High-Level Pan-Azole-Resistant Aspergillosis

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High-level pan-azole-resistant *Aspergillus fumigatus* was recovered from four patients with chronic lung disease. In one patient, the development of progressive resistance followed long-term azole therapy and switching between antifungal azoles. The high-level pan-azole-resistant phenotypes were not associated with a specific *cyp51A* gene mutation. New strategies that avoid the development of progressive azole resistance are needed.

Azole resistance in *Aspergillus fumigatus* is an emerging problem which is associated with treatment failure in patients with aspergillus diseases [1]. Resistance is commonly due to mutations in the *cyp51A* gene [1] that typically lead to high-level resistance (MIC, ≥8 mg/liter) against one azole and low-level resistance (MICs close to the resistance breakpoint) against others [2, 3].

We identified four *A. fumigatus* isolates with MIC of ≥8 mg/liter for all mold-active azoles, measured using EUCAST methodology [4]. We labeled this unique phenotype high-level pan-azole resistance. We analyzed the *cyp51A* gene sequence of the isolates, using previously published algorithms [5], and retrieved clinical data for these four patients (Table 1).

The first patient was a 22-year-old male with cystic fibrosis. After being diagnosed with allergic bronchopulmonary aspergillosis (ABPA), he commenced itraconazole and steroid maintenance therapy. Fungal sputum cultures after 8 months of itraconazole therapy revealed an *A. fumigatus* isolate with the high-level pan-azole-resistant phenotype. The patient continued itraconazole maintenance therapy, and the high-level pan-azole-resistant isolate was not recovered from repeat cultures.

The second patient was a 71-year-old male with a medical history of asthma, bronchiectasis, and intermittent culture positivity with itraconazole- and voriconazole-susceptible *A. fumigatus*. The patient did not meet diagnostic criteria for ABPA and was never treated with azoles. The high-level pan-azole-resistant *A. fumigatus* isolate was isolated once from a sputum sample; follow-up sputum cultures yielded azole-susceptible *A. fumigatus*.

The third patient was a 47-year-old female with severe pulmonary sarcoidosis, complicated by a pneumothorax with subsequent pleural empyema. From this empyema, an azole-susceptible *A. fumigatus* isolate was cultured (itraconazole and voriconazole MICs, 0.5 mg/liter; posaconazole MIC, 0.063 mg/liter). Treatment with itraconazole was started, later changed to voriconazole, and ultimately changed to posaconazole as a chronic suppressive therapy. After 18 months of azole therapy, the patient's disease progressed, and a sputum sample was ordered for fungal culture. This sample grew the high-level pan-azole-resistant isolate and induced a switch to liposomal amphotericin B therapy. Despite treatment, the patient's condition deteriorated, and she died of respiratory failure.

The fourth patient was a 39-year-old male diagnosed with chronic granulomatous disease and ABPA. The patient had been treated for multiple episodes of invasive pulmonary aspergillosis, and he received secondary prophylaxis with itraconazole. He then presented with arthritis of the sternoclavicular joint, and itraconazole-resistant *A. fumigatus* was cultured from biopsy specimens.

Treatment with voriconazole was initiated, but visual disturbances forced a switch to liposomal amphotericin B and anidulafungin. The patient responded and was discharged on posaconazole maintenance therapy. After 11 months, the patient presented with increasing dyspnea, dry cough, and fever. A chest computed tomography (CT) scan revealed a cavity and bronchiolitis in the right lower lobe. Bronchoalveolar lavage cultures were positive for *A. fumigatus*, which exhibited the high-level pan-azole-resistant phenotype and an M220R mutation in the *cyp51A* gene, absent in the patient's previous isolates. Micafungin was added to posaconazole, based on susceptibility test results (Table 1). After 1 and 7 days of treatment, follow-up cultures remained positive for the high-level pan-azole-resistant strain. After 10 days of therapy, the patient died of pulmonary hemorrhage. Microsatellite typing [4] showed that the isolates from this patient were isogenic.

The isolates cultured from these four patients reveal a new and highly worrisome phenotype characterized by high-level resistance to all mold-active azoles, including the new azole isavuconazole. Isavuconazole was shown to exhibit cross-resistance to voriconazole [6]. All patients had chronic lung diseases, and a chronic aspergillus disease was diagnosed in three patients. In patients 1 and 2, there were no clinical factors that might explain the single recovery of the high-level pan-azole-resistant *A. fumigatus* isolates. The high-level pan-azole-resistant isolates might have been acquired as such from the environment [7].

In patient 3, a persisting aspergillus infection was treated with various antifungal azoles. Although the initial isolate was azole susceptible, the high-level pan-azole-resistant isolate with an environmental resistance mechanism (TR$_{46}$/Y121F/T289A) was cultured during therapy. The high-level pan-azole-resistant phenotype may have developed during azole therapy or may have been acquired as such from the environment. Clear evidence for the development of progressive resistance was present in patient 4. As

TABLE 1 Patient characteristics, azole *in vitro* activities, and *cyp51A* resistance mechanisms in four high-level azole-resistant *A. fumigatus* isolates

Case	Sex[a]/age (yr)	Underlying condition	Aspergillus disease	Prior azole exposure	Origin of isolate	MIC (mg/liter) of[b]:						cyp51A mutation(s)
						AmB	AFG	ITC	VRC	POS	ISA	
1	M/22	Cystic fibrosis	ABPA[c]	ITC	Sputum	1	0.063	>16	16	>16	>16	TR$_{34}$/L98H
2	M/71	Bronchiectasis	None	None	Sputum	1	0.063	>16	>16	>16	>16	None detected
3	F/47	Sarcoidosis	Empyema	ITC, VRC, POS	Sputum	0.5	0.016	>16	>16	>16	>16	TR$_{46}$/Y121F/T298A, M172I, G448S
4	M/39	Chronic granulomatous disease	Chronic invasive aspergillosis	ITC, VRC, POS	BAL[d] specimen	0.5	0.063	>16	8	>16	16	M220R

[a] M, male; F, female.
[b] All MICs were determined using EUCAST methodology (2, 4). AmB, amphotericin B; AFG, anidulafungin; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; ISA, isavuconazole.
[c] ABPA, allergic bronchopulmonary aspergillosis.
[d] BAL, bronchoalveolar lavage.

treatment was switched between the azole compounds, *A. fumigatus* progressively developed resistance to each azole that was used for treatment. Possibly, the cavitary lesions or other difficult-to-reach sites with high fungal burdens facilitated the development of resistance (8).

We were unable to detect genetic changes in the *cyp51A* gene that paralleled the development of the high-level pan-azole-resistant phenotype (Table 1). We did not find known azole resistance mechanisms in the isolate of patient 2, indicating that non-*cyp51A*-mediated resistance mechanisms may have emerged in these isolates. Two isolates (of patients 1 and 3) harbored *cyp51A* mutations that have been associated with environmental azole exposure, TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A (1, 4). In the Netherlands, typical MICs of TR$_{34}$/L98H isolates are >16 mg/liter for itraconazole, 4 to 8 mg/liter for voriconazole, and 1 mg/liter for posaconazole, and those of TR$_{46}$/Y121F/T298A isolates are >16 mg/liter for voriconazole, variable for itraconazole (1 to >16 mg/liter), and 0.5 to 1 mg/liter for posaconazole (2). The high-level pan-azole-resistant phenotype, particularly the high posaconazole MIC, suggests that these isolates have accumulated additional non-*cyp51A* resistance mechanisms. These could include the recently described overexpression of *cyp51A*, *cyp51B*, and the cdr1B efflux pump; for these three mechanisms, MICs of ≥8 mg/liter for itraconazole, 1 to 8 mg/liter for voriconazole, and 0.125 to 2 mg/liter for posaconazole have been measured (9–11). Based on these reported phenotypes, particularly the low-level posaconazole resistance, these mechanisms are unlikely responsible for the high-level pan-azole-resistant phenotype observed in these four patients' isolates.

The M220R mutation (in patient 4) has not been previously reported, but M220K and M220T have been reported to lead to itraconazole MICs of >8 mg/liter but variable MICs for voriconazole (1 to 4 mg/liter) and posaconazole (0.5 to >8 mg/liter), so may equal the phenotype seen in patient 4 (12).

High-level pan-azole resistance has consequences for patient management. Dose escalation of voriconazole, (intravenous) posaconazole, and azole-echinocandin combination therapy may be effective against low-level azole-resistant *A. fumigatus* (3, 13). In a high-level pan-azole-resistant infection, dose escalation will be inadequate and the efficacy of combination therapy, at best, uncertain. The activity of amphotericin B and the echinocandins *in vitro* remained unaffected. Liposomal amphotericin B has shown good efficacy in a nonneutropenic murine model of acute azole-resistant invasive aspergillosis (14).

Direct detection of resistance mutations in clinical samples is increasingly studied (15, 16). These samples indicated a wild-type *cyp51A* gene or the M220R, TR$_{34}$/L98H, or TR$_{46}$/Y121F/T289A resistance mechanism, none of which corresponds to the high-level pan-azole resistance phenotype observed in these four patients.

We report the emergence of high-level pan-azole-resistant *A. fumigatus* isolates. This phenotype might originate from the environment but also may develop through switching between azole compounds in patients with chronic aspergillosis. There is a clear need to develop strategies in patients at risk for chronic aspergillosis that avoids high-level resistance development.

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