

Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania

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Objectives: Azole resistance in *Aspergillus fumigatus* isolates has been increasingly reported with variable prevalence worldwide and is challenging the effective management of aspergillosis. Here we report the coexistence of both TR₃₄/L98H and TR₄₆/Y121F/T289A resistance mechanisms in azole-resistant *A. fumigatus* (ARAF) isolates originating from Tanzania, Africa.

Methods: A total of 30 soil and woody debris samples from the surroundings of Kilimanjaro Christian Medical Centre, Moshi, Tanzania, were processed for detection of ARAF isolates and were investigated for susceptibility to itraconazole, voriconazole, posaconazole and isavuconazole. All ARAF isolates were subjected to a real-time PCR assay for detection of mutations and were genotyped by microsatellite typing.

Results: Of the 30 samples, 29 yielded 108 *A. fumigatus* isolates. Overall, 15 ARAF isolates were obtained, which included 4 ARAF harbouring the TR₄₆/Y121F/T289A mutation and 11 isolates carrying TR₃₄/L98H. All four TR₄₆/Y121F/T289A *A. fumigatus* isolates showed high MICs of voriconazole (>16 mg/L) and isavuconazole (8 mg/L). In contrast, the 11 TR₃₄/L98H *A. fumigatus* isolates were pan-azole resistant. The isolates were cross-resistant to azole fungicides. Notably, 20% of environmental samples harboured ARAF and the TR₄₆/Y121F/T289A resistance mechanism was found in 5.5% of the soil samples, where it coexisted with TR₃₄/L98H. The Tanzanian TR₄₆/Y121F/T289A strains had a genotype identical to Dutch clinical TR₄₆/Y121F/T289A isolates.

Conclusions: The present study reports the isolation of resistant *A. fumigatus* strains harbouring the TR₄₆/Y121F/T289A mutation from Africa. Recovery of TR₄₆/Y121F/T289A from the environment is worrisome and we must strive for effective surveillance of clinical and environmental sources to detect azole resistance in *A. fumigatus*.

Keywords: azole-resistant *A. fumigatus*, microsatellite typing, resistance mechanisms, Africa

Introduction

Aspergillus fumigatus, a ubiquitous opportunistic pathogen, is the global leading cause of aspergillosis. The triazole antifungals voriconazole, itraconazole and posaconazole are recommended for prophylaxis and treatment of both invasive and chronic aspergillosis. The emergence of azole resistance in patients with aspergillosis results in increased treatment failures.^{1,2} Recently, aspergillosis patients who acquired azole-resistant *A. fumigatus* (ARAF) strains from external environments have been reported at higher frequencies in azole-naïve patients from several European and Asian countries.^{3–5} This environmental mode of resistance development is commonly due to the TR₃₄/L98H mutation in the *cyp51A* gene of *A. fumigatus*. This resistance

mechanism is responsible for most of the multi-azole resistance reported both in patients and from their environment in many European countries, China, Japan, the Middle East and India.^{2–12} The cross-resistance of TR₃₄/L98H isolates to azole fungicides suggested that clinical isolates may have acquired resistance through exposure to azole fungicides in the environment.^{11,13,14} In addition, a new resistance mechanism has been described in *A. fumigatus*, consisting of a 46 bp tandem repeat in the promoter region along with substitutions of tyrosine to phenylalanine at codon 121 and threonine to alanine at codon 289 (TR₄₆/Y121F/T289A) of the *cyp51A* gene. These mutations, conferring high voriconazole and variable itraconazole MICs in *A. fumigatus* strains, have been reported in invasive aspergillosis patients and from their environment in Belgium, the Netherlands, India and, more

recently, Germany.^{15–18} Considering that azole antifungals are the mainstay of chronic and invasive aspergillosis therapy, the emergence of resistance, especially in resource-limited countries such as sub-Saharan African countries, will have profound impacts on morbidity and mortality with a subsequent increase in health costs. We report the coexistence of both TR₃₄/L98H and TR₄₆/Y121F/T289A resistance mechanisms in ARAF isolates originating from the surroundings of a large teaching hospital in Moshi, Tanzania, Africa.

Materials and methods

Processing of environmental samples

A total of 30 soil samples and woody debris in the neighbourhood of a tertiary care referral hospital, Kilimanjaro Christian Medical Centre (KCMC) Moshi, Tanzania, were collected from the hospital parking areas ($n=6$), gardens ($n=6$), tree trunk hollows ($n=9$) and wood samples from trees ($n=3$) along Sekon Toure Way and at the KCMC nursing school surroundings ($n=6$). The specimens were processed as described previously.¹¹ Briefly, ~2 g of soil sample was suspended in 8 mL of 0.85% NaCl, vortexed and allowed to settle. The suspension was then diluted 1:10 and 100 μ L of this dilution was plated in triplicate on Sabouraud dextrose agar (SDA) plates supplemented with 50 mg/L chloramphenicol and incubated at 37°C for 48 h. One gram of wood sample was suspended in 10 mL of 0.85% NaCl, vortexed and allowed to settle; 100 μ L of this suspension was then plated in triplicate on SDA plates and incubated at 37°C for 48 h. *Aspergillus fumigatus* isolates growing on SDA plates were identified by macro- and microscopic characteristics and growth at 50°C. All the *A. fumigatus* isolates were screened for resistance on two SDA plates one of which was supplemented with 4 mg/L itraconazole and the other with 1 mg/L voriconazole. The resistant isolates were confirmed as *A. fumigatus* by sequencing of the β -tubulin and calmodulin genes.

Antifungal susceptibility testing (AFST)

All of the resistant *A. fumigatus* were subjected to AFST against four medical triazoles, namely itraconazole (Lee Pharma, Hyderabad, India), voriconazole (Pfizer, Groton, CT, USA), posaconazole (Merck, Whitehouse Station, NJ, USA) and isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), three echinocandins and amphotericin B (Sigma, St Louis, MO, USA) using CLSI M38-A2 broth microdilution.¹¹ Additionally, 10 azole fungicides were tested for their activity against all the ARAF isolates.

The final concentrations of the drugs were 0.03–16 mg/L for itraconazole and voriconazole and 0.015–8 mg/L for posaconazole and isavuconazole. The azole fungicides tested were bromuconazole, cyproconazole, difenoconazole, epoxiconazole, penconazole, tebuconazole, triadimefon, metconazole (Sigma), hexaconazole (Rallis India, Mumbai, India) and tricyclazole (Cheminova India, Mumbai, India). The fungicides were dissolved in DMSO and the concentration range used for testing was 0.06–32 mg/L.

Mutation and microsatellite genotypic analysis

All of the resistant *A. fumigatus* strains were subjected to mixed-format real-time PCR assay as described previously for detection of mutations responsible for triazole resistance.¹¹ Genotyping of ARAF and wild-type isolates was performed with a panel of nine short tandem repeats (STRs) as described previously.¹¹ For phylogenetic analysis, the Tanzanian *A. fumigatus* isolates, ARAF isolates from the Netherlands (TR₄₆/Y121F/T289A, 14 clinical and 3 environmental; and TR₃₄/L98H, 2 clinical and 5 environmental), India (TR₄₆/Y121F/T289A, 6 environmental; and TR₃₄/L98H, 1 clinical and 3 environmental) and a wild-type reference strain (Af293) were included.

Results

Of the 30 samples, 29 yielded 108 *A. fumigatus* isolates on SDA plates. Out of 108 *A. fumigatus* isolates tested, 15 (13.9%) were found to be itraconazole and/or voriconazole resistant. Of these, 4 grew on voriconazole plates and 11 on both voriconazole and itraconazole plates. The MIC data on 15 ARAF isolates are given in Table 1. All four *A. fumigatus* isolates that grew on voriconazole plates showed high MICs of voriconazole (>16 mg/L) and isavuconazole (8 mg/L) with reduced susceptibility to itraconazole (range 1–2 mg/L) and posaconazole (range 0.25–0.5 mg/L). In contrast, the 11 *A. fumigatus* isolates revealing growth on both voriconazole and itraconazole plates were pan-azole resistant and exhibited high geometric mean (GM) MICs of itraconazole (GM 16 mg/L), voriconazole (GM 3.6 mg/L), isavuconazole (GM 8 mg/L) and posaconazole (GM 1.1 mg/L). All isolates were also cross-resistant to 10 azole fungicides tested (MICs >32 mg/L). *Aspergillus fumigatus* isolates that grew on voriconazole plates ($n=4$) had the TR₄₆/Y121F/T289A mutation whereas 11 isolates that grew on both itraconazole and voriconazole plates harboured the TR₃₄/L98H mutation detected by mixed format real-time PCR

Table 1. *In vitro* antifungal susceptibility profile of environmental *A. fumigatus* isolates from Tanzania against medical triazoles and other antifungals

Isolates	MIC/MEC (mg/L)							
	ITC	VRC	ISA	POS	AMB	CFG	MFG	AFG
TR ₃₄ /L98H ($n=11$)								
MIC ₅₀	16	4	8	1	0.25	0.125	0.015	0.015
MIC ₉₀	16	4	8	2	0.5	0.125	0.03	0.03
GM MIC	16	3.52	8	1.13	0.25	0.10	0.01	0.01
range	16 to >16	2–8	8 to >8	1–2	0.125–0.5	0.06–0.125	0.015–0.03	0.015–0.03
TR ₄₆ /Y121F/T289A ($n=4$)								
GM MIC	1.68	16	8	0.42	0.29	0.05	0.01	0.01
range	1–2	16 to >16	8 to >8	0.25–0.5	0.25–0.5	0.06–0.125	0.015–0.03	0.015–0.03

MEC, minimum effective concentration (of CFG, MFG and AFG); MIC₅₀, MIC at which 50% of the test isolates are inhibited; MIC₉₀, MIC at which 90% of the test isolates are inhibited; ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; AMB, amphotericin B; CFG, caspofungin; MFG, micafungin; AFG, anidulafungin; GM, geometric mean.

assay. The Tanzanian TR₄₆/Y121F/T289A strains had a single genotype identical to 7 of 14 Dutch clinical TR₄₆/Y121F/T289A isolates. In contrast, the Tanzanian TR₃₄/L98H revealed a cluster distinct from all TR₄₆/Y121F/T289A isolates but identical to the Indian TR₃₄/L98H genotype (Figure 1).

Notably, overall 20% (6/30) of environmental samples (18 soil samples and 12 woody debris samples) from Tanzania harboured ARAF. The TR₄₆/Y121F/T289A resistance mechanism was found in

5.5% (1/18) of the soil samples where it coexisted with TR₃₄/L98H. On the other hand, a much higher prevalence (20%) was observed for the TR₃₄/L98H mutation, which was found in 2 of 18 soil samples and 4 of 12 woody debris samples. The TR₄₆/Y121F/T289A *A. fumigatus* isolates originated from surrounding soil of the hospital parking areas and the remaining isolates harbouring the TR₃₄/L98H mutation were recovered from surroundings of KCMC parking soil (n=3), tree trunk hollows (n=7) and garden soil (n=1).

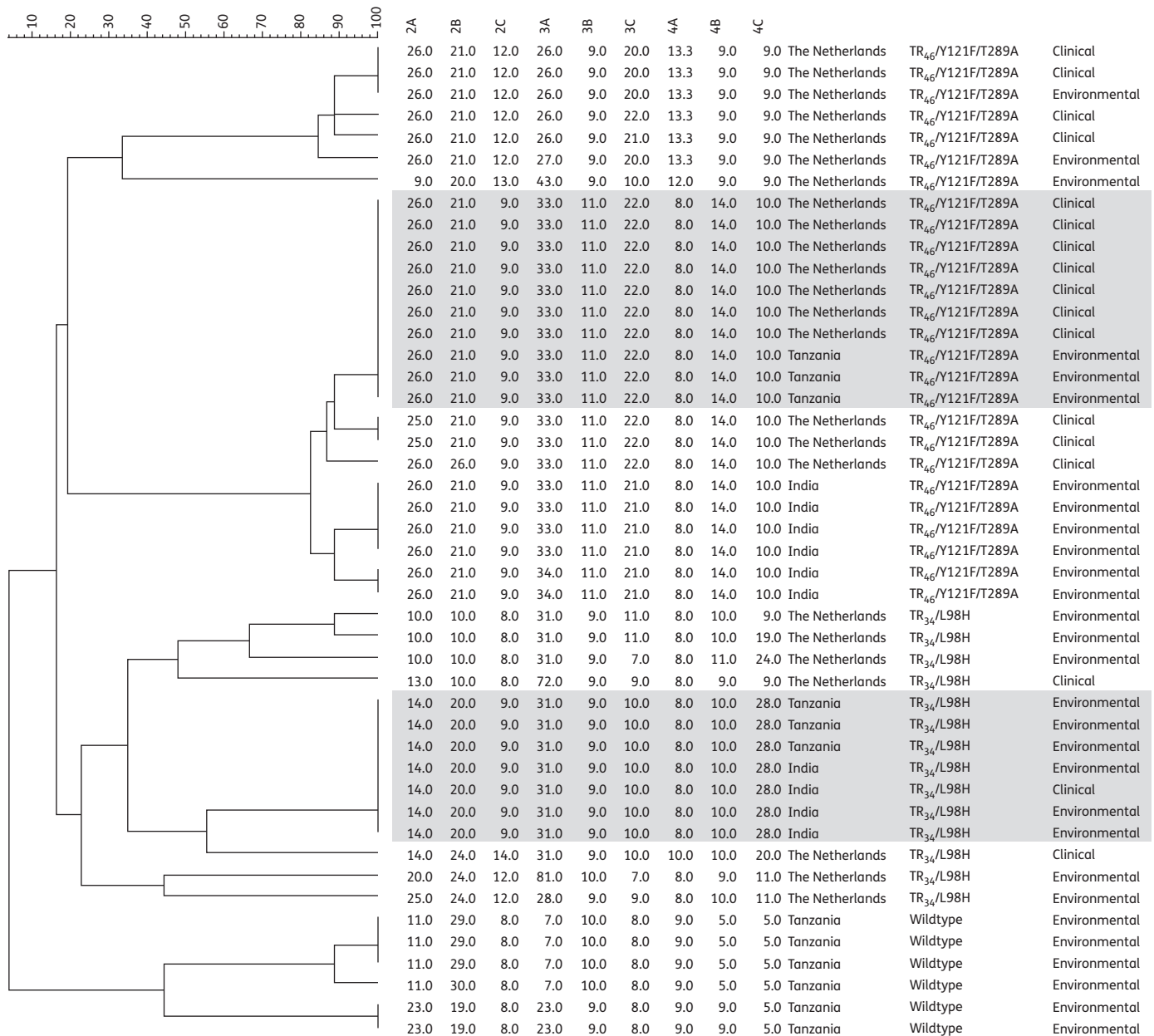


Figure 1. Genotypic relationship of *A. fumigatus* isolates from environmental samples collected from the surroundings of a Tanzanian hospital (including TR₄₆/Y121F/T289A, n=3; TR₃₄/L98H, n=3; and wild-type, n=6) with resistant isolates from the Netherlands (TR₄₆/Y121F/T289A, 14 clinical and 3 environmental; and TR₃₄/L98H, 2 clinical and 5 environmental), India (TR₄₆/Y121F/T289A, 6 environmental; and TR₃₄/L98H, 1 clinical and 3 environmental) and a wild-type reference strain, Af293. The dendrogram is based on a categorical analysis of nine microsatellite markers in combination with UPGMA clustering. The scale bar indicates the percentage identity. Identical genotypes of TR₄₆/Y121F/T289A and TR₃₄/L98H isolates from Tanzania, the Netherlands and India are shaded in grey.

Discussion

We report the detection of environmental azole resistance mechanisms in *A. fumigatus* isolates from soil samples in Tanzania and their genetic relatedness to resistant isolates from other parts of the world. Environmental azole resistance has been reported in various parts of the world, including Europe, the Middle East and Asia, and currently for the first time in the African continent. This finding has important medical implications as early diagnosis and effective treatment of azole-resistant aspergillosis is a challenge, especially in resource-limited areas. Populations at risk in Africa include primarily patients with chronic *Aspergillus* diseases. The high burden of tuberculosis in sub-Saharan Africa and complications such as aspergilloma and chronic pulmonary aspergillosis in these patients has recently been highlighted.^{19,20} As itraconazole, voriconazole and posaconazole are the recommended first-line drugs in the treatment and prophylaxis of aspergillosis, the high burden of environmental isolates harbouring azole resistance in such settings poses a therapeutic challenge.^{1,2,15} Furthermore, alternative treatment options, such as lipid formulations of amphotericin B, are costly and require appropriate medical infrastructure to allow intravenous administration.

Previously, ARAF strains have been isolated from various environmental niches, including flowerbeds, compost, leaves, plant seeds, soil samples of tea gardens, paddy fields and hospital surroundings, and aerial samples in hospitals from the Netherlands, Denmark, India, Iran and Kuwait.^{11,13,14,21} The outskirts of Moshi, with a tropical wet and dry climate, are known for extensive farming of maize, beans and sugar cane. In this study soil harboured ARAF isolates with both TR₃₄/L98H and TR₄₆/Y121F/T289A mutations. Little information pertaining to the usage of azole fungicides in this area is available, but fungicides might have played a role in providing an environment that enables azole-resistant isolates to persist.

The African environmental TR₄₆/Y121F/T289A *A. fumigatus* isolates were genotypically similar to Dutch isolates harbouring this resistance mechanism, as detected by STR typing. This typing assay has been reported to be robust, exhibits high discriminatory power to delineate unique genotypes and is recommended for typing of *A. fumigatus* isolates.²² However, future studies undertaking whole-genome sequencing may be confirmatory. Previously, Indian ARAF isolates carrying the TR₃₄/L98H mutation have been reported to be a cross between an azole-resistant strain from outside India and a native azole-susceptible strain.¹¹ The molecular epidemiology suggests that isolates harbouring these resistance traits migrate rather than develop *de novo* in different areas of the world. The other perspective could be that these resistance mechanisms may be a natural phenomenon occurring in the absence of fungicides and are further selected in the presence of the azole fungicides. Furthermore, the migration of resistance is facilitated by the capability of *A. fumigatus* to sporulate abundantly and survive in almost any environment. Identifying sources of resistance selection in the environment should be prioritized as elimination of these sources might enable us to control resistance and maintain the use of azoles for crop protection and for patient management.

Our study implies that azole resistance is becoming a global problem and a primary goal is to understand the route of resistance selection and migration in order to enable effective control

and prevention. This would require a multidisciplinary approach to understand the migration of *A. fumigatus*, identify applications and practices that are associated with a high risk of resistance selection, and develop alternative approaches to control fungal disease in food production and material preservation.

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Transparency declarations

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