) patients with positive PCR died.

Discussion

Zygomycosis is a life-threatening fungal infection with high mortality. Using a molecular method, in this study, 9.7% of the high-risk patients with suspected invasive fungal infections have presented zygomycosis. The etiologic agents were different and some of them were not reported previously in our region. The clinical significance of zygomycosis is dependent on patients' immune systems and pathogenicity of the etiologic agent. The diagnosis of zygomycosis is difficult. Early diagnosis and treatment can have an important role in the management and outcome of the patients. The precise identification of the etiologic agents from clinical samples can lead to appropriate treatment and decrease the rate of drug resistance fungal. Use of a sinus and chest CT and MRI imaging can be helpful in diagnosis but cannot help identified the etiologic agent. Isolation of etiologic agents (culture) from sterile samples and histopathology diagnostic methods are the

Table 4. The etiologic agents of zygomycosis based on DNA sequencing in Shiraz, southern Iran.

Etiologic agent	Number/ Percent	Accession numbers
Rhizopus oryzae	44 (42.7%)	MK288131, MK351263, MK351264, MK351552-59, MK351561, MK351564-66, MK503709, MK503711, MK504452, MK505671, MK506124, MK506125, MK506236, MK506237, MK506239-41, MK506243-48, MK512102-105, MK512360, MK512362, MK512365, MK512366, MK512378, MK512454, MK512573, MK512366, MK512678
Rhizopus microsporus	31 (30.1%)	-
Rhizopus stolonifer	15 (14.6%)	MK351258, MK351261, MK351262, MK351265, MK351560, MK351562, MK351563, MK351877, MK503667, MK503668-73
Saksenaea vasiformis	3 (2.9%)	MK499472, MK346253, MK346254
Basidiobolus ranarum	3 (2.9%)	MK501619, MK501321
Apophysomyces elegans	3 (2.9%)	MK500867, MK500893, MK500900
Sporodiniella umbellata	2 (1.9%)	MK500860, MK346255
Lichtheimia corymbifera	2 (1.9%)	KJ690940*
Total	**103 (100%)**	**64 (62.1%)**

* Similar previous accession number; ** One accession number was obtained from similar species.

gold standard for diagnosis. However, sterile samples collection like tissue in immunocompromised patients, especially hematologic malignancies is not validated (thrombocytopenia). Species identification in the histopathologic analysis is challenging. Culture and susceptibility pattern of the isolated fungi can lead to the best outcome of the patients. But, culture is time-consuming and needed at least 2–5 days for identification of the isolated zygomycetes and may be negative in some samples [10]. It is not suitable to accurately distinguishing all the isolated species by the routine culture. In a review of 929 cases of mucormycosis, only 15% had positive culture results [11]. Circulating antigen detection tests like mannan, galactomannan, and 1,3 Beta-D-glucan cannot be used for the evaluation of mucormycosis. According to the literature, diagnosis by molecular methods and sequencing may provide accurate information about this infection. Such methods can identify the etiologic agents in many samples (except endoscopic sinus surgery specimens) with culture-negative results [12,13]. PCR-based detection of zygomycetes in clinical material has focused mainly on rapid diagnosis and specific identification. Also, PCR has been utilized for the DNA detection of the culture-negative samples [13,14]. The limitations of PCR in the diagnosis of mucormycosis are the high environmental contamination rate, false-negative result due to low DNA copy number, using low-quality materials, improper sample collection/transport, degradation of nucleic acids, and any laboratory processing error. Therefore, the results of the PCR test should be interpreted by the clinical signs and symptoms of the patients. In some previous studies, diagnosis of mucormycosis was made by autopsy, therefore, there are limited data about the epidemiology of mucormycosis in the literature. The rate of mucormycosis in the other studies from Iran were reported by 9.7% in 2008 and 23.7% in 2014 [15]. The incidence rate of mucormycosis in France in patients with hematopoietic disorders from 1997 through 2006 increased from 0.7 to 1.2 cases/million persons [16]. The rate of infection is different in each region due to health care strategy and the number of high-risk population patients.

The most predisposing factors in the present study were hematologic disorders (57.2%), followed by diabetes (21.4%). Bitar and coworker reported hematologic malignancy was the most prominent underlying disease ranging between 38% and 62% in different studies [16] and in Ambrosioni *et al.* hematologic malignancies (42%) was the most frequent

predisposing factor [17]. In the patients living in central Europe, malignancy (63%) was the most common underlying disease followed by diabetes (17%) and solid organ transplantation (10%) [18]. Diabetes mellitus was the most frequent underlying disease with uncontrolled diabetes mellitus in Nashibi *et al.* (85%) [19] and in Hammond *et al.* (36%) [14]. Other risk factors reported include solid organ transplantation, prolonged steroid therapy, deferoxamine therapy, burns, injection drug use, and bone marrow transplantation [15, 20]. In India, trauma (N = 31, 10.2%) was reported as the common risk factor [20]. The common underlying diseases vary in different regions. The host immune system and portal of fungi entry may be responsible for the type of infection presentation.

In this study, the most prevalent site of infections was the respiratory system (lung and sinus). According to the literature, the site of infection is related to the patients' underlying diseases, e.g., in malignant patients, pulmonary infection and patients with diabetes mellitus, sino-orbital and rhinocerebral disease are the most infection presentations [21]. The most common etiologic agents according to sequencing in this study were *R. oryzae* followed by *R. microsporus*, *R. stolonifer*, *B. ranarum*, *A. elegans*, *S. vasiformis, S. umbellata*, and *L. corymbifera*. In Roden et. al. *Rhizopus*, *Mucor*, and *Lichtheimia* were the most etiologic agents (accounting for 70–80% of all cases) causing mucormycosis, and < 1–5% of reported cases were *Actinomucor*, *Cunninghamella*, *Rhizomucor, Syncephalastrum, Apophysomyces*, *Saksenaea*, and *Cokeromyces* [11]. In Petrikkos *et al.* the 8/24 and 16/24 patients were infected with *Rhizopus* spp. and *Mucor* spp., respectively [21]. The commonest fungi isolated from clinical specimens of mucormycosis infected patients were *R. oryzae* followed by *L. corymbifera* and *Mucor racemosus* [20]. In India, the second frequent pathogen identified from clinical specimens after *R. oryzae* was reported *A. elegans* [22]. In another study from India, *R. arrhizus* (51.9%) was the predominant agent identified, followed by *R. microsporus* (12.6%), *Apophysomyces variabilis* (9.2%), and *Rhizopus homothallicus* (2.5%) [23]. In some studies, especially in older studies, identifying the involved species was not performed and the term 'Mucor' always represents some isolated species.

According to our data, there is a significant relationship between the use of antifungal agents and zygomycosis but no significant relationship was observed between this infection and caspofungin or voriconazole monotherapy. It may be due to the low

number of patients treated with these antifungal monotherapies. The prophylactic use of voriconazole in high-risk patients was reported as a risk factor in the emergence of mucormycosis [24,25]. In the present study, the mean of the WBC count was 1,058 and only 14.1% of infected patients were in the WBC count normal range. In the study by Sarvestani and coworkers, the mean WBC count of patients was 19,260 (range 100 – 228,300) [26]. In Bhat *et al*. in all the cases studied, WBC count was increased [27]. The mean ESR in the present study was 65 mm/hour and in Bhat *et al.* was 79 mm/hour [27]. The mean ESR was elevated significantly in the study by Ghafur [28]. The mean value of CRP in the present study was 57 mg/L. "In healthy individuals, the CRP level is generally below 2 mg/L but can be up to 10 mg/L" [29]. The role of WBC, ESR, and CRP in the diagnosis of mucormycosis in immunocompromised patients is not clear. Because this population is at risk of many disorders like bacterial infections, malignancy, and prolonged staying in the hospital. Future studies need to evaluate the role of these parameters in patients suffering from zygomycosis.

In this study, the mortality rate was 21.4% of patients with positive PCR results. In Nashibi *et al.* five patients (25%) died [19] and in Prakash *et al.* In 388 mucormycosis cases, the overall mortality rate was reported 46.7% [20]. The mortality rate was reported to be significantly associated with the site of infection such as gastrointestinal (OR: 18.70, $p = 0.005$) and pulmonary infections (OR: 3.03, $p = 0.015$) [20]. In Skiada *et al.* according to the site of infection, the mortality rates were 58% (18/31), 56% (33/59), and 44% (8/18) in patients with the disseminated, pulmonary and diabetic disease, respectively [18]. This rate varies based on the study population, underlying disease, and regional health care system. In the present study, amphotericin B and/or posaconazole were used for treating the infected patients. Treatment of zygomycosis is always difficult despite the newer antifungal agents [2]. Early diagnosis and initiation of proper antifungal therapy have important roles in the outcome of patients. For proper antifungal therapy, the predisposing factors should be eliminated immediately. Necrotic tissue debrides and removes by the aggressive surgical methods as soon as the infection diagnosed. Lipid formulation of amphotericin B and amphotericin B lipid complex are the drugs of choice for zygomycetes therapy and given through the vein. Posaconazole and isavuconazole with broad-spectrum antifungal activity can be given for treatment of this infections through the vein and by mouth [30,31].

Treatment must be continued for several weeks and until the patient has shown signs of improvement. Combination antifungal therapy is not recommended in the treatment of zygomycetes [32].

Conclusions

Hematologic disorders were the most predisposing factor in our study. Successful management of zygomycetes (with nonspecific clinical manifestations) requires early diagnosis and treatment. Culture is not a suitable method for the identification of zygomycetes. Molecular methods and sequencing may have considered as the suitable tools in diagnose of this infection. *A. elegans*, *S. umbellata*, and *Saksenaea vasiformis* were diagnosed for the first time in the clinical samples of Iranian patients. Identification of the new species may be considered as the proper antifungal therapy and management of the respective patients. According to our results, significant relationship between prior antifungal therapy and hematologic disease and non-significant relationship between WBC count, CRP and ESR values with zygomycosis observed.

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