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High detection rate of azole-resistant *Aspergillus fumigatus* after treatment with azole antifungal drugs among patients with chronic pulmonary aspergillosis in a single hospital setting with low azole resistance

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Abstract

The prevalence of azole-resistant *Aspergillus fumigatus* (ARAF) among chronic pulmonary aspergillosis (CPA) patients treated with azoles in Japan is unknown. The aim of this study was to determine the detection rate of ARAF in isolates from CPA patients who were treated with azoles for varying durations. The potential mechanism of acquiring resistance was examined by sequencing *cyp51A* and *hmg1*, two genes associated with ARAF. *A. fumigatus* isolates ($n = 120$) were collected from CPA patients ($n = 104$) between February 2012 and February 2019, at National Hospital Organization Tokyo National Hospital. The isolates were tested for susceptibility to the azole drugs itraconazole (ITCZ) and voriconazole (VRCZ). The detection rate of ARAF among all isolates was 8.3% ($n = 10$). Of the 10 resistant isolates, eight were ITCZ-resistant and five were VRCZ-resistant. Among 47 isolates obtained from 36 CPA patients who were treated with ITCZ (for an average of 256 days) and/or VRCZ (for an average of 29 days), the resistance rates were 17.0% and 10.6%, respectively. In addition, 46.2% of 13 isolates obtained from CPA patients with ongoing azole treatment at the time of antifungal therapy failure were resistant to azoles. Among the 10 ARAF isolates, a point mutation was detected in *cyp51A* in seven isolates and in *hmg1* in two isolates. ARAF was detected at a high rate in CPA patients, particularly in those with ongoing long-term azole treatment, at the time of azole antifungal therapy failure.

Lay Summary

Aspergillus fumigatus can acquire azole resistance during long-term treatment with azole drugs in patients with chronic pulmonary aspergillosis (CPA). The aim of this study was to determine the detection rate of azole-resistant *A. fumigatus* (ARAF) in isolates from CPA patients who had been treated with azoles. In addition, a potential mechanism of acquiring resistance was examined by sequencing *cyp51A* and *hmg1*, two genes associated with ARAF. *A. fumigatus* isolates ($n = 120$) were collected from CPA patients ($n = 104$). The isolates were tested for susceptibility to the azole drugs itraconazole (ITCZ) and voriconazole (VRCZ). The detection rate of ARAF from all isolates was 8.3% ($n = 10$). Greater than 10% of the 47 isolates obtained from 36 CPA patients who had been treated with azoles exhibited resistance. Furthermore, 46.2% of 13 isolates

obtained from CPA patients with ongoing azole treatment at the time of antifungal therapy failure were resistant to azoles. Among the 10 ARAF isolates, a point mutation was detected in *cyp51A* in seven isolates and in *hmg1* in two isolates. ARAF was detected at a high rate in CPA patients undergoing long-term azole treatment at the time of antifungal therapy failure.

Key words: *Aspergillus fumigatus*, azole resistance, chronic pulmonary aspergillosis, *cyp51A*, *hmg1*.

Introduction

Chronic pulmonary aspergillosis (CPA) is a refractory fungal disease with life-threatening conditions and increasing morbidity.[1] The survival rate at 5 years is about 60%.[2] Treatment with oral antifungal drugs, for example, azoles, is recommended for at least 4–6 months for patients with CPA.[3–5] *Aspergillus fumigatus* is the most common cause of pulmonary aspergillosis.[6,7]

Azole-resistant *A. fumigatus* (ARAF) is an emerging problem, leading to high mortality rates.[2,8,9] Two routes to ARAF development are known: an environmental route supposedly caused by azole fungicides[10–12] and a patient route, developed after long-term azole treatments, particularly in patients with CPA.[13,14] Azole resistance via an environmental route may is due to tandem repeats in the promoter region of *cyp51A*.[10–12] On the other hand, the main mechanism associated with the patient route is point mutations in *cyp51A*,[8] which thwarts the ability of azoles to inhibit ergosterol synthesis.[15,16] In addition, mutations in *hmg1* in non-*cyp51A* ARAF result in resistance to multiple azoles with increased intracellular ergosterol levels.[17]

The detection rate for ARAF depends on the local epidemiology.[8,18–20] The reported ARAF detection rate in Japan ranges from low (<10%)[21–24] to relatively high (12.7%).[25] However, it is unknown how high the rate is among CPA patients treated with azoles for the long term. In this retrospective study, we calculated the detection rate of ARAF using *A. fumigatus* isolates from patients with CPA in a single hospital setting, particularly patients with a history of or ongoing azole treatment. In addition, the two main mechanisms of acquiring azole resistance, mutations in *cyp51A* and *hmg1*, were investigated.

Methods

Aspergillus fumigatus isolates

Aspergillus fumigatus isolates, identified morphologically, were consecutively collected from the lower respiratory tract, that is, sputum, endotracheal aspirate, bronchoalveolar lavage, and surgical samples at the National Hospital Organization (NHO) Tokyo National Hospital, Tokyo, Japan, from February 2012 to February 2019. The deposit of bronchoalveolar lavage after centrifugation was used for the culture. Surgical sample was diluted with BBL™ Trypticase™ Soy Broth (Becton, Dickinson and Company, Sparks, MD, USA) and homogenized. Each sample was cultured (10 μl and 100 μl of each sample) on Sabouraud dextrose agar (KANTO KAGAKU, Tokyo, Japan) or Potato dextrose agar (KANTO KAGAKU) at 35°C for the first 2 days and then at 22 ± 2°C for up to 14 days.[26] One or two culture plates were used per sample. One *A. fumigatus* CFU per culture was selected for further susceptibility testing and sequencing in this study.

Isolates were collected from patients with CPA at the time of diagnosis for CPA and different diagnosis (e.g., pneumonia), during follow-up, or in the case of azole antifungal therapy failure. Isolates were excluded from some CPA patients after ARAF was detected. A total of 120 *A. fumigatus* isolates, from 104 CPA patients, were included in this study.

The isolates were genetically identified as *A. fumigatus* by sequencing part of the β-tubulin gene[27] at the Medical Mycology Research Center, Chiba University, Chiba, Japan.

Susceptibility testing against itraconazole (ITCZ) and voriconazole (VRCZ)

Susceptibility testing against the azole drugs ITCZ and VRCZ was performed as described previously,[28] according to the Clinical and Laboratory Standards Institute (CLSI) M38 3rd edition[29] with partial modifications using a dried plate (Eiken Chemicals, Tokyo, Japan, catalogue number: 9DEF47). Quality control was confirmed by examining the following quality control and reference strains:[30] *Paecilomyces variotii* ATCC MYA-3630 (=IFM 58693), *A. flavus* ATCC 204304 (=IFM 56856), *A. fumigatus* ATCC MYA-3627 (=IFM 58690), and *A. fumigatus* ATCC MYA-3626 (=IFM 58689), which were provided through the National Bio-Resource Project (NBRP), Japan (http://www.nbrp.jp/). The minimum inhibitory concentrations (MICs) of ITCZ and VRCZ were determined, and the epidemiological cutoff values were defined as follows: ITCZ 1 μg/mL and VRCZ 1 μg/ml.[31] ARAF was defined as *A. fumigatus* isolates of which the MICs of ITCZ and VRCZ > 2 μg/ml.[31]

Sequencing *cyp51A* and *hmg1*

The promotor and entire *cyp51A* and *hmg1* genes were amplified with appropriately designed primers.[17] The products were sequenced and compared with reference sequences from GenBank (accession no. AF338659 for *cyp51A*) and the Fungi DB (http://fungidb.org/fungidb/; accession nos. AFUB_020770 for *hmg1*) to detect mutations.

Patient characteristics

The medical records of 104 patients with CPA were retrospectively reviewed. The clinical characteristics at the time of first isolate sampling including age, sex, underlying lung diseases, history, and duration of treatment with azoles (ITCZ and/or VRCZ) before ARAF isolation, and the reason for sample collection were analyzed. The form of ITCZ used, capsule or oral solution, was also recorded. CPA was diagnosed based on three guidelines detailed in the following publications: Guidelines for the Management of Deep-Seated Mycosis 2014, 3rd edition,[3] Infectious Diseases Society of America,[4] and the European Respiratory Society.[5] Briefly, CPA was diagnosed based on (1) chronic pulmonary symptoms lasting a few months, (2) positive *Aspergillus* precipitating antibody and/or galactomannan *Aspergillus* antigen results, and (3) thoracic imaging of cavitation, pleural thickening, pericavitary infiltrates, or a fungal ball.

Definition of terms

Antifungal therapy failure for CPA was considered to be deterioration in clinical and/or radiological findings;[5] hemoptysis, increased cough and sputum production, deteriorated fatigability, and breathlessness were clinical signs of deterioration. Radiological signs of deterioration included CT findings of an expanding cavity, increased pleural or cavitary wall, and increased consolidation.

Statistical analysis

The Mann-Whitney *U* test was used to compare the duration of ITCZ and VRCZ use prior to patient sampling, $P < .05$. Statistical analyses were performed using GraphPad Prism version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA).

Ethics

The Institutional Review Board of NHO Tokyo National Hospital (approval number: 130020) approved the retrospective study, and written informed consent was not required.

Results

Characteristics of CPA patients

Baseline characteristics of all CPA patients at the time of first isolate sampling are shown in Table 1. The prevalence of underlying pulmonary diseases revealed prior pulmonary tuberculosis as the major disease, followed by nontuberculous mycobacterial pulmonary infection, and chronic obstructive pulmonary disease. Thirty-six patients (34.6%) had been treated with azoles before first isolate sampling.

Table 1. Baseline characteristics of all CPA patients at the time of first isolate sampling.

	All patients, $n = 104$
Age (years)[a]	68.5 ± 11.4
Male/Female[b]	66 (63.5) / 38 (36.5)
Underlying pulmonary diseases[b,*]	
Prior pulmonary tuberculosis	38 (36.5)
Nontuberculous pulmonary infection	23 (22.1)
COPD	20 (19.2)
Interstitial lung disease	13 (12.5)
Bronchiectasis	7 (6.7)
Lung cancer	5 (4.8)
History of thoracic surgery	4 (3.8)
History of treatment with azoles[b,*]	36 (34.6)
ITCZ use	31 (29.8)
ITCZ capsule use	28 of 31 (90.3)
VRCZ use	8 (7.7)

Data are presented as [a]mean ± SD or [b]*n* (%). CPA, chronic pulmonary aspergillosis; COPD, chronic obstructive pulmonary disease; ITCZ, itraconazole; VRCZ, voriconazole.
[*]Includes duplicate cases.

Susceptibility to ITCZ and VRCZ

The detection rate of ARAF from all isolates ($n = 120$) was 8.3% ($n = 10$): 6.7% ($n = 8$) and 4.2% ($n = 5$) of tested isolates were resistant to ITCZ and VRCZ, respectively (Fig. 1A, B). Among 47 isolates obtained from 36 CPA patients who had been treated with azoles, 17.0% (8 of 47) of the isolates were resistant to ITCZ and 10.6% (5 of 47) of the isolates were resistant to VRCZ (Fig. 1C, D). The time of ARAF isolation is shown in Figure 2. Thirteen isolates were obtained from CPA patients at the time of azole antifungal therapy failure, revealing that 46.2% (6 of 13) of the isolates were ARAF.

Duration of azole antifungal use before ARAF isolation

The duration of patient azole antifungal agent exposure before isolate sampling is shown in Table 2. Forty-seven isolates exposed to azoles were obtained; 39 were exposed to ITCZ and 13 to VRCZ. The median duration of ITCZ or VRCZ use, including duplicate exposure, before ARAF isolation was 256 days (14–1675) and 29 days (8–1385), respectively. The ITCZ treatment times were significantly longer for ARAF isolates compared to azole-susceptible *A. fumigatus* isolates ($P = .042$), although there was no significant difference in comparison of duration of both ITCZ capsule and oral solution between ARAF isolates and azole-susceptible *A. fumigatus* isolates. No differences were observed among the two isolate groups for VRCZ treatment periods ($P = .39$).

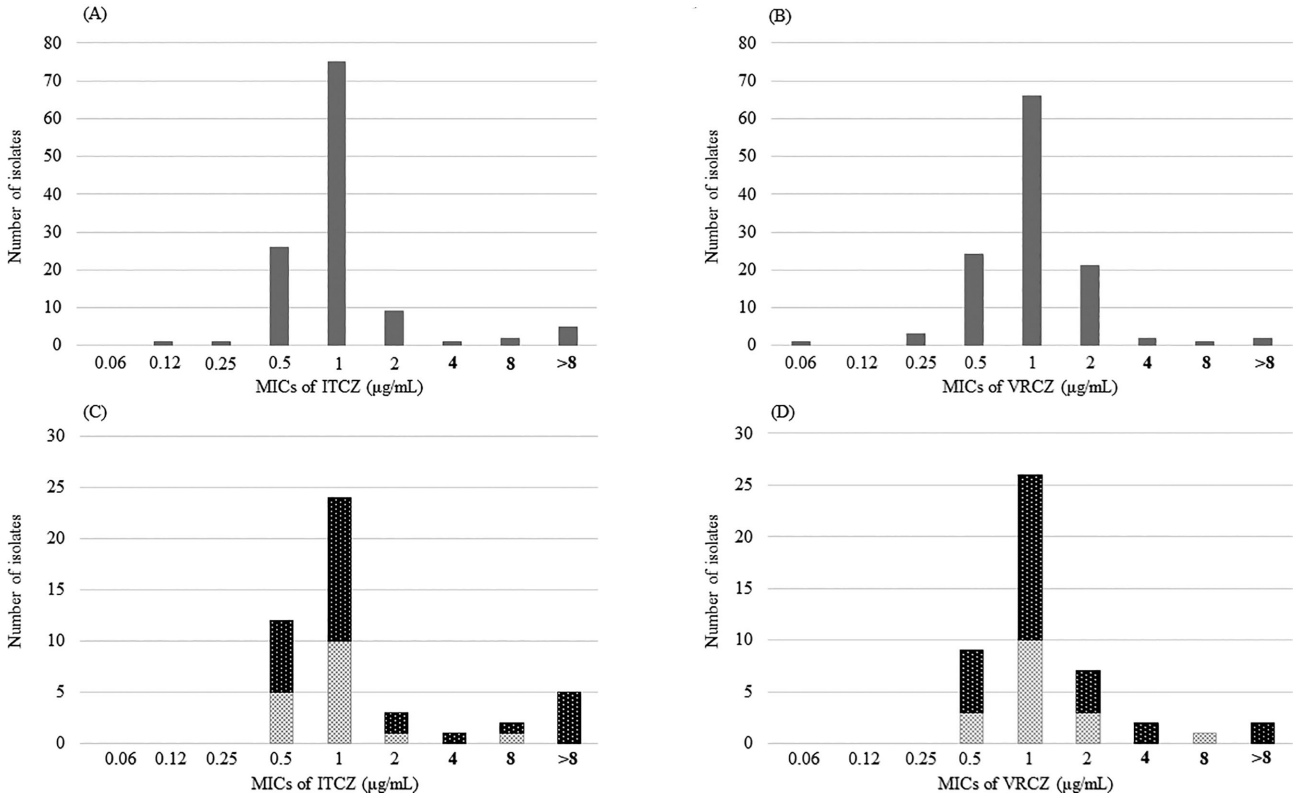

Figure 1. MICs for ITCZ and VRCZ. The detection rate of ARAF was 6.7% (8 of 120) for ITCZ (A) and 4.2% (5 of 120) for VRCZ (B) from 104 patients with CPA. The detection rate of ARAF was 17.0% (8 of 47) for ITCZ (C) and 10.6% (5 of 47) for VRCZ (D) from 36 patients with CPA who had been treated with azoles. MICs of resistance for azoles (>2 μg/ml) are shown in bold. ■ : Isolates obtained from patients with ongoing treatment with azoles. ▨ : Isolates obtained from patients with a history of treatment with azoles. ARAF, azole-resistant *Aspergillus fumigatus*; CPA, chronic pulmonary aspergillosis; ITCZ, itraconazole; MIC, minimum inhibitory concentration; VRCZ, voriconazole.

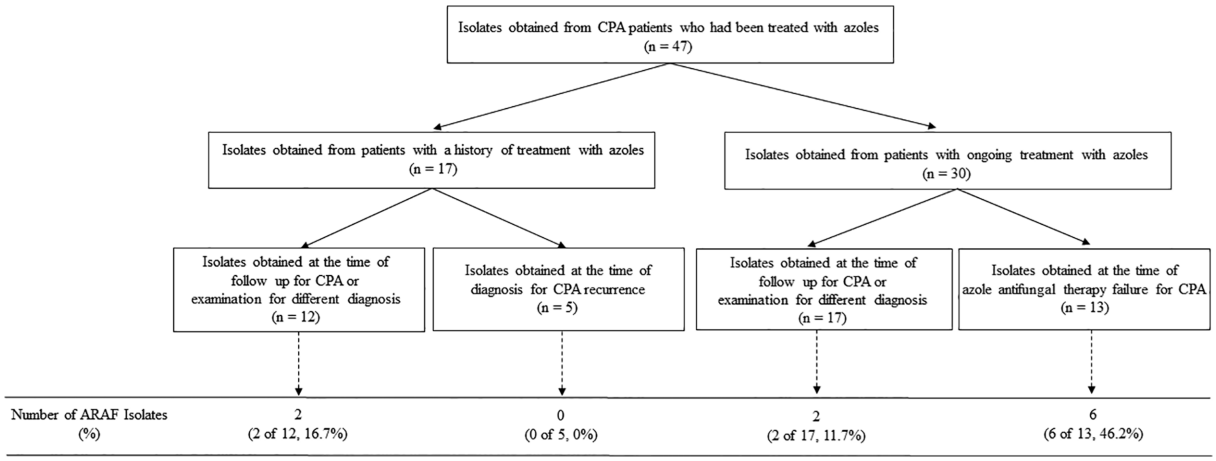

Figure 2. Time of ARAF isolation. Among 47 isolates from CPA patients who had been treated with azoles, 30 were obtained from patients with ongoing treatment with azoles. Thirteen isolates were obtained from patients at the time of azole antifungal therapy failure; 46.2% (6 of 13) were ARAF. ARAF, azole-resistant *Aspergillus fumigatus*; CPA, chronic pulmonary aspergillosis.

Azole resistant *Aspergillus fumigatus* CPA cases and gene mutations

Of the 10 ARAF isolated from CPA patients, eight (80.0%) were treated with azoles for more than 6 months; the shortest azole use was 21 days (Table 3, case 7). There were no ARAF isolates with tandem repeats in the promoter region of *cyp51A*. Seven of 10 (70.0%) ARAF isolates had a point mutation in *cyp51A*. Two of 10 (20.0%) ARAF isolates had a point mutation in *hmg1* (Table 3).

Table 2. Duration of patient azole antifungal agent exposure before isolate sampling.

	A. fumigatus isolates, $n = 47$*	Azole susceptible *A. fumigatus* isolates, $n = 37$*	ARAF isolates, $n = 10$*	*P*
	Time (range), days	Time (range), days	Time (range), days	
ITCZ exposure†	256 (14–1675), $n = 39$	164 (14–1675), $n = 30$	428 (90–1524), $n = 9$	.042
ITCZ cp exposure	306 (8–1675), $n = 32$	174 (8–1675), $n = 25$	687 (194–1524), $n = 7$	.053
ITCZ OS exposure	88 (14–428), $n = 10$	79 (14–400), $n = 8$	259 (90–428), $n = 2$	.40
VRCZ exposure	29 (8–1385), $n = 13$	20 (7–1385), $n = 9$	44 (21–384), $n = 4$	.39

Data are presented as median (range). ARAF, azole-resistant *Aspergillus fumigatus*; cp, capsule; ITCZ, itraconazole; OS, oral solution; VRCZ, voriconazole.
*Includes duplicate isolates exposed to both ITCZ and VRCZ.
†Includes duplicate isolates exposed to both ITCZ cp and ITCZ OS.

Discussion

The detection rate of ARAF in the population of azole-treated CPA patients was over 10%, even in a setting of known low azole-resistance. Among isolates from CPA patients with ongoing treatment with azoles at the time of antifungal treatment resistance, 46.2% of isolates were azole resistant. The median duration of treatment with the azole drugs ITCZ or VRCZ was 265 and 29 days, respectively. The longer exposure to ITCZ was related to increased detection of ARAF. The major mechanism of acquiring resistance appears to be point mutations in *cyp51A*.

Our findings are consistent with those of Tashiro et al., who reported that the ITCZ MIC increased in relation to the duration of ITCZ treatment, and this correlated with ARAF detection.[32] The median duration of ITCZ use in this study was approximately 6 months, which is the recommended treatment duration for CPA antifungal therapy.[4,5] Thus, the isolation of ARAF from CPA patients who had been treated with azoles for the recommended times suggests that *A. fumigatus* can acquire azole resistance during this time.[33]

The detection rate of ARAF among isolates obtained from CPA patients with ongoing azole treatment at the time of azole antifungal therapy failure was approximately 50%. The remaining 50% of azole susceptible isolates in CPA case at azole antifungal therapy failure may be explained in two ways. First, the plasma and tissue concentrations of azoles could have been low. Second, only azole-susceptible *A. fumigatus* was subjected to susceptibility testing during the process of sample isolation and subculturing, even when CPA was caused by mixed-infection of azole susceptible *A. fumigatus* and ARAF. To avoid prescribing ineffective drugs, next-round antifungal agents should be selected depending on the results of susceptibility tests and/or identified gene mutations associated with azole resistance.

The detection rate of ARAF is influenced by local epidemiology.[8,18–20] In Japan, several reports indicated that the detection rate was approximately 10%;[21–25] these studies analyzed isolates from clinical and/or environmental settings. In the current study, the rate of ARAF detection among all isolates was 8.3%. This rate suggests that this study was analyzed in a setting of low azole-resistance, although the isolates were collected only from patients with CPA at a single hospital.

One of the two main azole-resistance-acquiring mechanisms in *A. fumigatus* is a point mutation in *cyp51A*[34] caused by long-term azole exposure among aspergillosis patients. The position of the mutation might determine the azole resistant phenotype, that is, single, multi, or panazole resistance.[35,36] All of the *cyp51A* mutations detected in this study were known ones, and most of them were previously associated with azole resistance. For example, ARAF cases 4 and 10 exhibited alterations in *cyp51A* at codon 54 and were ITCZ resistant but VRCZ susceptible (Table 3), in agreement with previous reports.[13,14,36]

Two of 10 ARAF isolates (20%) had mutations in *hmg1*. Hagiwara et al.[17] reported 50% of ARAF isolates without *cyp51A* mutations had mutations in *hmg1*. In this study, one isolate displayed a point mutation in *hmg1* among the three ARAF isolates in which no *cyp51A* mutation was detected. Mutations in *hmg1* are the second most frequent mutations observed among ARAF isolates, after *cyp51A*. The L273F and S305P substitutions identified in ARAF were new *hmg1* mutations (Table 3), which might be associated with azole resistance because the mutations are located within the sterol-sensing domain.[17] Further studies will be needed to confirm the function of these point mutations by gene editing and protein homology studies.

Two ARAF isolates identified in this study did not have a mutation in either *cyp51A* or *hmg1*; they may use other azole-resistance-acquiring mechanisms possibly related to efflux pumps[37,38] or other unknown mechanisms. Furthermore, analysis of whole genome sequences of azole-susceptible and azole-resistant isolates from the same patients should help in elucidating a mechanism.

This study had several limitations. First, the plasma concentrations of azoles used were not obtained. Most patients were treated with ITCZ capsules, bioavailability of which is lower than that of oral solutions.[39] Additional pharmacokinetic and pharmacodynamic studies are needed to investigate ways to prevent ARAF development. Second, there were fewer isolates exposed to VRCZ than ITCZ. This may have influenced the

Table 3. Azole-resistant *Aspergillus fumigatus* CPA cases.

Case	Age	Sex	Underlying pulmonary disease	Azole use before isolation, days		Treatment status	ITCZ MIC	VRCZ MIC	*cyp51A* substitution	*hmg1* substitution
				ITCZ	VRCZ					
1	64	F	Prior pulmonary tuberculosis	cp, 194	29	Ongoing treatment with VRCZ	8	>8	**G448S**	–
2	61	M	History of thoracic surgery due to traumatic hemopneumothorax	cp, 1524	None	Ongoing treatment with ITCZ	>8	>8	**M220I**	S305P
3	79	F	Nontuberculous pulmonary disease	cp, 377	None	Ongoing treatment with ITCZ	>8	2	F219C	–
4	59	M	History of thoracic surgery due to pneumothorax	cp, 687	None	Ongoing treatment with ITCZ	>8	2	**G54W**	–
5	64	M	Prior pulmonary tuberculosis	cp, 732	None	Ongoing treatment with ITCZ	2	4	–	–
6	58	M	COPD, History of thoracic surgery due to pneumothorax	cp, 307	59	Ongoing treatment with VRCZ	2	8	–	–
7	55	F	Nontuberculous pulmonary disease	None	21	History of treatment with VRCZ	>8	1	**M220V**	–
8	65	M	Nontuberculous pulmonary disease, COPD	OS, 90	None	History of treatment with ITCZ	8	0.5	P216L	–
9	62	F	Bronchiectasis	cp, 898	384	Ongoing treatment with ITCZ	4	4	–	L273F
10	54	F	Bronchiectasis	OS, 428	None	Ongoing treatment with ITCZ	>8	1	**G54W**	–

Mutations previously known and associated with azole resistance are shown in bold. COPD, chronic obstructive pulmonary disease; cp, capsule; CPA, chronic pulmonary aspergillosis; ITCZ, itraconazole; MIC, minimum inhibitory concentration; OS, oral solution; VRCZ: voriconazole; '–': no mutation.

results showing no significant difference in the number of azole-susceptible and azole-resistant *A. fumigatus* isolates detected in VRCZ-treated patients.

In conclusion, it should be noted that azole-resistant *A. fumigatus* emerged at a high rate in patients with ongoing long-term azole treatment at the time of antifungal therapy failure. Antifungal drugs should be selected based on susceptibility testing results and/or known gene mutations, revealing single, multi-, or pan-azole resistance.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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