Short Report

Detection of Substitutions at 98th, 121st, and 289th Amino Acid Residues in Cyp51A using Cycling Probes

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ABSTRACT

Azole-resistant Aspergillus fumigatus containing unique mutation(s) of cyp51A with tandem repeats in the promoter region has emerged and has become dispersed in environments worldwide. For this study, we designed primers and cycling probes to detect mutations associated with tandem repeats. Substitutions at the 293rd nucleotide (leucine or histidine at the 98th amino acid residue), at the 362nd nucleotide (tyrosine or phenylalanine at the 121st amino acid residue), and at the 865th nucleotide (threonine or alanine at the 289th amino acid residue) in cyp51A were detected using these primers and probes. These results suggest that the primer and probe sets are helpful in detecting these mutations and in differentiating the types of tandem repeats in cyp51A.

Key words: Aspergillus fumigatus, azole, azole resistance, cyp51A, cycling probe

Introduction

Azole resistance in Aspergillus fumigatus presents a growing threat to global health1). Particularly, azole-resistant strains containing tandem repeats (TR) in the cyp51A promoter region are found in different environments around the world1,2), indicating that azole-naïve patients are increasingly exposed to azole-resistant A. fumigatus. Azoles have been used as first-line agents against aspergillosis. Strains carrying TR in cyp51A are multiple-triazole-resistant. Therefore, differentiation of whether isolates from patients contain TR or not is important before and during the azole treatment. Our earlier study revealed a set of primers and cycling probes that were useful in detecting TR3). Nevertheless, TR types were not differentiated in the system.

For this study, we designed new sets of primers and cycling probes to detect unique nonsynonymous mutation(s) coexisting with 34-bp (TR34) and 46-bp (TR46) tandem repeats in Cyp51A. A cycling probe is a chimeric oligomer probe consisting of a DNA-RNA-DNA sequence. After forming an RNA-DNA duplex with the target, RNase H recognizes and cuts the duplex. A mismatch on the target is specifically detected in the system. The system has been used for detection of single nucleotide polymorphism in bacteria4), viruses5), and others.

TR34 and TR46 are combined respectively with L98H mutation and Y121F and T289A mutations. Cycling probes for detection and primers for amplification were designed using Cycleave PCR Assay Designer (Takara Bio Inc., Shiga, Japan). These sequences are shown in Table 1. Real-time PCR was performed as described in an earlier report6).

Initially, we examined the detection using amplicons that include a target sequence. Targeted sequences were amplified from the genomic DNA of a susceptible strain (A. fumigatus OKH31), a TR34/L98H strain (OKH50)3, 6), and a TR46/Y121F/T289A strain (IFM63432) using each primer set. Sequences were then purified and quantified using Qubit dsDNA BR Assay Kit and a fluorometer (Qubit 3.0; Thermo Fisher Scientific Inc., Waltham, MA, USA). As shown in Fig. 1, wild-type sequences and all mutations were detected using cycling probes.

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Table 1. Primers and cycling probes used in this study

| Name         | Sequence (5’ → 3’)            |
|--------------|-------------------------------|
| 98/121-F     | CAGGAAACGAGTTATTTCT           |
| 98/121-R     | GACGAGCCTTGAAAGTT             |
| L98-probe†   | Eclipse-ATCCTTG(A)GCT-HEX     |
| H98-probe†   | Eclipse-TCCTTG(T)GCT-FAM      |
| Y121-probe†  | Eclipse-GACAATCAT(A)CA-HEX    |
| F121-probe†  | Eclipse-GACAATCA(A)AC-FAM     |
| 289-F        | GTCTTGACGAGTAAGAAGAC          |
| 289-R        | TACTGAGCGGAGGAGGAGAC          |
| T289-probe†  | Eclipse-TGATGATA(A)CC-HEX     |
| A289-probe†  | Eclipse-ATGATGATA(G)CC-FAM    |

†Parentheses indicate RNA residues. Probes were conjugated with an Eclipse quencher at the 5’-end and a fluorophore FAM or HEX at the 3’-end.

Fig. 1. Detection of mutated sites with each specific probe. Detection of L98 site (wild type, A), H98 site (mutated, B), Y121 site (wild type, C), F121 site (mutated, D), T289 site (wild type, E), and A289 site (mutated, F) in cyp51A. Copy numbers are shown in the graph legend.
method even with only 50 copies of the target sequences. Quantitative accuracy, however, was not stable at 50 copies. Conventional tubes and tips were used for the experiments. Using low-DNA-binding tubes (DNA LoBind Tubes; Eppendorf, Hamburg, Germany) and tips (Sorenson Bioscience, Salt Lake City, UT, USA) for DNA handling, however, stabilized the quantitative accuracy with 50-copy targets in each reaction (data not shown), suggesting that using low-DNA-binding tubes and tips improves the quantitative accuracy at lower copy numbers of targets.

Since the genome size of *A. fumigatus* is approximately 30 Mb, the other untargeted regions could be nonspecific amplifications. Therefore, we conducted real-time PCR using genome DNA templates (Fig. 2). The templates were purified using a conventional method based on phenol-chloroform phase separation and ethanol precipitation. The DNA concentrations were determined to be 1.5 ng/µL (WT), 0.14 ng/µL (TR34/L98H), and 2.1 ng/µL (TR46/Y121F/T289A). Each sequence was detected precisely from whole genome DNA (1 µL) using each specific probe. Estimated copy numbers of the target sequences based on real-time PCR were 4.8–14 × 10^4 copies/µL (WT), 2.5–9.8 × 10^3 copies/µL (TR34/L98H), and 6.6–11 × 10^4 copies/µL (TR46/Y121F/T289A). These copy numbers were consistent with the results estimated from the DNA concentrations (WT: 4.7 × 10^4, TR34/L98H: 4.5 × 10^3, and TR46/Y121F/T289A: 6.9 × 10^4 copies/µL). Future studies must examine detection in DNA purified from body fluid samples such as sputum and blood.

Targeted mismatches found in TR strains were specifically detected by the method shown in this study. The mismatches...

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**Fig. 2.** Detection of each non-mutated or mutated site in genomic DNA templates. Lines with black dots show results obtained using genomic DNA from a wild type strain. Lines with black triangles and cross marks respectively show results obtained using genomic DNA from a TR34/L98H strain and a TR46/Y121F/T289A strain.
could be monitored during real-time PCR and detected within two hours. Although a real-time PCR system is required for the method, gel electrophoresis following PCR and cycle sequencing are not necessary. TR34- and TR46-types were detected but could not be differentiated by the previously reported method. As shown by Snelders et al., these resistance patterns were different from each other. And recently, Sewell et al. showed that the frequency of these resistance genotypes was unevenly distributed across two A. fumigatus clades. To date, a limited number of kits such as AsperGenius kit and MycoGENIE kit are available for the detection of azole-resistant A. fumigatus. The method shown here can be a useful alternative for detection of azole-resistant A. fumigatus and differentiation between these resistant genotypes.

In summary, herein we designed primer sets and probes to detect mutations found in cyp51A with TR in the promoter and demonstrated that they specifically detect each mutation. These new primer sets and probes, in addition to the TR detection sets described in an earlier report, contribute to the easy and rapid detection and confirmation of azole resistance in A. fumigatus isolates.

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**Conflict of Interest**

Self-declared COI content: none.

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