



Identification of Fungal Species and Detection of Azole-Resistance Mutations in the *Aspergillus fumigatus cyp51A* Gene at a South Korean Hospital

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Purpose: With changing fungal epidemiology and azole resistance in *Aspergillus* species, identifying fungal species and susceptibility patterns is crucial to the management of aspergillosis and mucormycosis. The objectives of this study were to evaluate performance of panfungal polymerase chain reaction (PCR) assays on formalin-fixed paraffin embedded (FFPE) samples in the identification of fungal species and in the detection of azole-resistance mutations in the *Aspergillus fumigatus cyp51A* gene at a South Korean hospital.

Materials and Methods: A total of 75 FFPE specimens with a histopathological diagnosis of aspergillosis or mucormycosis were identified during the 10-year study period (2006–2015). After deparaffinization and DNA extraction, panfungal PCR assays were conducted on FFPE samples for fungal species identification. The identified fungal species were compared with histopathological diagnosis. On samples identified as *A. fumigatus*, sequencing to identify frequent mutations in the *cyp51A* gene [tandem repeat 46 (TR46), L98H, and M220 alterations] that confer azole resistance was performed.

Results: Specific fungal DNA was identified in 31 (41.3%) FFPE samples, and of these, 16 samples of specific fungal DNA were in accord with a histopathological diagnosis of aspergillosis or mucormycosis; 15 samples had discordant histopathology and PCR results. No azole-mediating *cyp51A* gene mutation was noted among nine cases of aspergillosis. Moreover, no *cyp51A* mutations were identified among three cases with history of prior azole use.

Conclusion: Panfungal PCR assay with FFPE samples may provide additional information of use to fungal species identification. No azole-resistance mediating mutations in the *A. fumigatus cyp51A* gene were identified among FFPE samples during study period.

Key Words: Aspergillosis, mucormycosis, polymerase chain reaction, resistance, fungus

INTRODUCTION

Mucormycosis (formerly known as zygomycosis) and aspergillosis are invasive fungal diseases that usually present as rhino-

orbital-cerebral or pulmonary infections.^{1,2} *Aspergillus* species are usually susceptible to voriconazole, and isavuconazole has also become a first-line targeted therapy.³ However, voriconazole shows no activity against mucorales.³ Moreover, con-

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cerns about changing epidemiology and azole resistance are rising. A study using a deterministic model estimated the annual incidence of invasive aspergillosis (IA) and mucormycosis in South Korea to be 4.48 and 0.14 cases/100,000 people, respectively. A retrospective study performed in 10 university hospitals identified 334 cases of invasive pulmonary aspergillosis between 2008 and 2010. Another study collected 102 clinical and 129 environmental *Aspergillus* isolates from patients with hematologic malignancies in South Korea and evaluated the prevalence of azole resistance. In the study, the resistance rate of *A. fumigatus* to azole was 5.3%.⁴⁻⁶

Higher rates of mortality have been demonstrated for patients treated with voriconazole in voriconazole-resistant IA than for voriconazole-susceptible IA.^{7,8} Rapid detection of fungal species and of azole-resistance in *Aspergillosis fumigatus* may benefit outcomes by guiding appropriate antifungal therapy.⁷

Azoles are inhibitors of 14 α -sterol demethylases, which are responsible for catalyzing a critical step in the biosynthesis of ergosterol, a component of the fungal membrane.⁸ Mutations in the *cyp51A* gene, which is responsible for encoding 14 α -sterol demethylase enzymes, is the most common azole-resistance mechanism in *Aspergillus* species.⁹ Moreover, isolates harboring tandem repeats in the promoter region of the *cyp51A* gene and point mutations leading to amino acid changes are also known to cause azole-resistance.⁹ Furthermore, the incidence of azole-resistant *Aspergillus* species has increased over recent years due to previous exposure and environment-associated resistance.^{8,10}

Molecular methods can now be used to rapidly identify fungal species in formalin-fixed paraffin-embedded (FFPE) tissue specimens,¹¹ and polymerase chain reaction (PCR)-based methods have been devised to detect azole-resistance in FFPE and bronchial alveolar lavage specimens.^{12,13} The objectives of this study were to evaluate performance of panfungal PCR assays on FFPE samples for fungal species identification, and the detection of azole-resistance mutations in the *A. fumigatus cyp51A* gene at a South Korean hospital.

MATERIALS AND METHODS

Clinical samples and DNA extraction

Histopathology reports consistent with aspergillosis or mucormycosis issued between January 2006 and January 2016 and relevant FFPE blocks were retrieved from the Department of Pathology at a tertiary referral hospital in South Korea. Ethics approval was obtained from the hospital's Institutional Review Board (Yonsei University Health System, Severance Hospital, IRB trial number: 4-2016-0262). Panfungal PCR assays were performed on these FFPE blocks to determine the presence of *Aspergillus* species and Mucorales. Samples testing positive for *A. fumigatus* by DNA sequencing were subjected to L98H, M220, and TR46 PCR assays and consecutive DNA

sequence analysis to determine the presence of azole-resistance mutations in the *A. fumigatus cyp51A* gene.

FFPE tissues were deparaffinized with mineral oil, and DNA was extracted with proteinase K using the ReliaPrep™ FFPE gDNA Miniprep System (Promega, Madison, WI, USA), according to the manufacturer's instructions. DNA isolation with deparaffinization using mineral oil was performed using the ReliaPrep™ FFPE gDNA Miniprep System. 500 μ L of mineral oil was added to the sections. Sample lysis was done by adding 200 μ L of lysis buffer to the sample, followed by centrifugation of 10000 rpm for 15 seconds. Then, 20 μ L of Proteinase K was added to the lower phase and incubated at 56°C and 80°C for 1 and 4 hours, each. 10 μ L of RNase A was added directly to the lysed sample in the lower phase and incubated at room temperature (20–25°C) for 5 minutes. Next, 220 μ L of BL Buffer and 240 μ L of ethanol (95–100%) were added to the lysed sample. After centrifugation at 10000 rpm for 15 seconds, the entire lower blue (aqueous) phase of the sample was transferred to a binding column/collection tube assembly. After transfer, centrifugation at 10000 rpm for 30 seconds was done. Next, 500 μ L of 1X wash solution was added to the binding column and centrifuged at 10000 rpm for 30 seconds. The flow-through was discarded, and 500 μ L of 1X wash solution was added to the binding column, followed by centrifugation at 10000 \times g for 30 seconds. The flow-through was once again discarded, followed by centrifugation at 16000 \times g for 3 minutes to dry the column. The binding column was transferred to a clean 1.5-mL microcentrifuge tube, to which 30–50 μ L of elution buffer was added. After centrifugation at 16000 \times g for 1 minute, the extracted DNA was stored at -20°C. Finally, agarose gel electrophoresis and PCR methods were used to assess DNA degradation.

Primers for PCR assays of *Aspergillus* species and Mucormycosis identification

Panfungal PCR was performed to amplify internal transcribed spacer (ITS) regions. The primers ITS5 (forward; 5'-GGAAGTAAAGTCGTAACG-3') and ITS4 (reverse; 5'-TCCTCCGCTTATTGATATGC-3') were used to amplify ITS 1 to ITS 2 regions (ITS 1–2), and the primers ITS3 (forward; 5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (reverse; 5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS 2 region.¹⁴ The ITS 1–2 and ITS 2 PCR products obtained were of 640 and 350 base pairs (bp), respectively (Supplementary Table 1, only online).

Primers for PCR assays of *cyp51A* gene mutations

To amplify L98H, M220, and TR46 mutations in the *cyp51A* gene, we used a previously described nested, one-step PCR assay.¹⁵ Three different primer sets were used to amplify these three mutations. To amplify L98H, we used 5'-AAAAAACCA-CAGTCTACCTGG-3' (forward), and 5'-GGAATTGGGACAAT-CATACAC-3' (reverse) to generate a 143-bp PCR fragment.¹⁶ For M220, we used 5'-GCCAGGAAGTTCGTTCCAA-3' (for-

ward) and 5'-CTGATTGATGATGTCAACGTA-3' (reverse) to generate a 173-bp PCR fragment.¹⁶ Nested PCR assay was performed to amplify TR46 in the promoter region of *cyp51A*: one primer pair was used to amplify a long DNA fragment, and a second primer pair was used to amplify an inner shorter fragment in a second PCR step. For the first step, the PCR primer pairs were 5'-AAGCACTCTGAATAATTACA-3' (forward) and 5'-ACCAATATAGGTTTCATAGGT-3' (reverse) to obtain a 240-bp DNA fragment, and in the second step, 5'-GAGTGAATA-ATCGCAGCACC-3' (forward) and 5'-CTGGAACCTACACCT-TAGTAATT-3' (reverse) were used to generate a 103-bp DNA fragment (Supplementary Table 2, only online).¹⁵

PCR assays and controls

To amplify ITS regions, PCR was performed in total volumes of 50 μ L, consisting of 1X reaction buffer, 0.1 μ M dNTPmix, 1.25 U of Taq DNA Polymerase (RBC Bioscience, Xindian City, Taiwan), 20 pmol of each primer, and 200 ng of DNA (1 μ L) per sample. PCR was performed using the following protocol: 95°C for 3 minutes, 35 amplification cycles of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 7 minutes.

To detect L98H and M220 alterations, PCR was conducted in a total volume of 50 μ L containing 2 μ L of template DNA (100 ng human DNA+unknown amount of *A. fumigatus* DNA), 1X reaction buffer, 0.1 μ M dNTPmix, 1.25 U of Taq DNA Polymerase (RBC Bioscience), and 20 pmol of each primer. The PCR amplification protocol was as follows: 5 min of initial denaturation at 94°C, 39 amplification cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min.

To detect TR46 alterations, PCR was conducted in a total volume of 50 μ L as described for L98H and M220 above. The PCR amplification protocol was as follows: 5 min denaturation at 94°C, 22 amplification cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min. For the second PCR step, we used a total volume of 50 μ L and 3 μ L of the first-step PCR mixture as template. Other components were as described for L98H and M220. The second step PCR amplification protocol was as follows: 5 min initial denaturation at 94°C, 34 amplification cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min.

To exclude cross-reactivity of the primers with human genomic DNA, samples containing a mixture of 100 ng of human DNA and 50 pg of *A. fumigatus* wild-type DNA were used as a negative control. An azole-resistant *A. fumigatus* strain (GenBank accession no. AF338659) harboring the TR34/L98H/S297T/F495L mutation in the *cyp51A* gene was used as a positive control for detection of the L98H mutation.¹⁷

Sequence analysis

To identify *Aspergillus* species and Mucorales, PCR products

were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). A minimum of 50 ng DNA was sequenced using the BigDye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems). Sequences were edited and aligned using Sequence Scanner Software 2, ver. 2.0 (Applied Biosystems), and product sequences were compared with reference sequences using the NCBI alignment service Nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The GenBank accession number for the *A. fumigatus* sequences determined in this study is CM000169.1.

To detect potential mutations in the PCR products subjected to DNA sequence analysis, sequences were compared with the *A. fumigatus cyp51A* wild-type sequence using the NCBI alignment service Nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS

During the 10-year study period, 75 patients received a histopathological diagnosis of mucormycosis or aspergillosis, and PCR amplification and identification was positive for 31 (41.3%) of the 75 FFPE samples. Sixteen FFPE samples had corresponding histopathology and PCR sequencing results. Fourteen cases of *Aspergillus* species were identified: *A. fumigatus* (n=9), *A. flavus* (n=2), *A. oryzae* (n=2), and *A. tamaritii* (n=1). Two cases with a histopathological diagnosis of mucormycosis were identified as *Rhizopus oryzae* by sequence analysis. The nine cases identified as *A. fumigatus* species were further analyzed for azole-resistance mutations in the *A. fumigatus cyp51A* gene.

The demographic and clinical data of the 16 cases identified as aspergillosis or mucormycosis by panfungal PCR are presented in Table 1. Case 1, a 77-year-old male, had a history of chronic obstructive pulmonary disease and was receiving steroids when he developed a brain abscess. An empirical antibacterial agent, but no antifungal agent, was administered. Aspergillosis was confirmed after death by pathologic diagnosis, and PCR sequencing confirmed *A. fumigatus*. Case 2 was an 81-year-old male patient who developed fungal pneumonia after surgery for renal cell carcinoma. Pathologic diagnosis conducted on transbronchial lung biopsy tissue revealed aspergillosis. The patient was treated with voriconazole but succumbed despite appropriate treatment. PCR sequencing identified *A. fumigatus*. Case 4 was a 75-year-old male patient who had undergone liver transplantation due to hepatocellular carcinoma and was receiving immunosuppressive therapy and oral itraconazole for fungal prophylaxis prior to developing acute maxillary sinusitis. Case 5 was a 62-year-old female and had undergone liver transplantation due to liver cirrhosis. A mass lesion developed in her chest wall area at 1 year after transplantation. Excisional biopsy revealed narrow branching

Table 1. Demographics of 16 Cases Identified as Aspergillosis or Mucormycosis and *cyp51A* Alterations in Nine Cases Sequenced as *Aspergillus fumigatus*

Case	Sex/ age	Underlying condition	Type of tissue	Histopathologic identification	Molecular identification	Culture	Serum GM	Prior azole use	Mortality	<i>cyp51A</i> alterations		
										L98H	M220	TR46
1	M/77	COPD	Brain	Aspergillosis	<i>A. fumigatus</i>	NG	Neg	No	Yes	Neg	Neg	NT
2	M/81	Renal cell carcinoma	Lung	Aspergillosis	<i>A. fumigatus</i>	NG	Neg	No	Yes	Neg	Neg	Neg
3	M/62	Chronic sinusitis	Maxillary sinus	Aspergillosis	<i>A. fumigatus</i>	NT	NT	No	No	Neg	NT	NT
4	M/75	Liver transplantation*	Maxillary sinus	Aspergillosis	<i>A. fumigatus</i>	NT	Neg	Yes	No	Neg	Neg	NT
5	F/62	Liver transplantation*	Soft tissue/bone [§]	Aspergillosis	<i>A. fumigatus</i>	NG	Pos	Yes	No	Neg	Neg	Neg
6	F/74	Chronic sinusitis	Maxillary sinus	Aspergillosis	<i>A. fumigatus</i>	NT	NT	No	No	NT	NT	Neg
7	F/78	ESRD	Maxillary sinus	Aspergillosis	<i>A. fumigatus</i>	NT	NT	No	No	NT	Neg	Neg
8	F/58	Chronic sinusitis	Maxillary sinus	Aspergillosis	<i>A. fumigatus</i>	NT	NT	No	No	Neg	Neg	Neg
9	M/54	Lung transplantation [†]	Lung	Aspergillosis	<i>A. fumigatus</i>	NG	Pos	Yes	No	Neg	Neg	Neg
10	M/73	Diabetes mellitus	Maxillary sinus	Aspergillosis	<i>A. flavus</i>	NT	NT	No	No			
11	M/71	Cervix cancer, CKD	Lung	Aspergillosis	<i>A. tamarii</i>	Fungus	Pos	Yes	Yes			
12	M/52	Chronic sinusitis	Maxillary sinus	Aspergillosis	<i>A. oryzae</i>	NT	NT	No	No			
13	F/62	Chronic sinusitis	Maxillary sinus	Aspergillosis	<i>A. oryzae</i>	NT	NT	No	No			
14	M/65	ESRD	Maxillary sinus	Aspergillosis	<i>A. flavus</i>	NT	NT	No	No			
15	M/62	Kidney transplantation [‡]	Stomach	Mucormycosis	<i>Rhizopus oryzae</i>	Fungus		No	Yes			
16	M/69	Diabetes mellitus	Maxillary sinus	Mucormycosis	<i>Rhizopus oryzae</i>	NT		Yes	Yes			

GM, serum galactomannan; M, male; F, female; COPD, chronic obstructive pulmonary disease; ESRD, end stage renal disease; CKD, chronic kidney disease; *A. fumigatus*, *Aspergillus fumigatus*; *A. flavus*, *Aspergillus flavus*; *A. tamarii*, *Aspergillus tamarii*; *A. oryzae*, *Aspergillus oryzae*; NG, no growth; NT, not tested (insufficient samples remaining); Neg, negative; Pos, positive.

*Liver transplantation due to hepatocellular carcinoma, [†]Lung transplantation due to idiopathic pulmonary fibrosis, [‡]Kidney transplantation due to diabetic nephropathy, [§]Biopsies of chest wall soft tissue and left rib.

fungal hyphae consistent with aspergillosis, and PCR sequencing demonstrated the presence of *A. fumigatus*. The patient was receiving fluconazole for fungal prophylaxis. Treatment, which involved excision and systemic voriconazole, was successful in this case. Case 9 was a 54-year-old male who had undergone a lung transplant due to idiopathic pulmonary fibrosis. He had a history of itraconazole use for fungal prophylaxis. Case 15 was a 62-year-old male who had undergone kidney transplantation due to diabetic nephropathy. Histopathologic diagnosis after stomach biopsy revealed mucormycosis. Despite treatment with liposomal amphotericin B, the patient expired. PCR sequencing results identified *R. oryzae*. Case 16 was a 69-year-old male with diabetes mellitus on insulin therapy when he developed maxillary sinusitis. Mucormycosis was confirmed by histopathology and was consistent with PCR results, which demonstrated *R. oryzae*.

The results of the *cyp51A* alterations of the nine samples confirmed as *A. fumigatus* are summarized in Table 1. Seven samples were positive by the L98H PCR assay alone, but no mutations were detected in sequence analysis. Seven samples were positive by the M220 PCR assay alone, but also revealed no mutations in sequence analysis. Six samples were positive by the TR 46 PCR assay alone, but similarly, no mutations were noted in sequence analysis.

Discordant PCR and histopathology results were obtained for 15 samples. Two specimens histopathologically diagnosed

as Aspergillosis were identified as Mucorales: *Lichtheimia ramosa* and *R. oryzae*. Five specimens with a histopathological diagnosis of aspergillosis were identified by PCR as *Epicoccum nigrum*, *Bipolaris zeicola*, *Fusarium solani*, *Nakataea oryzae*, and *Cladosporium cladosporioides*. Eight samples were identified by PCR sequencing as uncultured fungus clones. One brain sample diagnosed as mucormycosis by histopathology was identified by PCR as an uncultured fungus clone. *L. ramosa* was identified by PCR in buttock tissue. *R. oryzae*, *B. zeicola*, *F. solani*, and *N. oryzae* were all identified in sinus samples with uncultured fungus clones, and *E. nigrum* and *C. cladosporioides* were identified in lung samples.

DISCUSSION

The identification of fungal DNA in tissue samples by PCR improves diagnostic accuracies for fungal infections,³ and pan-fungal PCR conducted on FFPE tissues provides an alternative to culture-dependent identification methods.¹¹ Mucorales has been identified by PCR in paraffin-embedded tissue samples of patients with a fungal infection,^{14,18} and studies have shown that fungal organisms can be identified by amplifying fungal ITS 1 and 2 using panfungal primers.¹⁴ The results of the present study support research indicating that PCR amplification of the ITS 1 and 2 regions accurately diagnoses fungal species

in FFPE specimens.

In all 31 FFPE samples that produced amplifiable DNA results, fungi were identified to the genus or species level. In two cases with a histopathologic diagnosis of aspergillosis, Mucorales-specific DNA was identified by sequencing PCR products. Although this may have been due to tissue specimen contamination, the risk of misdiagnosis by histopathology cannot be excluded. Similar cases have been described in cases confirmed by culture.¹⁹

Two samples with a histopathologic diagnosis of aspergillosis were identified as Mucorales: *L. ramosa* and *R. oryzae*. The *Lichtheimia* species (formerly known as *Absidia*) are currently regarded as emerging pathogens among Mucoralean fungi.²⁰ In the present study, the male patient identified with *L. ramosa* infection had a history of hepatocellular carcinoma and had undergone liver transplantation prior to infection. In addition, he was under immunosuppressive medication. Biopsy from a buttock revealed mucormycosis by PCR product sequencing. Although it is generally known to have low virulence, cases of mucormycosis due to *L. ramosa* in immunocompromised hosts have been reported.^{20,21} Chaumont, et al.²² reported a case of cutaneous mucormycosis requiring aggressive surgical debridement.

The second case, initially diagnosed by histopathology as aspergillosis, was found to be due to *R. oryzae* by PCR. This patient had a history of aplastic anemia before fungal infection and displayed rapid clinical deterioration resulting in death. *R. oryzae* is the most common cause of zygomycosis and is a life-threatening infection that usually occurs in patients with diabetic ketoacidosis.²³

Four samples histopathologically diagnosed as aspergillosis produced ambiguous results. In addition to PCR results corresponding as *Aspergillus* species, three samples with a concomitant uncultured fungus was identified, and in the other sample, *N. oryzae* strain was identified. Because fungal ribosomal genes have many similarities, identification at the species level can only be performed by sequencing PCR products.²⁴ However, it has been shown that even targets of base pairs of less than 300 bp within 18S rDNA may not be sufficient to differentiate genera.²⁵ Furthermore, *A. fumigatus* cannot be identified at the species level by PCR targeting 18S rDNA, because target sequences show high analogy to several Ascomycota.²⁵ We believe this lack of specificity may have explained the uncultured fungus clones identified in the present study.

Since aspergillosis and mucormycosis respond to different antifungal agents, delayed diagnosis or treatment might lead to devastating results.²⁶ Mucormycosis is an aggressive and invasive disease, and early surgical debridement of involved tissues and initiation of proper antifungal agents are crucial.²⁷ Our study results demonstrate that PCR can be used to differentiate and identify fungal species, and thus, provide guidance regarding appropriate antifungal treatment strategies.

Mutations in the *cyp51A* gene have been documented in

clinical isolates of patients with a long history of exposure to azoles.^{28,29} The most frequent resistance allele is TR34 in combination with L98H substitution (TR34/L98H).⁸ Azole-resistant mutations harboring tandem repeats of various sizes in the promoter region of the *cyp51A* gene and point mutations leading to amino acid changes in the *cyp51A* gene have also been documented in azole-naïve patients.^{30,31} TR34/L98H and TR46/Y121F/T289A are commonly associated with azole-resistance linked to environmental use of azoles in agriculture and are often found in azole-naïve patients.^{32,33} In the present study, azole resistance was not detected in three cases (patients 4, 5, and 9) with a history of prior azole use. Thus, because sample numbers were small, we suggest larger scale studies be performed to investigate azole resistance in patients with a history of azole exposure.

Several limitations of the present study warrant mention. First, the amount of fungal DNA available is crucial when investigating clinical samples, and DNA degradation and the effects of formaldehyde may have reduced DNA amounts in samples. Specimens that were collected more than a decade before had undergone severe degradation and were lacking viable fungal DNA for analysis. Second, culture results or azole susceptibility profiles were not considered. Third, as the TR46 and M220 mutations have never been reported in South Korea, a positive control for isolates harboring these mutations could not be acquired. Finally, there are many mutations within *cyp51A* that can confer elevated minimum inhibitory concentrations/resistance to triazoles, not just the ones stated in this paper, and thus, there may be unknown methods of elevated minimum inhibitory concentrations to the triazoles not linked to *cyp51A* mutations that would not be detected by this assay.

Panfungal PCR assay with FFPE samples may provide additional information of use in fungal species identification. No azole-resistance mediating mutations in the *A. fumigatus cyp51A* gene were identified among FFPE samples during study period.

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AUTHOR CONTRIBUTIONS

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