MAJOR ARTICLE

Aspergillosis due to Voriconazole Highly Resistant *Aspergillus fumigatus* and Recovery of Genetically Related Resistant Isolates From Domiciles

Jan W. M. van der Linden,[1,2,a] Simone M. T. Camps,[1,2,a] Greetje A. Kampinga,[3] Jan P. A. Arends,[3] Yvette J. Debets-Ossenkopp,[4] Pieter J. A. Haas,[5] Bart J. A. Rijnders,[6] Ed J. Kuijper,[7] Frank H. van Tiel,[8] János Varga,[9] Anna Karawajczyk,[10] J. Zoll,[1,2] Willem J. G. Melchers,[1,2] and Paul E. Verweij[1,2]

[1]Department of Medical Microbiology, and [2]Nijmegen Institute for Infection, Inflammation and Immunity (N4i), Radboud University Nijmegen Medical Centre, [3]Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, [4]Department of Medical Microbiology, VU–University Medical Centre, Amsterdam, [5]Department of Medical Microbiology, University Medical Centre Utrecht, [6]Department of Internal Medicine, Division of Infectious Diseases, Erasmus Medical Centre, Rotterdam, [7]Department of Medical Microbiology, Leiden University Medical Centre, and [8]Department of Medical Microbiology, University Medical Centre Maastricht, The Netherlands; [9]Department of Microbiology, University of Szeged, Hungary; and [10]LeadParma Medicine B.V., Nijmegen, The Netherlands

(See the Editorial Commentary by Denning and Bowyer on pages 521–3.)

Background. Azole resistance is an emerging problem in *Aspergillus fumigatus* and complicates the management of patients with *Aspergillus*-related diseases. Selection of azole resistance may occur through exposure to azole fungicides in the environment. In the Netherlands a surveillance network was used to investigate the epidemiology of resistance selection in *A. fumigatus*.

Methods. Clinical *A. fumigatus* isolates were screened for azole resistance in 8 university hospitals using azole agar dilution plates. Patient information was collected using an online questionnaire and azole-resistant *A. fumigatus* isolates were analyzed using gene sequencing, susceptibility testing, and genotyping. Air sampling was performed to investigate the presence of resistant isolates in hospitals and domiciles.

Results. Between December 2009 and January 2011, 1315 *A. fumigatus* isolates from 921 patients were screened. A new *cyp51A*-mediated resistance mechanism (TR_{46}/Y121F/T289A) was observed in 21 azole-resistant isolates from 15 patients in 6 hospitals. TR_{46}/Y121F/T289A isolates were highly resistant to voriconazole (minimum inhibitory concentration ≥16 mg/L). Eight patients presented with invasive aspergillosis due to TR_{46}/Y121F/T289A, and treatment failed in all 5 patients receiving primary therapy with voriconazole. TR_{46}/Y121F/T289A *Aspergillus fumigatus* was recovered from 6 of 10 sampled environmental sites.

Conclusions. We describe the emergence and geographical migration of a voriconazole highly resistant *A. fumigatus* that was associated with voriconazole treatment failure in patients with invasive aspergillosis. Recovery of TR_{46}/Y121F/T289A from the environment suggests an environmental route of resistance selection. Exposure of *A. fumigatus* to azole fungicides may facilitate the emergence of new resistance mechanisms over time, thereby compromising the use of azoles in the management of *Aspergillus*-related diseases.

Keywords. *Aspergillus fumigatus*; azole resistance; voriconazole; prevalence; invasive aspergillosis.

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[a]J. W. M. v. d. L. and S. M. T. C. contributed equally to this work.

Correspondence: Paul E. Verweij, MD, Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands (p.verweij@mmb.umcn.nl).

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The medical triazoles itraconazole, voriconazole, and posaconazole are the primary antifungal agents currently used in the management of infections caused by the saprophytic mold *Aspergillus fumigatus*. These triazoles are clinically licensed for the prevention and treatment of both noninvasive *Aspergillus* diseases as well as invasive aspergillosis [1]. Acquired resistance in *A. fumigatus* has long been perceived as a manageable problem as resistance development during azole therapy has been reported with only a very low frequency [2–4]. However, culture may underestimate the presence of resistance [5], and *A. fumigatus* isolates that harbor a resistance mechanism are commonly resistant to multiple triazoles [6–9].

In the Netherlands. a second route of resistance development was suggested in which clinical *A. fumigatus* isolates may have become resistant through environmental exposure to 14α-demethylase inhibitors (DMIs) [6, 10–12]. DMIs inhibit fungal Cyp51A activity and are abundantly used for crop protection and material preservation. Five DMIs, from the triazole class, showed in vitro activity against *A. fumigatus* and were shown to have a molecule structure that was highly similar to that of the clinically licensed triazoles [11]. The environmental mode of resistance development is of major importance as >90% of Dutch clinical azole-resistant isolates are believed to have originated through this mode of resistance development [6, 7, 12]. The first resistance mechanism that is believed to be of environmental origin consists of a substitution at codon 98 in the *cyp51A* gene in combination with a 34 base-pair tandem repeat in the gene promoter (TR_{34}/L98H).

TR_{34}/L98H first emerged in clinical *A. fumigatus* isolates from Dutch patients in 1998 and a national surveillance study indicated that this resistance mechanism is now endemic in Dutch hospitals. *Aspergillus*-related diseases due to TR_{34}/L98H included noninvasive infections and invasive aspergillosis, and infections were found to occur both in azole-treated as well as in azole-naive patients [6, 7]. TR_{34}/L98H is increasingly reported in other European countries, and more recently also in China and India [6, 13–23]. Molecular typing studies indicate that the fungicide-driven route of resistance development carries the risk of geographical migration of this resistance trait, similar to azole-resistant phytopathogenic fungi [24].

In our current study, we investigated the emergence of a new azole resistance mechanism in *A. fumigatus*. We describe the epidemiology and clinical implications, and performed environmental sampling to determine if the new resistance mechanism was present in our environment.

METHODS

Surveillance Network

Between May 2009 and January 2011, all *Aspergillus* isolates cultured from clinical samples that were processed in medical microbiology laboratories of the 8 university medical centers in the Netherlands were routinely screened for the presence of azole resistance, irrespective of the clinical relevance of the culture result. An online questionnaire was completed in 7 of 8 centers for every collected isolate. The questionnaire included questions about isolate characteristics (species identification and date of isolation) and patient characteristics (age, sex, and underlying disease). In one center the questionnaire was only completed for patients from whom a resistant isolate was recovered.

Screening: 4-Well Azole-Agar Dilution Plates

Aspergillus colonies that grew in primary cultures were subcultured on a specially developed 4-well azole-agar dilution (4D) plate [25]. All wells contained Roswell Park Memorial Institute 1640 agar, and 3 wells were each supplemented with 1 of the azoles: itraconazole (4 mg/L), voriconazole (1 mg/L), or posaconazole (0.5 mg/L). The fourth well contained no azole and served as growth control. The 4D plates were incubated at 37°C and growth was assessed after 48 hours. For every isolate that was able to grow on any of the azole-containing wells, the primary culture isolate was sent to the Radboud University Nijmegen Medical Centre for further analysis. For those isolates that grew only on the control well, the Web-based questionnaire was completed, but the isolate was considered azole-susceptible and not further analyzed. At the screening sites, the *Aspergillus* isolates were identified to the species level by conventional methods, that is, the ability to grow at 48°C and macro- and microscopic culture morphology.

Analysis of *A. fumigatus* Isolates

All *A. fumigatus* isolates that grew on 1 or more azole-containing agar wells were investigated for their antifungal susceptibility to itraconazole, voriconazole, posaconazole, and the DMI tebuconazole using the Clinical and Laboratory Standards Institute M38-A2 broth microdilution reference method [26]. For resistant isolates that were confirmed to exhibit a non-wild-type phenotype, the full coding sequence of the *cyp51A* gene and promoter region was determined by polymerase chain reaction (PCR) amplification and sequencing to detect any mutations (reference *cyp51A* sequence: GenBank accession number AF338659) [27]. Molecular identification was performed by sequencing the highly conserved β-tubulin and calmodulin gene, as described previously [28].

Microsatellite genotyping was used to investigate genetic distances between the isolates by analysis of 6 microsatellites (STR*Af* 3A, 3B, 3C, 4A, 4B, and 4C), as described previously [6]. If multiple resistant isolates were obtained from 1 patient, only the first isolate was included. For every resistant isolate, 2 control isolates were selected that had been cultured between 1 month before and 1 month after the date of isolation of the resistant isolate. One control isolate harbored the TR_{34}/L98H resistance mechanism, whereas the other exhibited a wild-type phenotype. From the microsatellite data, allele sharing distance matrices were generated and these matrices were used as input for the Neighbor

program of the PHYLIP software package (Phylogeny Inference Package version 3.6, Department of Genome Sciences, University of Washington, Seattle) to produce the dendrogram [29].

Patient Characteristics

The Web-based database was used to retrieve clinical information of the patients with a culture yielding *A. fumigatus*. For patients harboring an azole-resistant isolate, the following additional information was recorded: the presence of *Aspergillus* disease, azole exposure within 12 weeks preceding the culture of the resistant isolate, treatment, and outcome at 12 weeks. Invasive aspergillosis was classified according to the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) consensus definitions [30]. Human experimentation guidelines from the Committee on Research Involving Human Subjects Arnhem–Nijmegen were followed in the conduct of this research.

Environmental Sampling

The presence of *A. fumigatus* resistant to medical triazoles in the environment was investigated through sampling at the Radboud University Nijmegen Medical Centre, the University Medical Centre Groningen, and 8 domiciles (6 in the Nijmegen area and 2 in the Groningen area). Indoor sites and 1 site in the direct outdoor proximity were sampled. Air samples were obtained using a Casella air sampler (Casella Measurement, catalog number E7627/Z-24, serial number 026510/026514, London, UK). Cultures of airborne viable fungi were performed on Sabouraud agar. For selection of azole-resistant fungi, Sabouraud agar supplemented with itraconazole (4 mg/L) or voriconazole (1 mg/L) were used. The volume of air that was sampled was 14 000 L (700 L/minute for 20 minutes) to detect azole-resistant spores. The plates were incubated at 37°C and inspected twice daily for 4 days.

Any colony that grew on the agar supplemented with azoles was subcultured on a Sabouraud agar slant and was identified as *A. fumigatus* using colony morphology and microscopic characteristics. *Aspergillus fumigatus* isolates were screened for the presence of an insertion in the promoter region of the *cyp51A* gene by previously described PCR primers and conditions [24]. Tandem repeats of different sizes could be identified on the basis of the size of the amplified PCR fragment. Isolates containing the TR_{34} were screened for the presence of the L98H substitution by using 2 PCR reactions: an L98- and an L98H-specific PCR (primers described elsewhere) [24]. Azole-resistant isolates without TR_{34}/L98H were selected for sequencing the *cyp51A* gene and promoter region as described above [27].

RESULTS

Emergence of the New Resistance Mechanism

In January 2010, a clinical *A. fumigatus* isolate originating from Nijmegen grew on the well containing voriconazole, and not on those containing itraconazole or posaconazole. In vitro susceptibility testing showed no activity of voriconazole (minimum inhibitory concentration [MIC] >16 mg/L), and attenuated activity of itraconazole (MIC 2 mg/L) and posaconazole (MIC 0.5 mg/L) (Table 1). Sequence analysis of the *cyp51A* gene showed the presence of 2 mutations leading to substitutions Y121F and T289A. In addition, a 46 base-pair tandem repeat was found in the gene promoter (TR_{46}/Y121F/T289A). A second isolate was cultured in January 2010 from a patient in Amsterdam that exhibited a similar voriconazole-resistant phenotype and identical TR_{46}/Y121F/T289A resistance mechanism (Table 1).

The culture collection of the surveillance network was then investigated for isolates with a voriconazole MIC of ≥16 mg/L. Since May 2009, when screening of isolates using the 4D plates had begun, 5 of 33 azole-resistant isolates were identified with a voriconazole MIC of ≥16 mg/L. Sequence-based analyses of the *cyp51A* gene of these isolates identified a third isolate, harboring the TR_{46}/Y121F/T289A resistance mechanism, that had been cultured on 31 December 2009 in Utrecht. This was considered to be the first clinical isolate from our surveillance network to harbor the TR_{46}/Y121F/T289A resistance mechanism.

Prevalence of the TR_{46}/Y121F/T289A Resistance Mechanism

From December 2009 to January 2011, 1315 *A. fumigatus* isolates from 921 patients were screened for resistance in 7 of 8 university centers. In one center, the total number of isolates screened was unknown, as this center did not complete the online questionnaire for susceptible isolates. The overall prevalence of azole resistance was 6.8% (63 of 921 patients). Forty-seven of 63 patients (74.6%) harbored the TR_{34}/L98H resistance mechanism and 13 patients (20.6%) TR_{46}/Y121F/T289A. No *cyp51A* mutations were found in azole-resistant *A. fumigatus* isolates from 3 patients (4.7%). The prevalence of TR_{46}/Y121F/T289A was 1.4% (13 of 921 patients) in this 14-month period. Besides this, 2 isolates with TR_{46}/Y121F/T289A and 2 isolates with TR_{34}/L98H were recovered from the center that had not recorded the total number of screened isolates. Therefore, within 14 months (December 2009 to January 2011), TR_{46}/Y121F/T289A was detected in 21 clinical *A. fumigatus* isolates obtained from 15 patients in 6 different university hospitals in the Netherlands (Table 1, Figure 1).

Characterization of TR_{46}/Y121F/T289A Isolates

The 21 TR_{46}/Y121F/T289A isolates were identified as *A. fumigatus* based on sequence analysis of the β-tubulin and calmodulin genes, and voriconazole showed no in vitro activity against any of the isolates (MIC ≥16 mg/L). The activity of itraconazole and posaconazole was attenuated in all isolates (Table 1). The DMI tebuconazole, which has been shown to exhibit activity

Table 1. Clinical Characteristics of 15 Patients From Whom an *Aspergillus fumigatus* Isolate Was Cultured That Harbored the TR_{46}/Y121F/T289A Resistance Mechanism

Sex/ Age	Month of Isolation/ Site	City	MIC (mg/L)			Underlying Condition	*Aspergillus* Disease [30]	Previous Azole Exposure[a]	Treatment	Outcome at 12 wk
			ITZ	VCZ	POS					
F/11	Dec 2009/sputum	Utrecht	4	>16	0.25	Relapse ALL, HSCT, GVHD	Probable IA	None	VCZ, CAS	Persistent infection
M/70	Jan 2010/ear	Amsterdam	>16	>16	2	Chronic otitis externa, sinusitis, and paralysis of abducens nerve	IA[b]	None	L-AMB, AND	Persistent infection
F/51	Jan 2010/ abdominal abscess	Nijmegen	2	>16	0.5	Kidney transplant	Proven IA	None	VCZ, POS	Died
F/9	Feb 2010/sputum	Amsterdam	4	>16	0.5	Cystic fibrosis	No IA	None	None	Alive
M/69	Feb 2010/sputum	Amsterdam	>16	>16	2	Lung carcinoma, radiation	No IA	None	None	Alive
M/54	Mar 2010/sputum	Groningen	1	>16	0.25	Multiple myeloma, autologous HSCT, relapse	Probable IA	None	VCZ, L-AMB	Died
F/54	Mar 2010/sputum	Groningen	16	>16	0.5	Cystic fibrosis, bilateral lung transplant	Proven IA	VCZ	L-AMB	Alive
F/65	May 2010/biopsy	Amsterdam	4	>16	1	Chronic otitis after cholesteatoma surgery	Proven IA	None	Surgery, L-AMB	Alive
M/76	May 2010/sputum	Amsterdam	>16	>16	1	Lung fibrosis	None	None	None	Alive
M/70	Jun 2010/sputum	Amsterdam	1	>16	0.25	High energetic trauma, ICU admission	None	None	None	Died
M/59	Jul 2010/brain biopsy	Amsterdam	4	>16	1	β-thalassemia and diabetes mellitus	Proven IA	None	VCZ, L-AMB, CAS	Died
F/21	Sep 2010/sputum	Nijmegen	2	>16	0.5	Cystic fibrosis	ABPA	VCZ	None	Alive
F/49	Oct 2010/sputum	Groningen	>16	>16	2	COPD, unilateral lung transplant	None	None	L-AMB, VCZ	Alive
F/64	Nov 2010/sputum	Leiden	>16	>16	2	COPD	No IA	None	None	Alive
F/50	Jan 2011/sputum	Utrecht	>16	>16	1	NH B-cell lymphoma, allo-SCT	Probable IA	VCZ	VCZ	Died

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; ALL, acute lymphoblastic leukemia; AND, anidulafungin; CAS, caspofungin; COPD, chronic obstructive pulmonary disease; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplant; IA, invasive aspergillosis; ICU, intensive care unit; ITZ, itraconazole; L-AmB, liposomal amphotericin B; MIC, minimum inhibitory concentration; NH, non-Hodgkin; POS, posaconazole; SCT, stem cell transplant; VCZ, voriconazole.

[a] Azole exposure within 12 weeks preceding the culture of the azole-resistant isolate.

[b] This patient could not be classified according to the European Organization for Research and Treatment of Cancer and Mycoses Study Group consensus definitions. The patient showed bone destruction of the skull on computed tomography scan and *Aspergillus fumigatus* was recovered repeatedly from the ear, without any other explanation.

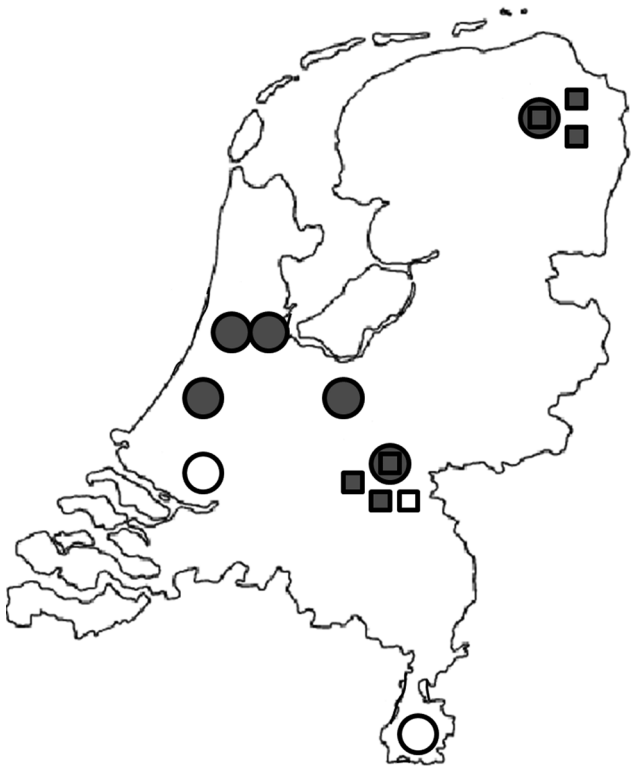

Figure 1. Geographical map of the Netherlands indicating screening sites and recovery of clinical and environmental TR_{46}/Y121F/T289A isolates. Circles indicate hospitals where screening of clinical isolates took place and squares indicate the sites where air sampling took place. Gray indicates the presence of TR_{46}/Y121F/T289A isolates and white the absence of TR_{46}/Y121F/T289A.

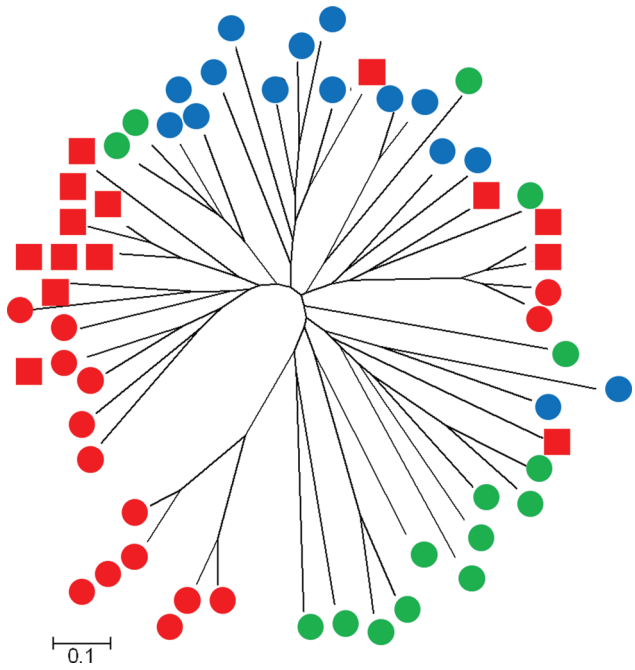

Figure 2. Allele sharing distance matrices of the microsatellite genotypes of the clinical and environmental resistant TR_{46}/Y121F/T289A isolates, compared with TR_{34}/L98H and wild-type controls. Red dots: TR_{46}/Y121F/T289A isolates from clinical origin; red squares: TR_{46}/Y121F/T289A isolates from environmental origin; blue dots: TR_{34}/L98H isolates; green dots: wild-type isolates.

against wild-type *A. fumigatus* isolates [11], showed no in vitro activity against TR_{46}/Y121F/T289A isolates. One isolate with TR_{46}/Y121F/T289A contained 2 additional substitutions, M172I and G448S. Recombinant experiments confirmed the association between the TR_{46}/Y121F/T289A resistance mechanism and the observed phenotype (data not shown).

Microsatellite genotyping showed that clinical TR_{46}/Y121F/T289A isolates clustered together. TR_{46}/Y121F/T289A and TR_{34}/L98H were separated into different clades and apart from wild-type control isolates (Figure 2).

Clinical Characteristics

Among the 15 patients identified with a TR_{46}/Y121F/T289A isolate, 8 were diagnosed with azole-resistant invasive aspergillosis (Table 1). Three of these patients were classified as having probable disease and 4 as proven. One patient could not be classified according to the EORTC/MSG consensus definitions [30]. This patient showed bone destruction of the skull on computed tomography scan and *A. fumigatus* was recovered repeatedly from the ear, without any other explanation. All patients with invasive aspergillosis due to TR_{46}/Y121F/T289A were azole-naive, except 1 patient with probable and 1 patient with proven invasive aspergillosis. At 12 weeks after recovery of the TR_{46}/Y121F/T289A isolate, 4 of 8 patients with invasive aspergillosis had died and 2 patients had a persisting infection. All patients who died had received primary therapy with voriconazole. In 4 patients, primary therapy was initiated with liposomal amphotericin B. In 3 of these patients, invasive aspergillosis was diagnosed, and all patients were alive at 12 weeks (Table 1).

Environmental Sampling

A total of 140 azole-resistant *A. fumigatus* colonies were identified, recovered from 21 locations at 9 different sites (outdoor and indoor). *Aspergillus fumigatus* colonies could not be recovered from 3 samples (entrance of 1 of the hospitals and 2 domiciles) due to abundant growth of zygomycetes. Analysis of the *cyp51A* gene and the promoter region showed that 126 (90%) isolates harbored TR_{34}/L98H, and 14 (10%) harbored the new TR_{46}/Y121F/T289A resistance mechanism. Both resistance mechanisms were found in the Nijmegen and Groningen areas (Figure 1). In 6 of 10 sampled sites, the TR_{46}/Y121F/T289A resistance mechanism was found, and TR_{34}/L98H was recovered from 9 of 10 sites (Table 2). The genotypes of 11 of the 14

Table 2. Recovery of Azole-Resistant *Aspergillus fumigatus* Isolates Through Environmental Air Sampling of 14 000 L per Location

Site	City	Location	No. of Resistant Colonies	TR_{46}/Y121F/T289A	TR_{34}/L98H
1	Nijmegen	Hospital restaurant	3	. . .	3
		Hospital pediatrics department	9	2	7
		Outside hospital	5	. . .	5
2	Schaijk	Living room	2	. . .	2
		Basement	3	. . .	3
		Back yard	7	. . .	7
3	Schaijk	Living room	1	. . .	1
		Conservatory	5	. . .	5
		Back yard	3	. . .	3
4	Overasselt	Kitchen	6	1	5
		Basement	7	. . .	7
		Back yard	7	. . .	7
5	Schaijk	Living room	6	. . .	6
		Basement	3	. . .	3
		Back yard	3	. . .	3
6	Berghem	Living room	. . .	. . .	. . .
7	Schaijk	Hall	13	3	10
		Balcony	6	. . .	6
8	Groningen	Hospital entrance[a]	. . .	. . .	. . .
		Outside hospital	29	2	27
9	Scharmer	Living room	4	1	3
		Back yard	6	3	3
10	Garmerwolde	Living room[a]	. . .	. . .	. . .
		Back yard	12	2	10

[a] For these sites the agar plates, supplemented with azoles, were rapidly overgrown with zygomycetes, thereby precluding the opportunity to select suspected azole-resistant *A. fumigatus* colonies.

environmental TR_{46}/Y121F/T289A isolates grouped together with the clinical TR_{46}/Y121F/T289A isolates, irrespective of the geographic site of recovery, whereas susceptible control isolates and TR_{34}/L98H isolates generally clustered in different clades (Figure 2).

DISCUSSION

We describe the emergence and migration of a new azole-resistance mechanism in *A. fumigatus*, a major cause of fungal diseases in humans. The new TR_{46}/Y121F/T289A resistance mechanism conferred high resistance to voriconazole and was associated with treatment failure in patients with invasive aspergillosis. Isolates harboring TR_{46}/Y121F/T289A were recovered from the environment, indicating that selection through a fungicide-driven route could have taken place.

TR_{34}/L98H was the first resistance mechanism that has been recovered from both clinical specimens and from the environment in the Netherlands. This resistance mechanism first emerged in clinical *A. fumigatus* isolates in 1998 [4, 6]. A Dutch survey performed between 2007 and 2009 showed that TR_{34}/L98H was widespread and that the prevalence varied between 0.8% and 9.5% [7]. TR_{34}/L98H was found in azole-resistant *A. fumigatus* isolates that were recovered from environmental sources, such as soil and compost [10]. A second resistance mechanism reported in the Netherlands was a 53 base-pair tandem repeat (TR_{53}) without mutations in the *cyp51A* gene. TR_{53} was associated with a pan-azole-resistant phenotype and was reported to have caused *Aspergillus* osteomyelitis in a pediatric patient in 2006 [31]. Although a TR_{53} isolate was recovered from the environment (Verweij and Melchers, unpublished observations), there is currently no evidence for migration of this resistance mechanism. Our current study describes TR_{46}/Y121F/T289A as the third resistance mechanism that has emerged in clinical and environmental isolates.

Similar to TR_{34}/L98H [11], the fungicide-driven route of resistance development could have caused the emergence of TR_{46}/Y121F/T289A. Both resistance mechanisms consist of a combination of genomic changes that include a tandem repeat [15, 32]. The new resistance mechanism included 3 genomic changes, and it appears unlikely that these would have evolved during azole therapy in all individual cases. Until now, only point mutations have been described to have developed in azole-resistant *A. fumigatus* isolates through patient therapy [14, 27]. Both TR_{34}/L98H and TR_{46}/Y121F/T289A were recovered from epidemiologically unrelated patients, most of whom were azole-naive, and both were recovered from the environment. Furthermore, genetic typing showed clustering of TR_{34}/L98H and TR_{46}/Y121F/T289A in separate clades apart from wild-type isolates.

The evolving epidemiology of TR_{34}/L98H indicates that this resistance mechanism is not restricted to the Netherlands but is increasingly being observed in other European Union member states [6, 13–19, 22, 23] and outside Europe [20, 21]. Genotyping indicates that in Europe, TR_{34}/L98H isolates represent offspring of a common ancestor [24] and could have developed locally, possibly in the Netherlands, and subsequently spread across countries through wind-dispersed conidia or ascospores. Given the rapid geographical migration of TR_{46}/Y121F/T289A in Dutch hospitals, it can be anticipated that this resistance mechanism will spread, similar to TR_{34}/L98H. Indeed, recently a lethal case of azole-resistant invasive aspergillosis due to TR_{46}/Y121F/T289A was reported in a patient from the neighboring country of Belgium [33].

Resistance threatens the outcome of patients with *Aspergillus*-related diseases, especially those with azole-resistant invasive aspergillosis. Voriconazole, which is recommended for the primary therapy of invasive aspergillosis, was uniformly inactive against TR_{46}/Y121F/T289A isolates, and treatment failed in all patients with proven or probable invasive aspergillosis who had received primary therapy with voriconazole. As the activity of itraconazole and posaconazole was also reduced in the majority of the isolates, the azole class appears not to be a treatment option in patients with infection with *A. fumigatus* harboring TR_{46}/Y121F/T289A.

There are limited clinical data regarding alternative treatment options in azole-resistant invasive aspergillosis. In vitro and experimental studies indicated that the combination of voriconazole and anidulafungin was synergistic against azole-susceptible infection but that in voriconazole-resistant disease, synergism was lost [34, 35]. There is concern that in infection with isolates where voriconazole shows no activity, such as the TR_{46}/Y121F/T289A strains, the efficacy of the combination will rely solely on that of anidulafungin, which is suboptimal [35]. In a murine model of disseminated aspergillosis, liposomal amphotericin B was shown to be effective against azole-resistant *A. fumigatus* with various resistance mechanisms, including TR_{46}/Y121F/T289A [36]. In our current study, patients who received primary therapy with liposomal amphotericin B appeared to respond better than those receiving voriconazole, although the number of patients was very limited and the underlying conditions diverse.

Systematic surveillance through a network of clinical microbiology laboratories proved to be a useful strategy to detect the emergence and spread of the new resistance mechanism in *A. fumigatus*. Given the observed spread of azole resistance across Europe we believe that international surveillance programs are warranted. We believe that our observations are very worrisome as they indicate that continued use of triazole DMIs in our environment with activity against *A. fumigatus* will not only help resistance traits to sustain in the environment, but will also cause new resistance mechanisms to emerge. The potential of geographical migration, as observed with TR_{34}/L98H, indicates that the fungicide-driven route of resistance selection will not remain a regional problem. Unless we are able to implement measures that prevent fungicide-driven resistance selection, the clinical use of azoles will become severely compromised. It is therefore important to understand the conditions that allow for selection of resistance in the environment and to investigate which preventive measures might be effective.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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