Short Communication

Nested Polymerase Chain Reaction with Specific Primers for Mucorales in the Serum of Patients with Hematological Malignancies

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SUMMARY: Mucormycosis is an opportunistic infection occurring in immunocompromised hosts with hematological malignancies. Mortality due to mucormycosis in patients with hematological malignancies is high. However, the clinical symptoms of mucormycosis are poorly characterized, and diagnosis is difficult due to the lack of specific culture or serological markers or antigens. We present two cases in which nested polymerase chain reaction with specific primers was used in the serum of patients with hematological malignancies.

Mucormycosis is an opportunistic infection caused by fungi of the order Mucorales, which predominantly affects immunocompromised hosts with hematological malignancies. Mucormycosis can occur in any part of the body and can result in mortality. The recommended first-line treatment is liposomal amphotericin B (L-AMB), which improves patient outcomes (1–4).

Early diagnosis is important to initiate appropriate treatment as soon as possible. However, the clinical symptoms of mucormycosis are poorly characterized. Test results for (1→3)-β-D-glucan are often negative, and neither a specific serological marker nor a specific antigen is available (5). In most cases, blood culture yields negative findings (6). To achieve pathological diagnosis, pathological tissues need to be collected. We describe two cases in which nested polymerase chain reaction (PCR) with specific primers for Mucorales were used in patients with hematological malignancies.

Our first case involved a 65-year-old man diagnosed with adult T-cell leukemia/lymphoma (ATL), for which a modified-LSG15 therapy regimen was administered. After the second course, pulmonary and central nervous system infiltration was detected. We added mogamulizumab and an intrathecal administration of cytarabine, methotrexate, and prednisolone. Although ATL was controlled, severe myelosuppression persisted. The patient developed right renal infarction and pyelonephritis, and methicillin-resistant Staphylococcus epidermidis was detected in the urine. A broad-spectrum antibiotic was administered, but the patient died on day 98 after administering modified-LSG 15 therapy. Neither levels of (1→3)-β-D-glucan nor aspergillus and candida antigen increased.

Pathological autopsy revealed mycelia of Mucorales in the heart, right lung, inferior margin of the liver, renal, cerebrum, bladder, spleen, and blood vessels. The cause of death was myocardial infarction due to Mucorales invasion of the coronary vessels (Fig. 1A-G). The Mucorales body detected in the lung and liver were examined by sequence, and we identified the fungal species as Cunninghamella bertholletiae.

Because we could not diagnose mucormycosis while the patient was alive, we retrospectively examined the highest disease stage at which Mucorales could be detected using serum. We performed nested PCR with specific primers for the 18 sRNA of Mucorales using formalin-fixed, paraffin-embedded tissue specimens (7–9) for serum samples. We used the following primers: 

Mucor1 (5’-WTTACCRTGAGCAAATCAGA-3’) and Mucor2 (5’-CAATCYAAGAATITCCACCTTAG-3’) for the first PCR (outer primer) and Mucor3 (5’-AGCATGGGATAACGGAATA-3’) and Mucor4 (5’-AGCATGGGATAACGGGAATA-3’) for the second PCR (nested primer). A PCR product of 124 base pairs was obtained. We extracted DNA from 200 μL of the patient’s residual serum and used a volume of 50 μL containing 100–500 ng of DNA. The PCR thermocycling conditions were 94°C for 10 min, followed by 94°C, 55°C, and 72°C for 30 s (35 cycles), and ending at 4°C. PCR covered the following Mucorales species: Lichtheimia spp., Rhizomucor pusillus, Mucor spp., Actinomucor elegans, Cokeromyces recurvatus, Saksenaea spp., Apophysomyces spp., Rhizopus spp., Syncephalastrum racemosum, and Cunninghamamella spp.

The PCR band appeared with the residual sera from 81 to 94 days (Fig. 2A). Two bands were obtained; the
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Fig. 1. (Color online) Pathological autopsy findings. Case 1: Infiltration of Mucorales hyphae were observed in the heart (A, B, C). Mucorales were observed in the right lung (D, E). The right renal artery and vein were totally occluded by mycelia and fibrin (F, G). Case 2: Mucorales were confined to a part of the left lower lobe of the left lung (H). Mucorales were found mainly in the bronchi, other interstitial tissues, and blood vessels (I).

Fig. 2. Nested polymerase chain reaction (PCR) for detecting Mucorales. (A): Patient 1. M, 100-bp DNA ladder; N, negative control (Water); P, positive control. The numbers show when the patient’s serum sampling was undertaken from the day of admission. (B): Patient 2. M, 100-bp DNA ladder; N, negative control (Water); P, positive control; 1, patient’s serum.
The patient’s clinical symptoms did not improve. Our second case involved a 77-year-old woman who was diagnosed with myeloid/natural killer cell precursor acute leukemia. The disease was resistant to chemotherapy, and we opted for palliative care for her. The patient’s condition worsened, and she was admitted to the hospital. Computed tomography revealed a shadow with a reversed halo sign in the left lower lobe on day 20. Invasive pulmonary aspergillosis was suspected, and 2.5 mg/kg of L-AMB was initiated. The patient’s clinical symptoms did not improve. We considered the possibility of mucormycosis and conducted nested PCR analysis using serum samples obtained on day 33 (Fig. 2B), 13 days after detecting the reversed halo sign. The L-AMB dose was increased to 7.5 mg/kg. A few days later, the patient developed renal dysfunction, and the L-AMB dose had to be reduced. Her general condition gradually worsened, and she died on day 82.

Pathological autopsy revealed extensive leukemic infiltrations in several organs, including the kidney, liver, heart, lung, and bone marrow, and this was thought to be the direct cause of death. Renal dysfunction may have been due to leukemic infiltration. Mucorales was found in the left lung, bronchi, and blood vessels (Fig. 1H and I). We identified the fungal species as *Mucor circinelloides* by sequence.

We describe two cases in which serum was tested using nested PCR, rather than quantitative PCR (qPCR). The characteristic features of our method were the ease with which specimens could be collected and the ability to obtain tissue specimens without having to perform biopsies. Additionally, our method usually requires 8 h in order to obtain results. Previous studies have investigated the use of qPCR with serum (10–13) and cerebrospinal fluid (13). The advantage of qPCR is the quantification of target DNA (14), which may be useful for assessing mucormycosis. Additionally, a previous report suggested that qPCR could be used for therapeutic monitoring (11–13). Compared with nested PCR, qPCR needs only one primer, but can result in a nonspecific product being produced. Because it uses multiple primers, nested PCR is known to be more sensitive than PCR using a single primer (15). Therefore, nested PCR may be a suitable screening tool.

Fungal DNA concentrations below the detection limits may result in false-negative results. In addition, exogenous contamination can cause false-positive results and lead to suboptimal treatment. To avoid false-positive results, it is important to compare histopathological findings, clinical data, radiological features, and other specimen cultures.

As suggested in previous reports (10,11,13), when a positive band is detected with qPCR and mucormycosis is suspected as the pathogen, anti-fungal therapy should be initiated. One report suggested that appropriate treatment for mucormycosis would have been administered from 1 to 23 days earlier if detected using a qPCR method (10).

Based on the issues raised in this report, further studies, particularly prospective studies with large patient populations, are warranted to validate the usefulness of nested PCR for detecting Mucorales infection and facilitating optimal management of infection.

**Acknowledgments** We would like to express our gratitude to Mr. Yuta Miharu and Mr. Masato Suzuki of the Department of Clinical Laboratory in Research Hospital, Institute of Medical Science, University of Tokyo.

**Conflict of interest** None to declare.

**REFERENCES**


