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Original article

In vitro resistance of *Aspergillus fumigatus* to azole farm fungicide

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ABSTRACT

Azole resistance in *Aspergillus fumigatus* is mainly due to a point mutation in the 14α-sterol demethylase (*CYP51A*) gene, which encodes the target of azole fungicides. Moreover, overexpression of *CYP51B* or multidrug resistance (MDR) gene is supposedly related to the mechanism of azole resistance in *A. fumigatus*. In this study, we tried to induce resistance to tetraconazole, an azole fungicide, in strains of *A. fumigatus* from a farm and then investigated mutation and expression of their *CYP51A*, *CYP51B*, and multidrug resistance (MDR) genes. Three tetraconazole resistant strains were induced and their minimum inhibitory concentration (MIC) for tetraconazole was 145 mg/L. However, the MICs of itraconazole (ITZ), posaconazole (POS), and voriconazole (VRZ) obtained by an E-test of the three tetraconazole resistant strains were 0.064–0.19 mg/L for ITZ, 0.023–0.32 mg/L for POS, and 0.047–0.064 mg/L for VRZ. No gene mutations were detected in the *CYP 51A* sequence amplified in these strains. RT-PCR of cyp51A and cyp51B indicated that the tetraconazole resistant strains more highly expressed these genes than the susceptible strain in tetraconazole containing medium.

1. Introduction

Azole resistance in *Aspergillus fumigatus* has been reported worldwide. Resistance is mainly due to a point mutation in the 14α-sterol demethylase (*CYP51A*) gene, the target of azoles [1]. The mutation induces resistance of *A. fumigatus* to some azoles, including itraconazole (ITZ), which is used commonly to treat human aspergillosis. The mutation has been confirmed in isolates from patients who received long-term azole therapy and from agricultural fields where large quantities of azole fungicides were used [1]. Environmental isolates of *A. fumigatus* from farms were first reported to be resistant in the Netherlands where large quantities of azole fungicides were used, and resistant strains have been reported from farms all over the world [1–6]. However, the relationship between farm environments and azole resistance of *A. fumigatus* is not well understood.

In our previous study, 50 isolates of *A. fumigatus* were obtained from a farm where tetraconazole (an azole fungicide) had been sprayed twice a year for more than 15 years [7]. The sequence of *CYP51A* of these isolates was confirmed to contain no gene mutations. Based on the antifungal susceptibility of isolates to tetraconazole, it was not believed that tetraconazole sprayed on the farm could easily induce resistance to tetraconazole or ITZ in *A. fumigatus*. However, the European Centre for Disease Prevention and Control issued a risk assessment report on azole resistance in Aspergillus spp., indicating that environmental usage of azole fungicides was possibly linked to azole resistance in *A. fumigatus* (http://www.life-worldwide.org/media-centre/article/ecdc-issues-risk-assessment-on-azole-resistance-in-aspergillus-from-environ/). The number of cases of serious human infection due to azole resistant Aspergillus seems to be increasing in several European countries, although the number of related studies is small in other countries. It has not been demonstrated that *A. fumigatus* in the environment receives sufficient azole challenge to select resistant strains or to gain azole resistance [6]. Therefore, it is required to examine whether azole fungicides induce mutation in *A. fumigatus*. In this study, we tried to induce tetraconazole resistance in strains of *A. fumigatus* from the environment and investigated the mutation and expression of their 14α-sterol demethylase genes (*CYP51A* and *CYP51B*) and multidrug resistance (MDR) gene.

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Table 1
Species and strains examined used in this study.

Species	Strain number	Origin
A. fumigatus	26	Isolated from a pumpkin farm[a]
A. fumigatus	35	Isolated from a pumpkin farm[a]
A. fumigatus	44	Isolated from a pumpkin farm[a]
A. fumigatus	50	Isolated from a pumpkin farm[a]
A. fumigatus	51	Isolated from a pumpkin farm[a]
A. fumigatus	44-1-2	Derived from 44 strain in this study
A. fumigatus	44-1-5	Derived from 44 strain in this study
A. fumigatus	44-1-14	Derived from 44 strain in this study

[a] Reported in Ref. [7].

2. Materials and methods

2.1. Strains examined

Six isolates (strains 6, 26, 35, 44, 50, and 51) of *A. fumigatus* from the farm in our College of Bioresource Sciences, Nihon University with a MIC value of 1.5 mg/L to ITZ were used in this study [7] (Table 1). They had no *CYP 51A* gene mutations and their MICs for tetraconazole were ≤36.25 mg/L, confirming that the spraying of tetraconazole did not induce tetraconazole and ITZ resistance in these strains [7].

2.2. Inducing tetraconazole resistance in strains

The procedure for inducing tetraconazole resistant strains is shown in Fig. 1. From each strain, 2.3×10^5 conidia were cultured on 9-cm diameter plates of potato dextrose agar (PDA) [8] with 38.7 mg/L tetraconazole and incubated at room temperature for 3 weeks. The dosage (3000× dilution; 38.7 mg/L) in Sabouraud's dextrose agar (SDA) was in accordance with the manufacturer's directions for the concentration of the farm fungicide. Then, three colonies, which developed only from strain 44, were pic ked and incubated on PDA containing 116.1 mg/L tetraconazole (1000× dilution) at room temperature. No colonies from 5 strains (strains 6, 26, 35, 50, and 51) grew on PDA containing 116.1 mg/L tetraconazole.

One week afterward, one colony (strain 44-1) that developed on the plate was picked and inoculated on a PDA plate containing 116.1 mg/L tetraconazole (1000× dilution) at room temperature. From the 44-1 strain, 8.0×10^5 conidia were cultured on four 9-cm diameter PDA plates containing 116.1 mg/L tetraconazole and incubated at room temperature. Two weeks afterward, 20 colonies had grown on the plate and were incubated on PDA plates containing 232.2 mg/L tetraconazole (500× dilution). Two weeks afterward, three colonies (strains 44-1-2, 44-1-5, and 44-1-14) had grown and were used for further investigation.

2.3. Susceptibility of strains to tetraconazole

A broth microdilution assay was performed to assess the susceptibility of the three strains (44-1-2, 44-1-5, and 44-1-14) to tetraconazole according to CLSI M38-A2 guidelines [9]. Stock inoculum suspensions were prepared from 7-day-old cultures grown on PDA at 30 °C. The final concentration of the suspensions was adjusted spectrophotometrically to an optical density in sterile 0.9% saline at 530 nm of 1.0. Tetraconazole concentrations were 580 to 1.132 mg/L in physiological saline. MIC values were determined after 72 h of incubation at 30 °C. The MIC of tetraconazole was defined as the lowest concentration showing 100% growth inhibition.

2.4. Susceptibility of strains to azoles

Tetraconazole resistant strains (44-1-2, 44-1-5 and 44-1-14) were examined using E-test gradient strips of ITZ, voriconazole (VRZ), and posaconazole (POS) obtained from AB Biodisk (Solna, Sweden). Stock inoculum suspensions were prepared as described in the E-test Technical Guide 10 [10]. For quality control, the strain *Candida parapsilosis* ATCC 22019 was used in each experiment to check the accuracy of drug dilution [10]. MIC values were determined after 72 h of incubation at 37 °C.

2.5. CYP51A gene sequences

The *CYP51A* genes of the three tetraconazole resistant strains were sequenced as described previously [11].

2.6. Real-time PCR of CYP51A, CYP51B, and MDR genes

Mycelia of strain 44 were obtained by culturing cells in Sabouraud's dextrose broth (SDB) at 28 °C for 3 days; mycleia of strains 44, 44-1-2, 44-1-5 and 44-1-14 were obtained by culturing in SDB containing 19.35 mg/L tetraconazole (6000× dilution) at 28 °C for 3 days. Mycelial samples were collected by centrifugation at 3000 rpm (1600 × *g*) for 5 min and then homogenized in liquid nitrogen. Total RNA was extracted from approximately 1 g of sample using the RNeasy total RNA kit (QIAGEN, Tokyo Japan). Reverse transcription of the poly(A)$^+$ RNA was performed using the Omniscript Reverse Transcriptase kit (QIAGEN).

Primer pairs were those used in previous reports; for *AfuMDR4*, the forward primer sequence was 5′-CTATATCGGGTCAGTCCTGG-3′

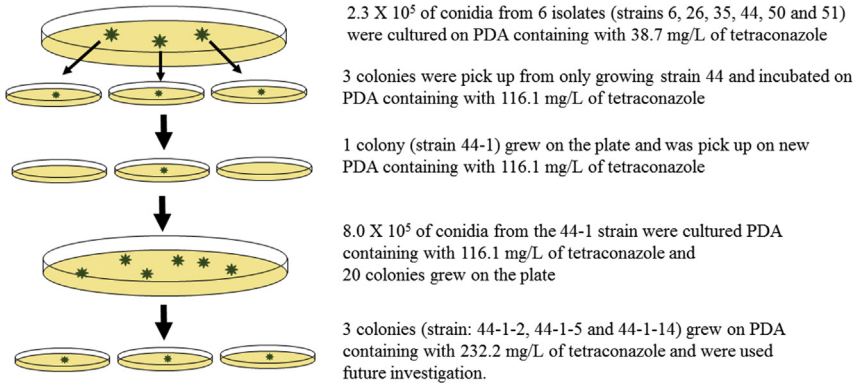

Fig. 1. Schema for inducing tetraconazole resistance in isolates.