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BRIEF REPORT

Intercountry Transfer of Triazole-Resistant *Aspergillus fumigatus* on Plant Bulbs

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We investigated whether plants imported to Ireland from the Netherlands might harbor triazole-resistant *Aspergillus fumigatus*. Samples of plant bulbs were positive for triazole-resistant *A. fumigatus* with *CYP51A* mutations. We hypothesize that this represents a route for intercountry transfer of an emerging resistance mechanism in a major opportunistic mold pathogen.

Keywords. *Aspergillus fumigatus*; triazole resistance; environment; intercountry transfer.

Triazole antifungal drugs are agents of first choice to treat infections due to *Aspergillus fumigatus* [1]. However, their efficacy is threatened by the emergence of triazole resistance in *A. fumigatus*, which was first reported in the Netherlands and is thought most likely to have arisen in the environment from intensive use of triazole fungicides in agriculture [2–4]. Two dominant mechanisms have emerged, TR_{34}/L98H and TR_{46}/Y121F/T289A, both of which are recognized mutations in the target *CYP51A* gene. These mutations may be associated with itraconazole, voriconazole, posaconazole, and isavuconazole resistance for the TR_{34}/L98H mutation, and itraconazole (variable), voriconazole, posaconazole (variable), and isavuconazole resistance for the TR_{46}/Y121F/T289A mutation [5]. They have been reported among both clinical and environmental *A. fumigatus* isolates across 6 continents [4, 6] and typically infect triazole-naive immunocompromised patients, who most likely become infected through airborne acquisition from soil reservoirs [7]. The recovery of triazole-resistant isolates from flower fields suggests a link to floriculture [3] that includes the practice of dipping plant bulbs in fungicides (see http://ecdc.europa.eu/en/publications/Publications/risk-assessment-impact-environmental-usage-of-triazoles-on-Aspergillus-spp-resistance-to-medical-triazoles.pdf).

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Because the first reports of triazole-resistant *A. fumigatus* came from the Netherlands [4], which exports plant bulbs internationally, we were prompted to investigate whether plant products imported to Ireland from the Netherlands might be a harbinger of triazole-resistant isolates as plants are regularly present in the grounds of hospital campuses and in patients' dwellings. This coincided with documentation of our first cases of invasive aspergillosis due to triazole-resistant *A. fumigatus* in 2 allogeneic stem cell transplant recipients (isolate numbers D4 and D5, Table 1). Triazole-resistant isolates had previously, and have subsequently, been detected from air (isolate numbers D2 and D3, Table 1) and soil samples (isolate number D6, Table 1) on our hospital campus as part of an ongoing surveillance program. This raised the possibility of patient exposure to local healthcare environmental triazole-resistant isolates.

We also investigated whether the plant bulb, clinical, and environmental isolates from our center could be genetically linked to isolates from a Dutch database of triazole-resistant *A. fumigatus*.

MATERIALS AND METHODS

Plant Bulb Sampling

Sealed packages of tulip and narcissus bulbs labeled as originating from the Netherlands were purchased from a local garden center in Dublin, Ireland. The surface of each bulb was swabbed and plated onto Sabouraud dextrose agar supplemented with 50 mg/L chloramphenicol (SAB) (E&O Laboratories, United Kingdom), washed and 100 µL plated onto SAB agar, and finally dissected and a piece of the bulb placed onto SAB agar. All plates were incubated at 48°C for 48–72 hours.

Air Sampling

Air sampling was performed using a dual-head surface air sampler (VWR International, Milan, Italy). Air was sampled (1000 L/sample) twice monthly from both clinical (n = 7) and nonclinical areas (n = 1) within the hospital and outside areas (n = 5) on the hospital campus.

Soil Sampling

Soil samples were taken at 5 sites around our hospital. Each sample was taken from a 10-cm depth. Additionally, samples were taken from commercial compost. Two grams of soil or compost was suspended in 8 mL of 0.85% sodium chloride, vortexed, and allowed to settle for 30 seconds. A volume of 100 µL was plated in duplicate onto SAB agar and incubated at 48°C for 48–72 hours.

Aspergillus fumigatus Identification

All *A. fumigatus* isolates were identified by macroscopic and microscopic morphology. Identification was further confirmed using polymerase chain reaction (PCR) and sequencing of the

Table 1. Sources of Triazole-Resistant *Aspergillus fumigatus* and Associated *CYP51A* Resistance Mechanisms

Sample No.[a]	Date of Sample	Type of Sample	Origin	No. of Triazole-Resistant/Total *A. fumigatus* Colonies/Plant Bulb Pack	MIC, mg/L[b] Itraconazole (ECV = 1) [9]	Voriconazole (ECV = 1) [9]	Posaconazole (ECV = 0.5) [9]	Resistance Mechanism
D1	Jan 2014	Commercial compost (2 g)	Local garden center, Dublin, Ireland	…	0.5	>8	0.25	TR_{46}/Y121F/T289A
D2	Jan 2014	Air (1 m^3)	Outside hospital building, Dublin, Ireland	…	2	2	0.5	TR_{34}/L98H
D3	Feb 2014	Air (1 m^3)	Outside hospital building, Dublin, Ireland	…	2	2	0.25	TR_{34}/L98H
D4	May 2015	Sputum	Allogeneic stem cell transplant patient 1, Dublin, Ireland	…	16	2	0.5	TR_{34}/L98H
D5	July 2015	Sputum	Allogeneic stem cell transplant patient 2, Dublin, Ireland	…	2	4	1	TR_{34}/L98H
P1	Jan 2016	Double mixed tulip bulbs (30[c])	Lisse, the Netherlands	1/5	0.5	>8	0.5	TR_{46}/Y121F/T289A
P2, P3	Jan 2016	Bastogne tulip bulbs (6[a])	Lisse, the Netherlands	2/3	1	4	1	TR_{46}/Y121F/T289A
					8	>8	0.5	TR_{34}/L98H
P4	Jan 2016	Triumph tulip bulbs (6[c])	Breezand, the Netherlands	1/4	8	4	0.5	TR_{34}/L98H
P5, P6	Jan 2016	Narcissus bulbs (8[c])	Breezand, the Netherlands	2/5	0.5	>8	1	TR_{46}/Y121F/T289A
					8	4	0.5	TR_{34}/L98H
P7	Jan 2016	Tall Triumph mixed tulip bulbs (10[c])	The Netherlands (region not specified)	1/2	1	>8	1	TR_{46}/Y121F/T289A
D6	Feb 2016	Soil (2 g)	Hospital campus, Dublin, Ireland	…	8	4	0.5	TR_{34}/L98H

Abbreviations: ECV, epidemiological cutoff value; MIC, minimum inhibitory concentration.

[a]D1, D6 = Irish compost/soil samples; D2, D3 = Irish air samples; D4, D5 = Irish clinical samples; P1–P7 = plant bulbs originating from the Netherlands.

[b]MIC testing performed using the commercial broth microdilution Sensititre plates and YeastOne broth (TREK diagnostics, Serosep, Ireland).

[c]Number of plant bulbs tested.

internal transcribed spacer (ITS), β-tubulin and calmodulin regions [8]. Amplicons were sequenced by Sourcebioscience (Tramore, Ireland). Sequence results were searched using the Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and confirmed as *A. fumigatus*.

Triazole Resistance Screening

Triazole resistance screening was performed by collecting conidia and suspending in sterile water. Using a densitometer, the turbidity was adjusted to 0.5–1 McFarland standard. A volume of 25 µL of the solution was transferred to each well of an in-house 4-well azole resistance screening plate. The wells contained RPMI agar with 3-(N-morpholino)propanesulfonic acid (MOPS) and 2% glucose alone and either (1) no antifungal (control well) or (2) 4 mg/L itraconazole, or (3) 2 mg/L voriconazole, or (4) 0.5 mg/L posaconazole. Plates were incubated at 37°C for 48 hours. Growth in any of the triazole containing wells indicated resistance to that triazole. Any isolate that grew in 1 or more of the triazole-containing wells was tested for the minimum inhibitory concentration (MIC) of each triazole using the commercial broth microdilution Sensititre plates and YeastOne broth (TREK diagnostics, Serosep, Ireland), which are designed to produce comparable results to the Clinical and Laboratory Standards Institute (CLSI) method (Table 1). Considering there are no CLSI breakpoints for the triazoles and *A. fumigatus*, epidemiological cutoff values (ECVs) were used to distinguish wild-type from non-wild-type isolates. These ECVs define non-wild-type isolates as showing MICs of >1 mg/L for itraconazole and voriconazole and >0.5 mg/L for posaconazole [9].

Resistance Mechanism Screening

PCR of the promoter region and a portion of the coding region of the *CYP51A* gene was performed as previously described [7]. Amplicons were sequenced by Sourcebioscience. Sequence results were compared to the wild-type *A. fumigatus CYP51A* sequence (GenBank accession number AF338659) using BLAST.

STR*Af* Genotyping

Microsatellite genotyping of all *A. fumigatus* isolates was performed as previously described [7].

RESULTS

Samples of bulbs from 5 of 6 bulb packages were culture positive for triazole-resistant *A. fumigatus* with typical *CYP51A*

mutations associated with an environmental source (isolate numbers P1–P7, Table 1).

STR*Af* genotyping of the triazole-resistant plant bulb isolates and the clinical and local environmental isolates showed genotypes that were distinct from each other and were also distinct from a representative database of triazole-resistant isolates from the Netherlands (Supplementary Figure 1).

DISCUSSION

Researchers in the Netherlands first drew attention to the link between triazole resistance in clinical cases of invasive aspergillosis and environmental fungicide use [4], and have reported rates of triazole resistance in clinical isolates of *A. fumigatus*, as high as 30%, depending on the denominator used [10], as well as finding resistant isolates in patients' homes [7]. A recent study reported recovery of genetically identical TR_{46}/Y121F/T289A isolates from a deceased patient and from environmental in-house cultures 4 months after his death [7]. This suggests that resistant isolates are able to persist for many months in the environment. This is particularly concerning as triazoles continue to be the preferred agents for treatment and prevention of invasive aspergillosis [1]. Isolates with these mutations and itraconazole resistance may, as we found, be cross-resistant to voriconazole and/or posaconazole. Our resistant isolates showed posaconazole MICs in some cases at or below the ECV (Table 1); however, these MIC values (range, 0.25–0.5 mg/L) do not necessarily predict clinical efficacy, as plasma posaconazole levels after oral administration for prophylaxis of invasive aspergillosis may not protect against breakthrough infection where MICs are close to the ECV.

We have demonstrated the presence of triazole-resistant *A. fumigatus* on Dutch plant bulbs imported into Ireland, a finding that identifies another way in which susceptible patients may have environmental exposure. Although triazole-resistant *A. fumigatus* has been reported in soil samples from flower fields [11], compost, potted plants, and even soils of ornamental plants [11, 12], it has not been reported from plant bulbs themselves. In 2014, the Netherlands exported 2.48 billion tulip bulbs worldwide [13], creating the opportunity for accelerated distribution and establishment of this triazole-resistant pathogen in soil habitats in recipient countries. The lack of similarity between our clinical and environmental isolates and the plant bulb and Dutch database isolates may be explained by the genetic diversity that has been found among European *A. fumigatus* isolates to date [8]. We suggest that decorative bulbs should not be planted in or near healthcare facilities or in immunocompromised patients' dwellings until their potential role in the spread of triazole-resistant *A. fumigatus* has been clarified.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. This study was designed by T. R. R., J. F. M., and K. D. Samples were collected by K. D. and N. P. Experimental work was done by K. D., N. P., and F. H. Data were analyzed and interpreted by T. R. R., K. D., N. P., F. H., and J. F. M. The manuscript was drafted by T. R. R. and K. D. and was reviewed by all authors.

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References

1. Patterson TF, Thompson GR 3rd, Denning DW, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis **2016**; 63:e1–e60.
2. Chowdhary A, Kathuria S, Xu J, Meis JF. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. PLoS Pathog **2013**; 9:e1003633.
3. Le Pape P, Lavergne RA, Morio F, Alvarez-Moreno C. Multiple fungicide-driven alterations in azole-resistant *Aspergillus fumigatus*, Colombia, 2015. Emerg Infect Dis **2016**; 22:156–7.
4. Verweij PE, Chowdhary A, Melchers WJ, Meis JF. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? Clin Infect Dis **2016**; 62:362–8.
5. Meis JF, Chowdhary A, Rhodes JL, Fisher MC, Verweij PE. Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus*. Philos Trans R Soc Lond B Biol Sci **2016**; 371. doi:10.1098/rstb.2015.0460.
6. Wiederhold NP, Gil VG, Gutierrez F, et al. First detection of TR34 L98H and TR46 Y121F T289A *CYP*51 mutations in *Aspergillus fumigatus* isolates in the United States. J Clin Microbiol **2016**; 54:168–71.
7. Lavergne RA, Chouaki T, Hagen F, et al. Home environment as a source of life-threatening azole-resistant *Aspergillus fumigatus* in immunocompromised patients. Clin Infect Dis **2017**; 64:76–8.
8. Abdolrasouli A, Rhodes J, Beale MA, et al. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. MBio **2015**; 6:e00536.
9. Espinel-Ingroff A, Diekema DJ, Fothergill A, et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). J Clin Microbiol **2010**; 48:3251–7.
10. Verweij PE, Lestrade PP, Melchers WJ, Meis JF. Azole resistance surveillance in *Aspergillus fumigatus*: beneficial or biased? J Antimicrob Chemother **2016**; 71:2079–82.
11. Prigitano A, Venier V, Cogliati M, De Lorenzis G, Esposto MC, Tortorano AM. Azole-resistant *Aspergillus fumigatus* in the environment of northern Italy, May 2011 to June 2012. Euro Surveill **2014**; 19:20747.
12. Haas D, Lesch S, Buzina W, et al. Culturable fungi in potting soils and compost. Med Mycol **2016**; 54:825–34.
13. Centraal Bureau voor de Statistiek. Goederensoorten naar land; natuur, voeding en tabak. Available at: http://statline.cbs.nl/Statweb/publication/?DM=SLNL&PA=81267NED&D1=a&D2=1323-1371&D3=0-6&D4=l&HDR=T&STB=G2,G1,G3&VW=T. Accessed 4 August 2016.