

Special Real-Time PCR and Conventional PCR for Detection of Rhino-Orbital-Cerebral Mucormycosis

Naghmeh Bahrami^{1,2}, Hamidreza Jamaati³, Behrooz Farzanegan^{4,5}, Rezvan Azim Nejad⁶, Farnoush Mohammadi^{2,7}, Mraj Naghdi⁸, Seyedeh Zahra Fotook Kiaei⁹ and Abdolreza Mohamadnia^{3,10,*}

¹Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

²Craniofacial Research Center, Tehran University of Medical Sciences, Tehran, Iran

³Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁴Department of Anaesthesiology, Critical Care Quality Improvement Research Center, Shahid Modarres Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁵Tracheal Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁶Department of Biology, Faculty of Sciences, Islamic Azad University, Tehran, Iran

⁷Department of Oral and Maxillofacial Surgery, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

⁸Islamic Azad University of Medical Sciences, Tehran, Iran

⁹Department of Pulmonary and Critical Care, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

¹⁰Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding author: Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Email: mohamadnia.ar@gmail.com

Received 2024 January 22; Accepted 2024 February 03.

Abstract

Background: Mucormycosis is an invasive fungal infection that occurs in immunodeficiency patients and belongs to infections caused by mucoral fungi, such as *Rhizopus* and *Rhizomucor*.

Objectives: This study investigated the identification and detection of the mucor fungus in tissue samples and other samples by the real-time polymerase chain reaction (PCR) method.

Methods: This was a cross-sectional-descriptive study. A total of 80 tissue samples were collected from referring patients to diagnose fungi with the opinion of a specialist doctor. After extracting deoxyribonucleic acid (DNA) from the samples, PCR and real-time PCR tests were performed using specific primers for mucor and universal-pan and compared to culture. The results were confirmed by sequencing.

Results: In this study, 80 samples were examined. In the PCR method, 74 and 75 cases of fungi were confirmed from the DNA obtained from the colonies and tissues, respectively. By using a specific primer for mucor, 12 patients were reported as positive for mucor. Additionally, 76 and 77 cases of fungi were confirmed in the real-time PCR method from the DNA obtained from the colonies and tissues, respectively, and positive cases were reported. By using specific primers and the real-time PCR test for mucor, 16 cases of mucor were reported from colonies, and 15 cases of mucor were reported positive from tissues.

Conclusions: This study showed that real-time PCR and PCR are high-speed and more accurate methods than the culture test in identifying its species and have acceptable results in clinical cases.

Keywords: Mucor, Detection, PCR, Real-time PCR

1. Background

Mucormycosis is an invasive and rapidly progressing fungal infection with significant mortality that occurs in immunocompromised patients, and its clinical characteristics and treatment methods are incomplete. Mucormycosis is a term that refers to infections caused by mucoral fungi, such as *Rhizopus* and *Rhizomucor*.

After *Aspergillus* species, mucormycetes are the second most common cause of mycosis in hematological patients (1, 2). Therefore, finding a fast and accurate method to identify mucor species is very important, especially in its clinical samples (3, 4). For this reason, the use of new, fast, and sensitive methods, including

molecular methods of deoxyribonucleic acid (DNA) detection in various samples, such as serum, BAL, and tissue, is currently being investigated (5, 6).

Mucormycosis has various clinical manifestations, the most common of which is the classical form of rhinocerebral-orbital. Successful treatment of mucormycosis is performed with a timely diagnosis. For this reason, the accurate diagnosis of the underlying factors seems to be very necessary to determine the appropriate clinical protocol and the prognosis of the disease. Meanwhile, molecular methods give us more accurate results due to high sensitivity and specificity.

Molecular tracking of mucormycosis is conducted

based on different methods, such as polymerase chain reaction (PCR) and real-time PCR. Real-time PCR is one of the best molecular detection techniques, primarily due to the reduction of time, and is suitable for large-scale work (7-9). Therefore, the PCR method is used in the diagnosis and follow-up of the treatment of many diseases, such as cancer (10-14).

2. Objectives

In this study, real-time PCR was used to identify mucor and some of its species in clinical samples.

3. Methods

This was a cross-sectional-descriptive study in 1402. After obtaining permission from the Ethics Committee (IR.SBMU.NRITLD.REC.1402.219), 80 tissue samples were collected from referring patients for fungus diagnosis with the opinion of a specialist doctor.

The inclusion criteria for the study were pathology (smear or histopathology) and clinical signs consistent with rhino-orbital-cerebral mucormycosis. The exclusion criteria were the failure to complete the hospital file accurately and the patients who, for any reason, were not given diagnostic and therapeutic measures and left the hospital.

In this study, real-time PCR and PCR methods were employed as molecular techniques for diagnosis. For this purpose, DNA extraction must be performed first. There are different methods for DNA extraction. Commercial DNA extraction kits were utilized to obtain better results and save time. Deoxyribonucleic acid extraction steps were performed using a GeNet Bio kit, and DNA quality and purity were evaluated by NanoDrop.

Before performing real-time RT-PCR and PCR, a specific primer was prepared for mucoral group fungi, and the specifications of the primer are shown in Table 1. To determine efficiency, a real-time RT-PCR reaction was performed with different dilutions of the primer, and the slope of the standard curve was obtained, indicating the primer's efficiency.

Using databases and the powerful Blast tool confirmed the binding site of the primer pair to the relevant gene. Primers were made by Gene Fanavaran company (Iran). The samples were analyzed by real-time PCR using specific primers to identify mucor (Table 1).

Real-time PCR was conducted in a final volume of 20 mL with the content of 12 mL Ampliqon master mix, 0.3 mL of each of F and R primers, 2 μ L DNA, and distilled water. By employing a rotor-gene cycler, the test was carried out in 40 cycles.

Table 1. Specifications of Mucor and Universal-Pan Primers

Parameters	Universal-Pan	Mucor
Forward primer	GCAGYAGATCTCTTAGATCTGA	CTACGACAGTCACATTGGTG
Length	22	20
Reverse primer	AACGTTTCGTTGACTCAATGG	ATCGGATCTAGAGATCTAGTC
Length	20	21
product length	122	90
Annealing	62	64

Temperature and time conditions included an initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 15 seconds, primers connection at 56°C for 60 seconds, and amplification at 72°C for 25 seconds (for 40 cycles). The final amplification was performed at 72°C for 5 minutes. Sequencing was conducted for several samples for final approval.

3.1. Statistical Analysis

The sample size was estimated according to the favorable ratio of the tests and considering 5% type 1 error and 20% type 2 error. The results were analyzed using SPSS software (version 22), and the mean and standard deviation were calculated. A paired *t*-test was utilized for data analysis. A P-value ≤ 0.05 was regarded as statistically significant.

4. Results

This study evaluated 80 tissue samples that were suspected of fungi in the pathology investigations. The average age of the patients was 56.8 ± 8.6 years. All the samples were cultured on Sabouraud dextrose agar (SDA). A total of 76 samples were culture-positive, and 14 of them were reported to be mucor-positive by examining microscopic smears and specific cultures.

Deoxyribonucleic acid extraction was performed from tissue samples and cultured colonies. Moreover, the molecular test was performed using the PCR method with the pan-fungi-specific primer. The PCR method of the DNA obtained from the colonies confirmed 74 cases of fungi. In addition, the PCR test of the DNA obtained from the tissues showed that 75 patients were positive for fungi. Then, using a specific primer for mucor, a PCR test was performed, and 12 mucor-positive cases were reported in the DNA obtained from the colonies and in the DNA obtained from the tissues.

In the next step, a molecular test was performed using the pan-fungi-specific primer by real-time PCR method. The real-time PCR method of DNA obtained from colonies

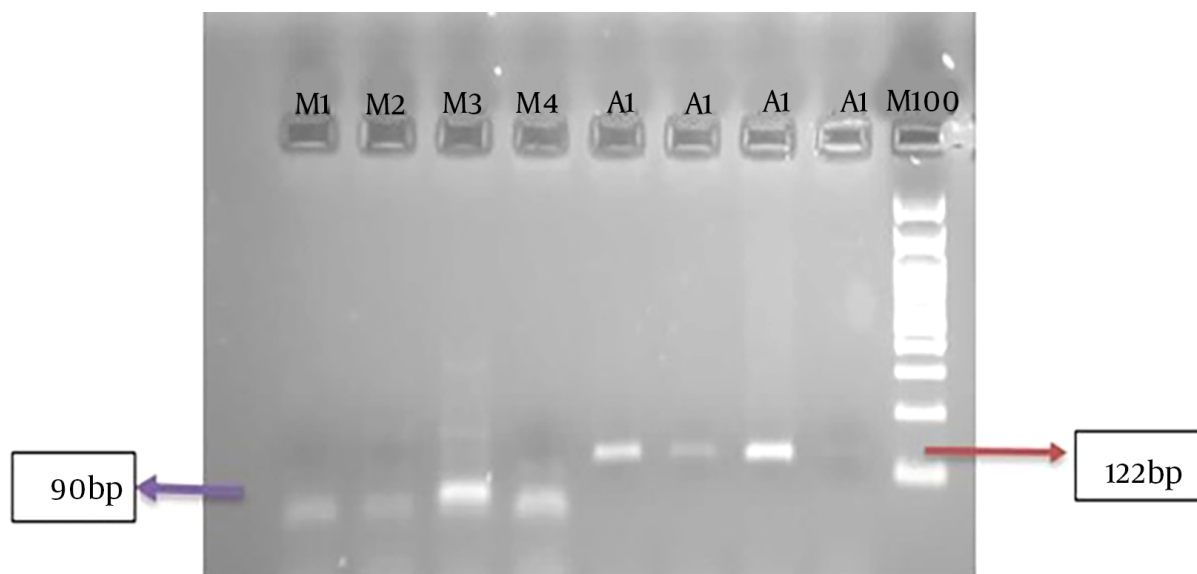


Figure 1. Agarose gel of pan-fungi and mucor; A3 is a positive control for pan-fungi, and M3 is a positive control for mucor. A1 and A2 are examples of positive pan-fungi. M1 and M2 are examples of positive mucors.

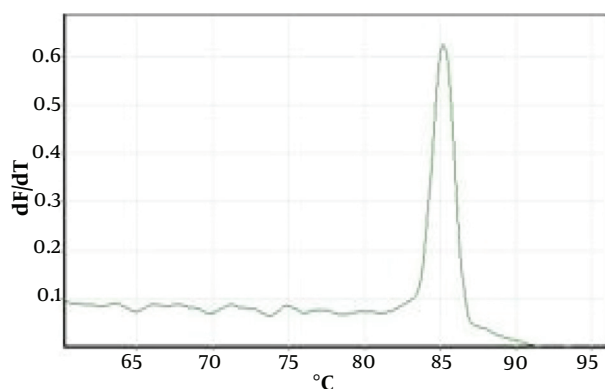


Figure 2. An example of a mucor melting curve

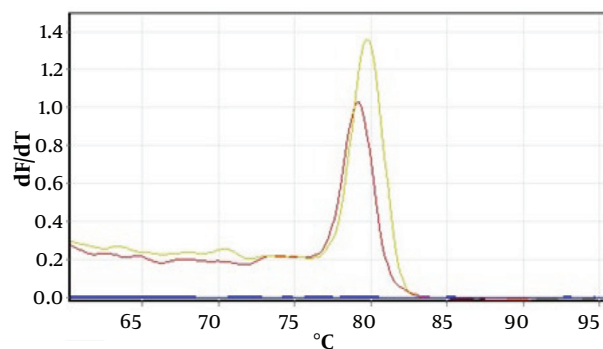


Figure 3. An example of a universal-pan-fungi melting curve

confirmed 76 cases of fungi. Furthermore, with real-time PCR tests of DNA obtained from tissues, 77 cases were positive for fungi.

Then, a real-time PCR test was performed using a specific primer for mucor, and 16 mucor-positive cases were reported in the DNA obtained from the colonies. Additionally, 15 mucor-positive cases were reported in the DNA obtained from the tissues.

Figure 1 shows an example of the PCR method for agarose gel. Figures 2 and 3 show the melting curves of the real-time PCR method for pan-fungi and mucor.

In the next step, the products of the positive samples

from real-time PCR were sent to Gene Fanavaran for sequencing, and the results were compared to the results of real-time PCR tests. The sequence results confirmed the number of 12 cases of real-time PCR results in the case of mucor.

5. Discussion

In recent years, the problems of increasing treatment-resistant fungal organisms on the one hand and the lack of definitive diagnostic methods and tools on the other hand have caused concern about the timely diagnosis and progress of invasive fungal diseases (6).

In recent decades, there have been various molecular methods to identify fungi and other diseases (15).

The real-time PCR method, which is used in clinical samples, is one of the best methods to identify and diagnose mucor. In the present study, 80 samples were examined, and PCR confirmed 74 and 75 cases of fungi from DNA obtained from colonies and tissues, respectively. By using specific primers and PCR tests for mucor, 12 cases were mucor and were reported as positive.

In addition, 76 and 77 cases of fungi were confirmed in the real-time PCR method from the DNA obtained from the colonies and tissues, respectively. By the use of specific primers and the real-time PCR test for mucor, 16 cases of mucor and 15 positive cases were reported from the tissues. These differences might be due to the different growth conditions of some fungi in the culture medium, the use of other substances in pathological staining, the use of other DNA extraction kits with different sensitivity, or different mixes used in the PCR process and/or real-time PCR (16).

In a study by Sani et al., the clinical symptoms of the ear, nose, and throat nearly 70% included most of the clinical signs, which is in line with the results of the present study, and the patients had similar clinical signs and were positive for mucor in laboratory tests (17).

It seems that the cause of mucormycosis is different in different cultures, such as mucor spp. and *Phizopus* spp. (18). Therefore, further research on different molecular diagnostic methods for different causes of mucormycosis and rare pathogens is needed to be able to identify both mucormycosis agents and their subspecies (19).

Millon et al.'s study showed that micromycetes DNA could be identified in at least 81% of patients with possible mucormycosis (20). In a recent study, 80/77 (96%) samples were diagnosed with mucor.

In any case, considering the results and efficiency of molecular methods in a short time in timely diagnosis for microbial and fungal infections, these tests are recommended for medical centers because these molecular methods have good sensitivity and specificity compared to other methods. However, further studies with larger samples and other specific primers are suggested to obtain results and accurately identify mucor factors and their subspecies.

Footnotes

Authors' Contribution: Abdolreza Mohamadnia: The corresponding author; Farnoush Mohammadi: Statistical analysis; Seyede Zahra Fotook Kiaei: Writing the article; Hamidreza Jamaati, Behrooz Farzanegan and Rezvan Azim Nejad: Data collection; Mray Naghdi: Executed laboratory

assays; Naghmeh Bahrami: Conducted the study. All authors read and approved the final manuscript.

Conflict of Interests: The authors declare that there is no conflict of interest.

Data Reproducibility: It was not declared by the authors.

Ethical Approval: Permissions were obtained from the Ethics Committee (IR.SBMU.NRITLD.REC.1402.219) of Masih Daneshvari Hospital.

Funding/Support: The study was supported by Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences.

Informed Consent: Written informed consent was obtained from all participants.

References

1. Slavin M, van Hal S, Sorrell TC, Lee A, Marriott DJ, Daveson K, et al. Invasive infections due to filamentous fungi other than *Aspergillus*: epidemiology and determinants of mortality. *Clin Microbiol Infect.* 2015;21(5):490 ei-10. [PubMed ID: 25677259]. <https://doi.org/10.1016/j.cmi.2014.12.021>.
2. Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin Infect Dis.* 2010;50(8):1091-100. [PubMed ID: 20218877]. <https://doi.org/10.1086/651263>.
3. von Hertwig AM, Sant'Ana AS, Sartori D, da Silva JJ, Nascimento MS, Iamanaka BT, et al. Real-time PCR-based method for rapid detection of *Aspergillus niger* and *Aspergillus welwitschiae* isolated from coffee. *J Microbiol Methods.* 2018;148:87-92. [PubMed ID: 29580982]. <https://doi.org/10.1016/j.mimet.2018.03.010>.
4. Walsh TJ, Wissel MC, Grantham KJ, Petraitiene R, Petraitis V, Kasai M, et al. Molecular detection and species-specific identification of medically important *Aspergillus* species by real-time PCR in experimental invasive pulmonary aspergillosis. *J Clin Microbiol.* 2011;49(12):4150-7. [PubMed ID: 21976757]. [PubMed Central ID: PMC3232976]. <https://doi.org/10.1128/JCM.00570-11>.
5. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization 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) Consensus Group. *Clin Infect Dis.* 2008;46(12):1813-21. [PubMed ID: 18462102]. [PubMed Central ID: PMC2671227]. <https://doi.org/10.1086/588660>.
6. Rouzbahani H, Badiie P, Barkhordar M, Badiie A, Faramarzi A, Moghadami M, et al. [Etiologic Causes of Invasive Fungal Sinusitis among Patients of, University Hospitals in Shiraz, Iran]. *J Isfahan Med Sch.* 2011;29(153):1286-96. Persian.
7. Lass-Flörl C. In vitro susceptibility testing in *Aspergillus* species: an update. *Future Microbiol.* 2010;5(5):789-99. [PubMed ID: 20441550]. <https://doi.org/10.2217/fmb.10.34>.
8. Walsh TJ, Petraitis V, Petraitiene R, Field-Ridley A, Sutton D, Ghannoum M, et al. Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. *J Infect Dis.* 2003;188(2):305-19. [PubMed ID: 12854088]. <https://doi.org/10.1086/377210>.

9. Karimi S, Bahrami N, Sharifi K, Daustany M, Baghbani-Arani F, Kazempour M, et al. Investigating gene expression level of MUC1 and CEA in pleural fluid of NSCLC lung cancer patients with real-time RT-PCR method. *Minerva Respir Med.* 2017;**56**(1):18–24. <https://doi.org/10.23736/s0026-4954.16.01772-7>.
10. Ghadimi K, Bahrami N, Fathi M, Farzanegan B, Naji T, Emami M, et al. Diagnostic value of LunX mRNA and CEA mRNA expression in pleural fluid of patients with non-small cell lung cancer. *Minerva Respir Med.* 2017;**56**(2):90–5. <https://doi.org/10.23736/s0026-4954.16.01773-9>.
11. Bahrami N, Malekolkottab F, Ebrahimi-Barough S, Alizadeh Tabari Z, Hamisi J, Kamyab A, et al. The effect of purmorphamine on differentiation of endometrial stem cells into osteoblast-like cells on collagen/hydroxyapatite scaffolds. *Artif Cells Nanomed Biotechnol.* 2017;**45**(7):1343–9. [PubMed ID: 27686538]. <https://doi.org/10.1080/21691401.2016.1236804>.
12. Bahrami N, Gholami M, Jamaati HR, Mohamadnia A, Dargahi H, Kazempour Dizaji M, et al. Expression of two essential mRNA biomarker in the peripheral blood as possible biomarkers for diagnosis of non-small cell lung carcinoma. *Minerva Pneumol.* 2016;**55**(3):31–6.
13. Bayat H, Shahabinejad H, Bayat M, Shirian S, Mohamadnia A, Alijani M, et al. Osteogenic differentiation of follicular stem cells on nano-Saghez scaffold containing BMP2. *J Orthop Surg Res.* 2019;**14**(1):442. [PubMed ID: 31842947]. [PubMed Central ID: PMC6916075]. <https://doi.org/10.1186/s13018-019-1507-0>.
14. Jamaati H, Bahrami N, Abniki M, Tabarsi P, Farzanegan B, Doroudinia A, et al. Real-time RT-PCR Detection of HCN4 and ADAM8 genes in ventilator-associated pneumonia patients Hospitalized in intensive care unit. *J Cell Mol Anesth.* 2016;**1**(4):163–7. <https://doi.org/10.22037/jcma.v1i4.13372>.
15. Heydar H, Mansouri K, Norooznezhad M, Norooznezhad F, Mohamadnia A, Bahrami N. Bevacizumab Inhibits Angiogenic Cytokines in Head and Neck Squamous Cell Carcinoma: From Gene to the Protein. *Int J Hematol Oncol Stem Cell Res.* 2018;**12**(2):136–41. [PubMed ID: 30233776]. [PubMed Central ID: PMC6141426].
16. Emami N, Mohamadnia A, Mirzaei M, Bayat M, Mohammadi F, Bahrami N. miR-155, miR-191, and miR-494 as diagnostic biomarkers for oral squamous cell carcinoma and the effects of Avastin on these biomarkers. *J Korean Assoc Oral Maxillofac Surg.* 2020;**46**(5):341–7. [PubMed ID: 33122459]. [PubMed Central ID: PMC7609927]. <https://doi.org/10.5125/jkaoms.2020.46.5.341>.
17. Sani AT, Fata AM, Arian M. Presenting features and outcome of rhino-orbital-cerebral mucormycosis in two referral center in Mashhad. *Tehran Univ Med J.* 2014;**72**(1).
18. Skiada A, Pagano L, Groll A, Zimmerli S, Dupont B, Lagrou K, et al. Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. *Clin Microbiol Infect.* 2011;**17**(12):1859–67. [PubMed ID: 21199154]. <https://doi.org/10.1111/j.1469-0691.2010.03456.x>.
19. Klimko N, Khostelidi S, Shadrivova O, Volkova A, Popova M, Uspenskaya O, et al. Contrasts between mucormycosis and aspergillosis in oncohematological patients. *Med Mycol.* 2019;**57**(Supplement 2):S138–44. [PubMed ID: 30816977]. <https://doi.org/10.1093/mmy/myy116>.
20. Millon L, Herbrecht R, Grenouillet F, Morio F, Alanio A, Letscher-Bru V, et al. Early diagnosis and monitoring of mucormycosis by detection of circulating DNA in serum: retrospective analysis of 44 cases collected through the French Surveillance Network of Invasive Fungal Infections (RESSIF). *Clin Microbiol Infect.* 2016;**22**(9):810 e1–8. [PubMed ID: 26706615]. <https://doi.org/10.1016/j.cmi.2015.12.006>.