Comparison of histopathological analysis, culture and polymerase chain reaction assays to detect mucormycosis in biopsy and blood specimens

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ABSTRACT

Background and Objectives: The aim of this study was to compare direct microscopic examination with culture and PCR for the diagnosis of *Mucorales* infection in blood and tissue specimens.
Material and Methods: Blood samples and tissue specimens were obtained from 28 patients (total 58 samples) with suspected invasive fungal infection and cultured on proper media. Direct smear of tissue samples was done with potassium hydroxide, hematoxylin and eosin, and methenamine silver staining. DNA extracted from blood and tissue specimens were used for semi-nested PCR targeting 18S rDNA of *Mucorales* species.
Results: Mucormycosis was documented in 7/28 (25%) of tissue specimens with positive findings by direct smear, of which PCR and culture were positive in 6 (86%) and 5 (70%) specimens, respectively. The etiologic agents were *Mucor* spp. and *Rhizopus* spp. However, culture and PCR results for all blood specimens were negative.
Conclusions: As the orders of *Mucorales* do not have well growth in culture media, PCR with tissue specimens is more sensitive than tissue or blood culture methods. Unfortunately, there is no alternative method for direct smear, which is an invasive method. Molecular methods may be helpful in these cases.

Keywords: Invasive fungal infection, *Mucoracea*, Semi-nested PCR, 18S rDNA

INTRODUCTION

Invasive fungal infection (IFI) is one of the main causes of mortality and morbidity among hematological malignancies and immunocompromised patients (1, 2). The most common fungi in such infections are *Aspergillus* and *Candida* species; however, a number of other organisms such as members of the *Mucoracea* are increasing frequently (3). Mucormycosis should be considered in transplant recipients and patients with hematological malignances, renal failure and diabetes mellitus (4), and immunocompromised patients including those with HIV infection (5). In

the past decades, the incidence of IFI has increased. Tiraboschi *et al.* (2012) reported incidence rates of mucor-mycosis of 0.13%, between 1980 and 2004 (10 cases), and 0.86% between 2005 and 2010 (7 cases) patient-years (6). In another report, 7% of patients at autopsy and 20 patients per 100 000 admissions were diagnosed with mucormycosis, and the mortality rate for these patients was about 50% to 100% (7). The highest incidence of mucormycosis (more than 90%) is related to *Rhizopus* spp., followed by *Mucor* spp., *Lichtheimia* spp., and, rarely, some other species (8, 9). Given the high mortality rate and rapid progression, early diagnosis and effective treatment are important.

There are many different methods for the diagnosis of IFI, such as considering signs and symptoms, serologic tests, culturing and microscopic examination, imaging procedures, histopathological methods, and polymerase chain reaction (PCR) (10-12). The documented diagnosis of IFI by mold fungi

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requires either the presence of hyphae in the involved tissues or growth of the fungus from sterile tissue in plate cultures (3). The diagnosis of mucormycosis is more difficult than other fungal infections, and its treatment is challenging because under normal laboratory conditions, sporulation fails and culture results from the biopsies are often negative due to unviable organism in necrotic tissues (13). Moreover, morphological identification of the hyphae can not help distinguish between different fungal species (14,15). Although culture is considered the gold standard for determining the causative agent, observing *Mucor*-like hyphae in the involved tissues by histopathologic examination can also establish mucormycosis (16). No serological test is available, radiological methods are not specific and very few studies have used molecular techniques such as PCR to detect this infection. Given very few studies on molecular method of mucormycosis diagnosis, in this study, we evaluated the semi-nested PCR method for the detection of zygomycete fungi in blood and tissue specimens, and compared it with direct smear as the gold standard.

MATERIALS AND METHODS

During two years (2010-2011), 28 patients with suspected IFI and for whom blood and tissue specimens were available, were enrolled in this study. Suspected IFI was defined as fever that developed or progressed during broad-spectrum antibacterial therapy after 96 hours, or radiological symptoms including focal lung lesions with or without surrounding halo, opacification of the sinuses as detected by high-resolution computed tomography, or typical endobronchial lesions detected by bronchoscopy, major peripheral embolization in echocardiography, especially in patients undergoing lung transplantation or those receiving mechanical ventilation, and heart failure. Biopsy specimens were carefully divided by cutting with a knife. Equal parts were used for histopathological analysis, culture, and molecular testing by examiners who were unaware of the microbiological, histopathological and clinical data. Blood samples were cultured on BACTEC medium (Becton-Dickinson, Sparks, MD, USA) and tissue cultures from sinus, heart (infected valve or vegetation) and lung were cultured on Sabourau dextrose agar (Merck, Darmstadt, Germany).

Direct smear of tissue samples was done with hematoxylin and eosin staining and methenamine silver staining for histopathological examination, and with potassium hydroxide in the mycology lab for the detection of hyphae in the tissues. The hyphae of zygomycetes appeared as wide, ribbon-like, pauci septate hyphae with right-angle branching, as described in the literature (17). Each tissue sample (transported in saline) was grown on three plates of Sabouraud dextrose agar and incubated at 30°C for 21 days. The result was considered positive if two out of three plates grew the same isolated fungi. Genus determination of the fungus was done by direct smear with lactophenol cotton blue.

DNA was extracted from the sera and tissues with QIAmp DNA Minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations. Extracted DNA concentrations were determined with a NanoDropND-1000 UV/Vis spectrophotometer (NanoDrop, USA).

The PCR program, master mix making, and primers of 18S rDNA from *Mucorales* were based on Rickerts *et al.* (18). These primers were divided into two groups: ZM1 (5´-ATT ACC ATG AGC AAA TCA GA-3´) and ZM2 (5´-TCC GTC AAT TCC TTT AAG TTT C-3´) for amplification of a 407-408 bp fragment (for the first round of PCR), and ZM1 and ZM3(5´-CAA TCC AAG AAT TTC ACC TCT AG-3´) for amplification of a 176-177 bp fragment (for the second round of PCR). All primers were made by Bioneer (South Korea). The PCR products were separated by 2% agarose gel electrophoresis (voltage 70, 1 hour), stained with ethidium bromide, and visualized with the Gel doc system (Gel Logic 2000, Kodak, USA).

To determine the lowest detection limit of PCR, different concentrations of the DNA extracted from *Mucor* isolates were subjected to PCR (extracted DNAs were diluted for 4, 2, 1, 0.5, and 0.25 ng/µL) (Fig. 1). Positive and negative controls for each PCR set were included.

Statistical analysis was performed with SPSS software for Windows (Statistical Package for Social Sciences, version 15.0, SPSS Inc, Chicago, IL, USA). This research study conformed to the Helsinki Declaration and local legislation, and was approved by the local ethics committee.

RESULTS

Of the 28 patients suspected to have IFIs, non

Table 1. Results of different methods in patients with mucormycosis.

No.	Sex/ age (years)	Tissue smear	Tissue culture	Blood culture	PCR Blood	PCR Tissue	Outcome
1	Female/11	Non septated hyphae	-	-	-	+	Survived
2	Male/66	Nonseptated hyphae	+	-	-	-	Died
3	Female/67	Non septated hyphae	-	-	-	+	Survived
4	Male/51	Non septated hyphae	+	-	-	+	Survived
5	Female/75	Nonseptated hyphae	+	-	-	+	Died
6	Female/19	Non septated hyphae	+	-	-	+	Survived
7	Male/31	Nonseptated hyphae	+	-	-	+	Survived

septated hyphae were detected histopathologically in 7 (25%) tissue samples. Their median age was 47 years (range, 11–75 years) and the females to males ratio was 8/20. According to the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group of the National Institute of Allergy and Infectious Diseases (3), all 7 patients had proven mucormycosis. None of the blood cultures was positive, and no false positives were detected in the tissue cultures. Tissue cultures from 5 patients were positive for the etiological agent *Rhizopous* spp. in 4 cases and *Mucor* spp. in 1 case. All positive samples were from sinuses. The lower limit of PCR detection was 0.25ng of DNA (Fig. 1).

The PCR test results for all serum samples (28 sera) were negative. For the tissue specimens, 7 positive findings on direct smear but 6 were positive with PCR (Table 1). The PCR results were negative in all samples with negative culture results except for 1 case, from which *C. albicans* was isolated.

In this study, direct smear was considered the gold standard (16). Compare with the gold standard test, the sensitivity, specificity, positive and negative predictive value was 70%, 100%, 100% and 91% for tissue culture and 86%, 96%, 86% and 96% for

tissue PCR. Table 1 presented the Results of different methods in patients with mucormycosis.

Once the specimens arrived at the lab, the required tests were performed on them promptly and the results were sent to the clinicians. That's why the mortality rate (2/7; 28.6%) was low due to early diagnosis.

DISCUSSION

In this study, we were able to increase the rate of etiologic diagnosis from 70% by culture method to 86% by PCR assay in patients with proven invasive mucormycosis. A confirmed diagnosis of mucormycosis requires the presence of characteristic hyphae in the involved tissues or other specimens by histopathologic staining or direct smear with potassium hydroxide (19). The presence of septated hyphae in the histopathology smear may mistakenly lead the pathologist to rule out zygomycosis, whose diagnosis requires professional pathological expertise, since it was previously thought that zygomycete hyphae are aseptate rather than pauciseptate. The presence of necrosis or fragmentation, and poor staining of thin hyphal walls, can make microscopic visualization difficult (20).
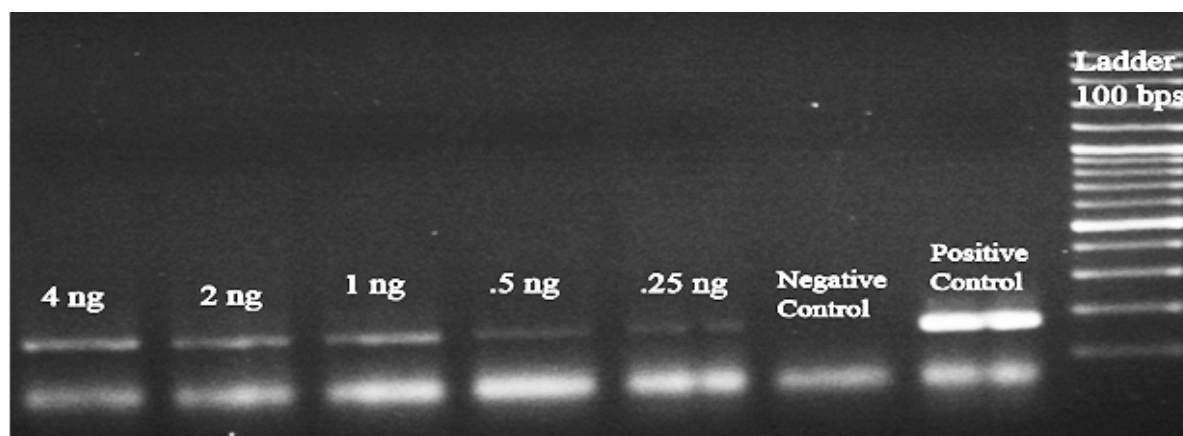
Fig. 1. Serial dilutions of DNA extracted from clinically isolated *Mucor* species. The first lane is the 100-bp DNA size marker. The lower limit of detection of the PCR assay was 0.25ng/μL extracted DNA with 176 base pairs.

Fungal culture is necessary to identify the genus. However, the recovery of zygomycetes can be difficult in culture (13, 19). Our results show that the sensitivity for tissue culture was 70% and blood culture specimens were all negative. Blood and urine cultures are rarely positive for zygomycetes (19), and the rates of successful tissue culture for histopathologically positive smears were reported as 33% and 50% (21, 22). The negative results for specimen culture may be due to: A) The initial processing of hyphal elements by grinding, which may damage the large, pauciseptate hyphae and make them nonviable (21, 22), B) The presence of hyphal elements in only a small portion of the specimens (23), C) Prolonged antifungal pretreatment, or D) Suboptimal standard culture conditions for the recovery of this organism from necrotic tissues (23). In the present study, morphological examination was the gold standard because it yielded the highest rate of diagnosis of the infection.

The diagnosis of animal molds from tissue specimens by PCR is more reliable than by culture (24). Our semi-nested PCR method in tissue samples had a sensitivity of 86% and a specificity of 96% and none of the blood samples was positive for mucormycosis. The sensitivity of real-time PCR in tissues for detecting *Mucorales* was reported to be 100% (25), in 2 tissue samples; however, the limited number of samples compromises the accuracy of this result. In contrast, the sensitivity in formalin-fixed and paraffin-embedded tissues was reported to be 57% (25). It is likely that extensive cross-linking of tissue proteins after fixation in formaldehyde increased fragmentation of the nucleic acids and inhibited the PCR process (26). Freshness of the tissue specimens is thus critical for the accuracy of PCR assays.

In conclusion, new molecular and antigenic tools are required for the early detection of mucormycosis and therapeutic monitoring. For an accurate and dependable diagnosis of this infection, PCR in fresh tissue samples is more sensitive than tissue or blood culture methods. Unfortunately, there is no alternative method for examination by tissue direct smear, which is an invasive procedure.

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