

# **Antifungal susceptibilities of *Aspergillus fumigatus* clinical isolates in Nagasaki, Japan**

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## ABSTRACT (75 words)

We investigated triazole, amphotericin B, and micafungin susceptibilities of 196 *A. fumigatus* clinical isolates in Nagasaki, Japan. The percentages of non-wild-type (non-WT) isolates for itraconazole, posaconazole, and voriconazole were 7.1%, 2.6%, and 4.1% respectively. G54 mutation in *cyp51A* was detected in 64.2% (9/14 isolates) and 100% (5/5 isolates) of itraconazole and posaconazole non-WT isolates, respectively. Amphotericin B MICs of  $\geq 2$   $\mu\text{g/ml}$  and micafungin MECs of  $\geq 16$   $\mu\text{g/ml}$  were recorded for 2 and 1 isolates, respectively.

The clinical importance of *Aspergillus* infection has increased as the number of immunocompromised patients has risen (16). Recommended antifungals to treat patients with invasive pulmonary aspergillosis (IPA) or chronic pulmonary aspergillosis (CPA) are triazoles, amphotericin B, and echinocandins (13, 15, 37). Patients with CPA often need years of treatment (13, 37). Although oral therapy is important for carrying out long courses of treatment, azoles (with the exception of fluconazole) are the only class of oral drugs licensed for the treatment of aspergillosis (14, 37).

*Aspergillus fumigatus* is the most common and pathogenic species of *Aspergillus* (34, 37). Antifungal resistance of *A. fumigatus* especially to azoles is one of the concerns in treatment of aspergillosis. During the last decade, many cases of treatment failure due to azole-resistance *Aspergillus* infection have been reported, and in the past few years a growing body of papers has been accumulating about antifungal susceptibilities of *A. fumigatus* (1, 3-6, 9, 10, 12, 18, 23-27, 31-33, 35, 36). Even though an increased rate of azole-resistance has been reported recently in the Netherlands and the United Kingdom, prevalence rate of azole resistance reportedly remains low in other countries (1, 3, 6, 9, 12, 23, 25, 33).

The azole target protein, lanosterol 14 $\alpha$ -demethylase of *Aspergillus* is encoded by the *cyp51A* gene, and mutations of *cyp51A* are a major mechanism of azole

resistance (8, 17, 19, 20, 22, 32). Some mutational hotspots, such as G54, M220, and TR/L98H, have been identified as cause of azole resistance (2, 21, 22). Of these mutations, TR/L98H was especially prevalent in the Netherlands. An environmental origin (resulting from agricultural antifungal drug usage) is suspected, in spite of the fact that the mechanism(s) of mutation induction have not been shown definitively (24, 31, 32).

We studied the antifungal susceptibility of 196 *A. fumigatus* clinical isolates obtained in the Pneumology Department of Nagasaki University Hospital, Nagasaki, Japan. The isolates were collected between February 1994 and April 2010. All of the isolates were subjected to susceptibility testing and *cyp51A* sequence analysis. All isolates were identified as *A. fumigatus* by macroscopic colony morphology, micromorphological characteristics, and the ability to grow at 48°C. Non-wild-type (non-WT) isolates were subjected to additional molecular identification by amplification of ribosomal internal transcribed spacers (ITSs) and ribosomal large-subunit D1-D2 sequencing as described previously (11). MICs of itraconazole, posaconazole, voriconazole, and amphotericin B and minimum effective concentrations (MECs) of micafungin were determined using the Clinical and Laboratory Standards Institute (CLSI) M38-A2 broth microdilution method. Assays were performed using

RPMI 1640 broth (0.2% dextrose), final inoculum concentrations ranging from  $0.4 \times 10^4$  to  $5 \times 10^4$  CFU/ml, and 48h of incubation at 35°C (7). MIC was defined as the lowest drug concentration that produced complete growth inhibition; MEC was read as the lowest concentration of drug that led to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well.

Susceptibility tests of non-WT isolates were performed at least three times for each isolate; each test was performed on different days. Because clinical breakpoints have not been established yet, we adopted the use of epidemiological cutoff values (ECVs) to evaluate azole susceptibility (9, 25, 29). Wild-type (WT) isolates of *A. fumigatus* ( $MIC \leq ECV$ ) were distinguished from non-WT isolates ( $MIC > ECV$ ) which may exhibit acquired low susceptible mechanisms. ECVs used in this study were as follows: itraconazole, 1 µg/ml; posaconazole, 0.5 µg/ml; voriconazole, 1 µg/ml as previously suggested (9, 25).

For sequence analyses, genomic DNA was extracted from non-WT isolates using the MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI). The full coding region of the *cyp51A* gene was amplified as previously described (32). DNA sequences were determined using a BigDye Terminator version 1.1 cycle sequencing kit (ABI) and an ABI 3100xl DNA analyzer. Sequence alignments were

performed against the sequence from an azole-susceptible strain (GenBank accession no. AF338659) using MacVector10.0 software (MacVector, Inc., Cary, NC) (20).

Mutations were confirmed three times by repeating the PCR and sequencing the relevant region using the closest primer.

In this study, using the ECVs, the percentages of non-WT isolates for itraconazole, posaconazole, and voriconazole were 7.1%, 2.6%, and 4.1% respectively (Table 1). To exclude the possibility of increased proportions of non-WT isolates due to clonal spread (notably, the potential repeated isolation of a drug-resistant strain originating from one patient), we confirmed those proportions on a per-case basis, which (for non-WT isolates) were 7.5%, 4.3%, and 6.5% for itraconazole, posaconazole, and voriconazole, respectively. These proportions of non-WT isolates were not that much different from other previous data from any regions, with the exception of data for the Netherlands and the United Kingdom (3, 9, 12, 23, 25, 33). All the itraconazole-resistant isolates (MIC  $\geq 4\mu\text{g/ml}$ ) were obtained from 1998 to 2001. No consistent trend of increased proportion of non-WT isolates was observed. Amphotericin B MICs of  $\geq 2\mu\text{g/ml}$  were recorded for 1.0% of the isolates (2/196); micafungin MECs of  $\geq 16\mu\text{g/ml}$  were recorded for 1.0% of the isolates (2/196) (Table 1). For these antifungals, the proportions of resistant isolates were low and similar to those of previous reports (3, 10,

23, 26).

In Japan, posaconazole has not been approved for clinical use; nonetheless, non-WT isolates to posaconazole already existed (Table 1). Resistance in these isolates might reflect native biological variability. Alternatively, this phenomenon could be associated with cross-resistance between itraconazole and posaconazole, because 80% (4/5) of posaconazole non-WT isolates were also itraconazole non-WT isolates (Table 2). In addition, non-WT isolates of itraconazole tended to be more resistant to posaconazole, though not to voriconazole (Table 1). Cross-resistance between itraconazole and posaconazole, but not with voriconazole, may result from the G54 mutation of *cyp51A*, which was present in 64.2% (9/14) of the itraconazole non-WT isolates and also present in 100% (5/5) of the posaconazole non-WT isolates (Table 2). There is a known structural basis for the association of the G54 mutation with this pattern of cross-resistance among the azoles: unlike voriconazole, itraconazole and posaconazole have long side chains that clash with the amino acid side chain of the residue replacing G54 in the mutated CYP51A protein (8, 27, 32, 38).

Among mutations of the *cyp51A* gene, TR/L98H has received the most attention, notably because this mutation was seen in a specific country and found in *A. fumigatus* isolated from environment (17, 22, 24, 31-33). Recently, likewise in China,

TR/L98H was detected in a multi-azole resistant isolate (17), suggesting that the TR/L98H mutation could be selected in Asia as well as in Europe. Of all 22 non-WT isolates in our study of Japanese isolates, CYP51A mutations were detected as follows: G54W, two isolates; G54R, one isolate; I266N, two isolates; G54E + I266N, seven isolates (Table 2). No TR/L98H-bearing isolates were detected. The I266N mutation, which has (to our knowledge) not been reported previously, also was seen in other azole-susceptible isolates; therefore, it might not be directly related to azole resistance. Of 21 non-WT-isolates, 9 isolates had no CYP51A substitution (Table 2). Interestingly, most voriconazole non-WT isolates did not possess *cyp51A* mutation. Although Bueid et al. reported an increase of frequency of azole-resistant isolates without *cyp51A* mutations, other possible resistant mechanisms (e.g. up-regulation of efflux pump) are not fully revealed yet (6, 28, 30). Further analysis is warranted.

Only a few previous analyses have examined changes in susceptibility over time; therefore, it is not clear that the frequency of azole-resistant *A. fumigatus* is increasing world-wide (12, 25, 33). Nevertheless, mechanisms of resistance induction in clinical settings or the environment (e.g., selection following agricultural antifungal exposure) remain poorly understood. Given that azole usage varies from one country to another, the mechanism of azole resistance may differ between regions.



In this study, we found low prevalence of resistance to triazoles in Japanese isolates of *A. fumigatus*, a clinically important fungus of increasing concern in respiratory medicine. The proportions of non-WT isolates were similar to those previously reported for other countries. In the future, Japanese *A. fumigatus* isolates may develop azole resistance by different mechanisms (such as TR/L98H); therefore, we urge the continued monitoring of azole susceptibility in this species.

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TABLE 1. MIC and MEC distributions of five antifungals

Antifungal agent	No. of isolates <sup>a</sup>											% of MICs >ECV
	Total	With MIC or MEC <sup>b</sup> (µg/ml):										
		≤0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	≥16	
<u>Triazoles</u>												
Itraconazole	182 (14)			1	28	107	46	(8)	(4)	(1)	(1)	7.1
Posaconazole	182 (14)	14	108	35	24 (2)	(8)	(1)	1 (2)			(1)	2.6
Voriconazole	182 (14)			1 (3)	20 (7)	101 (2)	53 (1)	7 (1)				4.1
<u>Polyene</u>												
Amphotericin B	182 (14)		6	8 (1)	19 (1)	90 (7)	57 (5)	2				–
<u>Echinocandin</u>												
Micafungin	182 (14)	177 (14)	2	1							2	–

<sup>a</sup> MIC distributions for each agent were obtained by subtracting from the total isolates tested the 14 non-WT isolates of itraconazole. The MIC distribution of each agent for those 14 isolates is also provided within parentheses.

<sup>b</sup> MICs are shown for amphotericin B, itraconazole, posaconazole, and voriconazole. Minimum effective concentrations (MECs) are shown for micafungin.

TABLE 2. MICs and Cyp51A substitution of 22 non-WT *Aspergillus fumigatus* isolates

Isolate no.	MIC ( $\mu\text{g/ml}$ )			Cyp51A substitution
	itraconazole	posaconazole	voriconazole	
MF-452	>8	0.5	0.5	I266N
MF-469	8	1	0.25	G54E, I266N
MF-460	4	2	0.25	G54E, I266N
MF-357	4	0.5	0.5	I266N
MF-468	4	0.5	0.25	G54E, I266N
MF-329	4	0.5	0.25	no substitution
MF-331	2	>16	0.25	G54W
MF-327	2	2	0.12	G54R
MF-439	2	0.5	0.25	G54E, I266N
MF-473	2	0.5	0.25	G54E, I266N
MF-454	2	0.5	0.12	G54E, I266N
MF-472	2	0.5	0.12	G54E, I266N
MF-843	2	0.25	2	no substitution
MF-748	2	0.25	1	ND <sup>a</sup>
MF-1011	1	2	0.12	G54W
MF-855	1	0.25	2	no substitution
MF-336	1	0.25	2	no substitution
MF-486	1	0.25	2	no substitution
MF-520	1	0.25	2	no substitution
MF-1091	0.5	0.25	2	no substitution
MF-474	0.5	0.25	2	no substitution
MF-303	0.5	0.12	2	no substitution

<sup>a</sup> ND, not determined.

Table 2. Tashiro et al.