Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use?

Paul E Verweij, Eveline Snelders, Gert H J Kema, Emilia Mellado, Willem J G Melchers

Invasive aspergillosis due to multi-azole-resistant *Aspergillus fumigatus* has emerged in the Netherlands since 1999, with 6·0–12·8% of patients harbouring resistant isolates. The presence of a single resistance mechanism (denoted by TR/L98H), which consists of a substitution at codon 98 of c*yp51A* and a 34-bp tandem repeat in the gene-promoter region, was found in over 90% of clinical *A fumigatus* isolates. This is consistent with a route of resistance development through exposure to azole compounds in the environment. Indeed, TR/L98H *A fumigatus* isolates were cultured from soil and compost, were shown to be cross-resistant to azole fungicides, and genetically related to clinical resistant isolates. Azoles are abundantly used in the environment and the presence of *A fumigatus* resistant to medical triazoles is a major challenge because of the possibility of worldwide spread of resistant isolates. Reports of TR/L98H in other European countries indicate that resistance might already be spreading.

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Introduction

Invasive aspergillosis is an infectious disease that is difficult to manage. Patients become infected by inhaling airborne *Aspergillus* spp conidia that are released by saprophytic moulds that inhabit the environment, typically in soil and decaying organic matter (eg, compost). Despite preventive measures in hospitals, aimed at reducing the levels of airborne fungal spores, specific patient populations remain at risk. These include patients with haematological malignancy, pulmonary diseases (eg, chronic obstructive pulmonary disease), solid-organ transplant recipients, and patients treated with corticosteroids. The incidence varies from below 1% in autologous haemopoietic stem-cell transplant recipients to up to 27% in allogeneic haemopoietic stem-cell transplant patients.[1] The incidence in critically ill patients was found to be 2·7–6·3%.[1] In most high-risk patients, deposition of aspergillus spores and even subclinical infection probably happen in the community, with invasive aspergillosis becoming clinically detectable after immunosuppressive treatment during admission to hospital.[2–4] Patient-to-patient transmission of invasive aspergillosis is extremely uncommon, and only reports of two clusters have been published (transplantation of a contaminated allograft and a surgical wound infection).[5,6]

Starting adequate antifungal treatment early is thought to improve outcome. Voriconazole is recommended for the primary treatment of invasive aspergillosis,[7] and another triazole, posaconazole, was shown to reduce the number of invasive fungal infections in neutropenic patients with acute myeloid leukaemia, myelodysplastic syndrome, and in patients with severe graft-versus-host disease when given prophylactically.[8,9] The role of azoles has therefore increased in the management of patients with invasive fungal diseases, most notably of invasive aspergillosis.

The increasing role of azoles might be threatened by the acquisition of resistance by opportunistic moulds. Evolution of drug resistance seems to be less prominent in fungi than in bacteria, in which horizontal transfer of genes and accessory genetic elements across taxa provides a major source of genetic variation.[10] In fungi, the evolution of drug resistance is more likely to proceed by the sequential accumulation of adaptive mutations.[10] However, fungi are capable of rapidly adapting in response to environmental challenges by antifungal drugs. In the setting of infection in individuals coinfected with HIV, *Candida albicans* has evolved various resistance mechanisms in response to short periods of azole exposure. Acquired resistance was also seen in *Aspergillus fumigatus* isolates cultured from patients with aspergilloma during treatment with azoles.[11–13]

We recently reported the rapid emergence of azole resistance in *A fumigatus* isolates cultured from patients with invasive aspergillosis.[14,15] Azole resistance has emerged since 1999 in clinical *A fumigatus* isolates from Dutch hospitals, and 6·0–12·8% of patients were found to harbour an azole-resistant isolate.[14–16] The phenotype of the resistant isolates was characterised by in-vitro resistance to itraconazole, and reduced activity of voriconazole and posaconazole, compared with wild-type isolates.[14,15] Patients with azole-resistant invasive aspergillosis presented with primary or breakthrough infection, and failed to respond to itraconazole or voriconazole treatment.[14,15,17–20]

We believe that, in addition to resistance development in azole-treated patients, resistance in *A fumigatus* could also develop in the environment. An environmental route of resistance development confronts us with a major challenge with worldwide dimensions, because efficient reproduction and spread of resistant fungus in the environment can be anticipated. We discuss the evidence that supports an environmental route of resistance development in *A fumigatus*.

Resistance development through patient exposure

Resistance has been shown to develop in patients with aspergilloma and cavitary lung disease who have been treated with azoles.[11–13] Because in-vitro susceptibility testing of aspergilli is not routinely done in many clinical

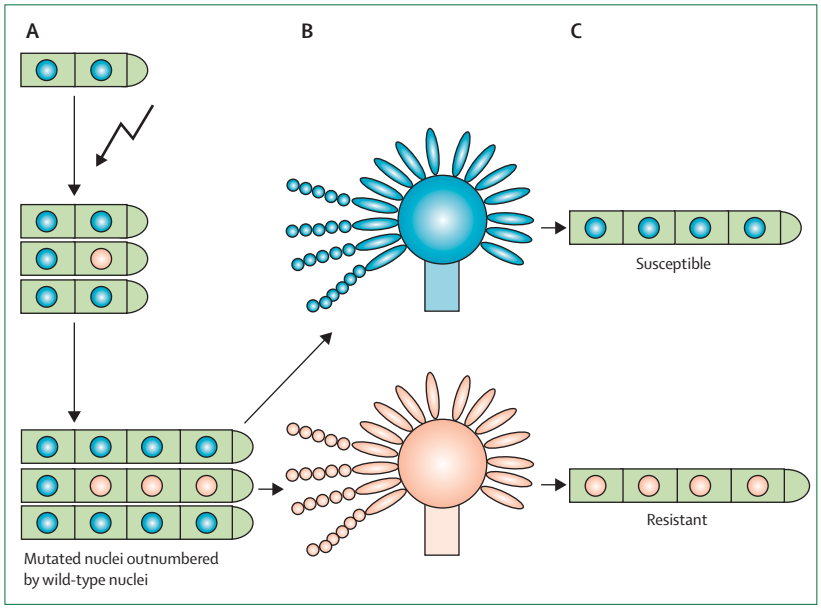

Figure 1: **Relation between mutations and phenotypic expression in aspergillus**
Hyphal growth, as present in invasive aspergillosis. Mutations will happen that have limited or no effect on the phenotype because they are vastly outnumbered by wild-type nuclei (A). From the hyphae, asexual propagative structures might be formed by which conidiospores (conidia) are produced via mitosis. This might happen in patients with aspergilloma. Mutations are expressed in conidia and in colonies from conidia (B). After germination of the conidia (C), hyphae and colonies with a susceptible (blue nuclei) or a resistant (brown nuclei) phenotype are formed.

microbiology laboratories, the prevalence of azole resistance is probably underestimated. Recently, Howard and colleagues[13] reported an increasing frequency of resistance in *A fumigatus* in predominantly azole-treated patients with aspergilloma. Pathogenesis involves infection of the patient with an azole-susceptible *A fumigatus* isolate that becomes resistant due to azole pressure. Importantly, the risk of development of phenotypic resistance seems to be dependent on the mode of reproduction of the moulds. Development of resistance might happen in patients with aspergilloma as aspergilli grow within the lung cavity by asexual reproduction through sporulation that allows multiple generations to form. This facilitates adaptation of the mould to the selective pressure of azoles.[21]

In patients with acute invasive aspergillosis, asexual reproduction by sporulation does not happen, and the infection progresses through hyphal elongation. Spontaneous mutations are likely to happen in nuclei within the hyphae. For example, in *Aspergillus niger*, stress (ie, high temperature or ultraviolet light) increases the mutation frequency.[22] Since the human body can be thought to be a hostile environment for any fungus because of exposure to host defences and antifungal drugs, mutations can be expected to happen during invasive fungal infection. However, the hyphae will contain millions of nuclei. Therefore, mutations might have little effect on the phenotype of the fungus even if the mutation results in a gain of function, primarily because the mutated genes in the pool would be vastly outnumbered by wild-type genes (figure 1). However, mutations would be expressed in uninucleate conidia and in all colonies that develop from those conidia. Therefore, asexual reproduction seems to be essential for phenotypic expression of mutations, including those predisposing for resistance.[22] As a consequence, development of phenotypic resistance during treatment of patients with acute invasive aspergillosis should be seen as highly unlikely, unless the fungus undergoes asexual reproduction, for instance when the infection progresses to a cavitary lesion.

Alternative sources of patient exposure to azoles include absorption of azole residues that are present in food. Several studies have investigated the presence of azole residues in food, most notably wines, because grapes are especially vulnerable to fungal infection and azole fungicides are intensively used in vineyards. Azole residues were found in more than 75% of wines, but all concentrations were below the required maximum residue concentrations.[23] Although long-term exposure of consumers to azoles could happen if food contains azole residues, in general, the risk of exposure is thought to be negligible in high-income countries.[24] Therefore, development of resistance to medical triazoles in *A fumigatus* is unlikely to be caused by azole residues in foods.

Resistance development through environmental exposure

Our hypothesis focuses on another route of resistance development through exposure of *A fumigatus* to azole compounds in our environment. Exposure of saprophytic fungi to azole compounds could take place in agriculture, where such compounds are commonly used for plant protection. The fungicides are applied repeatedly over a long period of time and could thereby create a persistent pressure of azole compounds on saprophytic fungi. We have previously suggested that an environmental route of resistance development in *A fumigatus* should be considered because a single mechanism of azole resistance was found in 94% of clinical isolates from different hospitals in the Netherlands.[15] The presence of such a dominant resistance mechanism is difficult to explain if resistance had developed exclusively through treatment of epidemiologically unrelated patients, because we would expect multiple mechanisms of resistance to develop. Substantial diversity of cytochrome P450 14-α sterol demethylase gene (*cyp51A*)-related resistance mechanisms was noted in the study by Howard and colleagues,[13] which was shown to be associated with azole therapy and suggests a different mode of resistance development than that seen in the Netherlands. Furthermore, spread of resistance is highly unlikely in invasive aspergillosis, because person-to-person transmission is very uncommon and a patient will either respond to treatment (precluding spread of the isolate) or

the treatment will fail.[15] In the latter case, the (resistant) fungus might survive, but its spread directly to another patient or through the environment seems highly unlikely. However, our finding of a dominant resistance mechanism could be explained by resistance development in the environment as the saprophytic growth of aspergilli enables the phenotypic expression and spread of mutations.

Furthermore, an environmental route of resistance development would be in line with the finding of primary invasive aspergillosis due to azole-resistant *A fumigatus* in azole-naive patients.[10,14] Breakthrough aspergillosis in patients on azole prophylaxis would also be consistent with an environmental route: patients would inhale both azole-susceptible and azole-resistant conidia, but the resistant conidia would have a selective advantage, thus allowing their germination in the lungs and subsequent invasive disease.[10,15]

Use of azoles as pesticides

Fungicides are widely used for plant protection in the European Union. At present, slightly less than half of the total European Union acreage under cereals and grapevine are treated yearly with azole fungicides.[25] This compares with less than 5% of the total crop area treated yearly in the USA.[25] Azole fungicides, also referred to as demethylation inhibitors, inhibit the biosynthesis of fungal ergosterol by inhibition of cytochrome P450 14-α sterol demethylase. The most important demethylation inhibitors used in agriculture are imidazoles and triazoles. Related groups of compounds that are used to a much less extent are derivatives of piperazines, pyridines, and pyrimidines.[26] The mode of action of these compounds is similar to that of azoles.[27] However, the azoles are the only class of compounds that are used both in agriculture and in clinical medicine.

Although the overall use of pesticides in most European Union member states has declined over the past decades, the use of fungicides has remained stable in the Netherlands in recent years. However, the volume of triazole fungicides sold has almost doubled between 1995 and 2007 (figure 2). For example, the volume of azoles and azole-like agricultural fungicides that was used in the Netherlands in 2004 was about 320-times higher than that of mould-active azoles used in clinical medicine (about 130 000 kg *vs* 400 kg). Because plant pathogenic moulds share their natural environment with *A fumigatus*, both are exposed to a strong and persistent pressure from azole fungicides.

Mechanisms of azole resistance

Resistance to fungicides is well-known in agriculture and is often first recognised when expected levels of disease control are no longer achieved. Depending on the mechanism of resistance, complete failure of disease control can happen, as has been found in benzimidazole fungicides, and a more gradual loss of control, which has been reported for triazoles. This failure of control corresponds with a sensitivity shift among plant pathogens towards reduced susceptibility to triazoles, which is thought to be happening in all European countries. Moreover, field isolates that develop resistance in response to exposure to a triazole commonly show a multi-azole-resistant phenotype.[28]

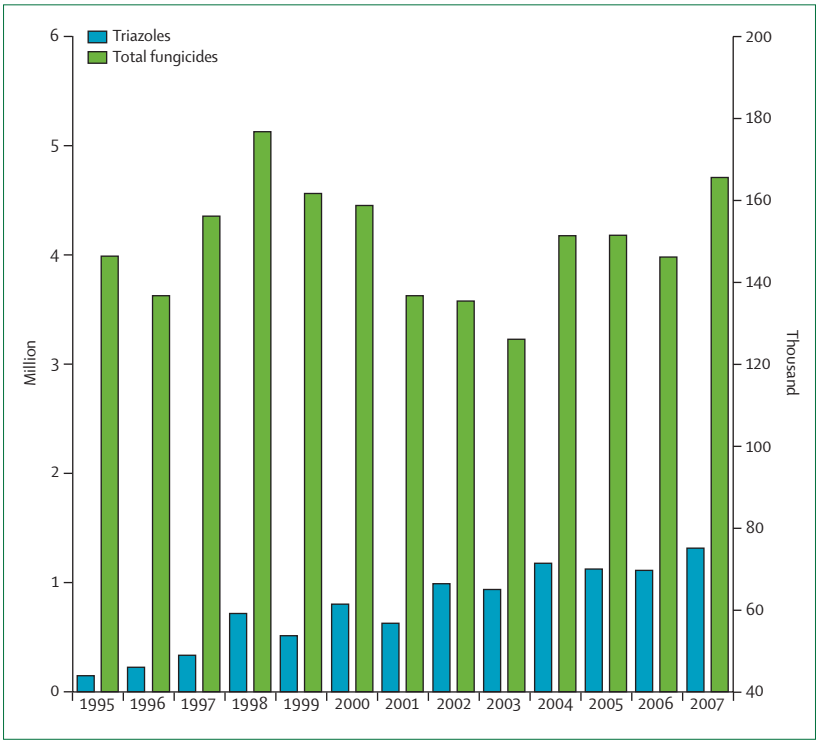

Figure 2: **Total volume of fungicides and triazoles sold in the Netherlands between 1995 and 2007**
Data from the Dutch Foundation for Phytofarmacy (Nefyto, Nederlandse Stichting voor Fytofarmacie).

Mechanisms of azole resistance that have been described in many plant pathogens include aminoacid substitutions in the *cyp51A* gene or the presence of transcriptional enhancers, which cause overexpression of the *cyp51A* gene (table).[11,13,28–44] The *cyp51B* gene of *A fumigatus* has not been shown to be a target for substitutions that are associated with resistance. The described mechanisms of fungicide resistance represent a stable, heritable trait in plant pathogenic moulds and have no apparent adverse affect on the fitness of the fungus.

In clinical *A fumigatus* isolates, multiple aminoacid substitutions in the *cyp51A* gene have been described that correspond to azole resistance (figure 3).[45] Specific mutations in *cyp51A* have been associated with different susceptibility profiles in *A fumigatus*: cross-resistance to itraconazole and posaconazole has been associated with aminoacid substitutions at glycine-54 (G54),[41] and a pattern of itraconazole resistance and increased minimum inhibitory concentrations for other azoles has been linked to different aminoacid substitutions at

	Azole drug resistance phenotype (Cyp51A aminoacid substitutions)*	cyp51A promoter alterations	Increased expression of Cyp51A
Penicillium digitatum[28,29]	Triflumizole, imazalil	126 bp tandem repeat (5 times)	Yes
Blumeria jaapii[30]	Fenbuconazole	Truncated non-long terminal direct repeats	Yes
Uncinula necator[31]	Triadimenol (Y136F)	..	..
Erysiphe graminis (formerly *hordei*)[32]	Benzimidazol (Y136F)	..	..
Mycosphaerella graminicola[33-35]	Tebuconazole (I381V), difenoconazole, triadimenol (Y137F)	..	No
Monilina fructicola[36]	Propiconazole	65 bp insertion	Yes
Venturia inaequalis[37]	Myclobutanil	553 bp insertion	Yes
Blumeria graminis (formerly *hordei, tritici*)[38]	Propiconazole (K147Q), triadimenol (Y136F)	..	..
Ustilago maydis[39]	Propiconazole (G464S)	..	..
Tapesia yallundae[40]	Prochloraz, trifumizol, flusilazol, triadimenol (S35T, Q34H, N224S)	..	..
Aspergillus fumigatus[11,13,41-44]	Itraconazole and posaconazole (G54V, G54R, G54W, G54E); multi-azole resistant (M220V, M220T, M220I, M220K, G448S, G138C)	..	..
	Multi-azole resistant (L98H)	34 bp insertion	Yes

*If detected.

Table: **Main resistance mechanisms to azole fungicides found in plant pathogens and clinical *Aspergillus fumigatus* isolates**

methionine-220 (M220).[42] Single mutations at codon 138, which was associated with an aminoacid substitution (G138C), and at codon 448 were also seen in a clinical *A fumigatus* isolate that had a multi-azole-resistant phenotype.[11,13,43]

The dominant azole-resistant phenotype of the *A fumigatus* isolates reported in Dutch hospitals was associated with a different resistance mechanism, which consisted of an aminoacid change in the *cyp51A* gene and the presence of a tandem repeat in the gene promoter (TR/L98H; figure 3).[15,44] Both alterations were shown to be necessary to display the azole-resistant phenotype, with the tandem repeat acting as a transcriptional enhancer.[44]

The resistance mechanism seems to be indicative of the mode of resistance development. The resistance mechanisms found in isolates thought to have become resistant through azole treatment consist of point mutations, whereas the presence of a tandem repeat is an important mechanism found in plant pathogenic moulds that develop resistance through exposure to azole fungicides (table). The insertion of a tandem repeat might happen more readily during sexual reproduction of the mould compared with asexual reproduction. Sexual reproduction was recently described in *A fumigatus*,[46] but this mode of reproduction is rarely associated with fungal diseases in human beings, unlike asexual reproduction. As a consequence, the development of the 34-bp tandem repeat in *A fumigatus* is most likely to be an event that has taken place in the environment. The second alteration found in the TR/L98H isolates, the substitution at codon 98, probably arose through a separate event.

The risk of opportunistic fungi becoming resistant because of azole antifungal use for plant protection has been acknowledged by investigators,[47,48] and by governmental authorities such as the Scientific Steering Committee of the Health and Consumer Protection Directorate-General of the European Commission,[25] but there has been little evidence to support a direct link between environmental and clinical resistant isolates. The reports were published when azole resistance in fungi was restricted to *Candida* spp, and was clearly associated with repeated azole treatment of immunocompromised patients with oropharyngeal candidiasis. Since then, a Swiss study has reported susceptibility to azoles used in clinical medicine of *A fumigatus* isolates from compost sites, vineyards, and crop areas. Among 150 environmental isolates, 32 showed increased minimum inhibitory concentrations of azoles, but no triazole cross-resistant isolates were identified.[49] However, we recently reported the presence of *A fumigatus* resistant to medical triazoles in the indoor hospital environment as well as in outdoor soil samples.[50] Azole-resistant isolates were never found in natural soil, only in cultivated soil samples. Furthermore, isolates resistant to medical triazoles were recovered from commercial compost and seeds obtained from a garden centre and plant nursery.[50] 13 of 15 (86%) environmental resistant isolates harboured the same resistance mechanism as found in clinical isolates, and genetic typing confirmed that these environmental and clinical resistant isolates were clustered.[50] This provides evidence that patients might become colonised and subsequently infected by resistant isolates originating from the environment.

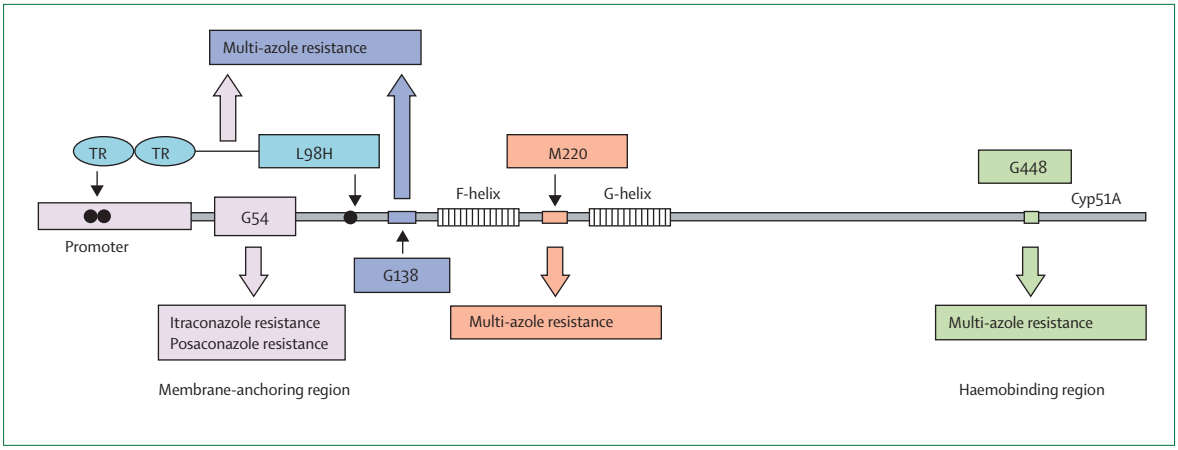

***Figure 3: Aspergillus fumigatus* cyp51A-related resistance mechanisms to azole antifungals**
The position of the different mutations are shown with the associated phenotypes. MIC=minimum inhibitory concentration. TR=tandem repeat.

A new route of antifungal drug-resistance development?

We believe that evidence is accumulating that resistance to medical triazoles might develop because of exposure of *A fumigatus* to azole fungicides in the environment. This mode of resistance development would imply that resistance development against azole fungicides causes cross-resistance to medical triazoles. The principle has been shown for the maize pathogen *Colletotrichum graminicola*, which developed cross-resistance to itraconazole and voriconazole after exposure to the fungicide tebuconazole.[51] Preliminary studies with TR/L98H isolates from clinical and environmental origins indicate that these isolates are cross-resistance to tebuconazole and metconazole, again supporting a role for fungicides in resistance development.[50]

We seem to be at the verge of a new era in resistance development in moulds, although many questions remained unanswered. The recent emergence of azole resistance in clinical *A fumigatus* isolates (after 1999) is difficult to explain by environmental exposure because azole fungicides have been used for several decades. A possible explanation might be that the early azole fungicides showed no activity against *A fumigatus*, and that only recently have new compounds been introduced that have activity against *A fumigatus*. However, the time between exposure and phenotypic resistance development is unknown, although the polyallelic nature of the TR/L98H resistance mechanism suggests a multistep development process. The dominance of the TR/L98H resistance mechanism is also unexplained because one would expect that azole exposure in the environment would result in multiple resistance mechanisms to emerge. Maybe the TR/L98H isolates have an advantage with respect to fitness compared with other mutated isolates, which would facilitate its survival and spread in the environment.

Compost might play a key part in the development and spread of azole resistance. Compost is an ecological niche for *A fumigatus*, and azole residues have been reported to be present in commercial compost.[52] Compost, which is used in domestic gardens and for indoor plants, would also provide a mode of exposure of susceptible hosts. If the environmental route of resistance development proves to be correct, we anticipate that azole resistance in *A fumigatus* is only the first of many, with multiple yet unknown resistance mechanisms having developed in other *Aspergillus* spp and in other clinically relevant moulds.

Future directions

To prove a relation between the use of demethylation inhibitors and resistance development in *A fumigatus*, all azole fungicides need to be tested for activity against *A fumigatus* isolates that are susceptible and resistant to medical triazoles, and against isolates harbouring different resistance mechanisms. The year of first field use of each fungicide will help to establish temporal relations between use of a specific fungicide and the recorded emergence of resistance in clinical isolates. For those fungicides that show cross-resistance, induction experiments should be done to establish whether resistant mutants show cross-resistance to medical triazoles. The ability of demethylation inhibitors to select the TR/L98H resistance mechanism in *A fumigatus* would prove such a relation. This will also allow us to identify which compounds are responsible for resistance development. However, even if a relation between the use of azole fungicides and the development of resistance in *A fumigatus* is proven, whether we can prevent further development and spread remains unclear, because resistant isolates are already present in the environment. Limiting the use of those fungicides that induce resistance might prevent the development of azole resistance, but preventing further spread of the TR/L98H resistance mechanism might not be feasible. Spread already seems to be happening, and TR/L98H has been found in other

European countries such as Norway,[15] the UK,[13,53] Belgium,[54] France, and Spain.[44] This might be the first sign of the global spread of azole resistance in aspergilli.

Contributors

PEV, ES, and WJGM wrote the first draft of the paper. PEV made the revisions that were reviewed by all authors. All authors contributed to the drafts and final version of the paper.

Conflicts of interest

PEV has received grants from Basilea, Cephalon, Gilead, Pfizer, and Schering-Plough. The other authors declare that they have no conflicts of interest.

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