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Title: Azole-resistant *Aspergillus fumigatus* is highly prevalent in the environment of Vietnam, with marked variability by land use type

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Running Title: Azole-resistant *Aspergillus fumigatus* in Vietnam

Originality-Significance Statement

Unprecedented levels of environmental azole-resistant *Aspergillus fumigatus* detected in the Mekong Delta region of Vietnam raise concerns for underappreciated threat of antifungal resistant infections in clinical practice, and call for the rational use of azole fungicides in agriculture.

Keywords: *Aspergillus fumigatus*; *cyp51A*; TR_{34}/L98H; antifungal resistance; chronic pulmonary aspergillosis; azoles; Vietnam

Summary

Azole-resistant environmental *Aspergillus fumigatus* presents a threat to public health but the extent of this threat in Southeast Asia is poorly described. We conducted environmental surveillance in the Mekong Delta region of Vietnam, collecting air and ground samples across key land-use types, and determined antifungal susceptibilities of *Aspergillus* section *Fumigati* (ASF) isolates and azole concentrations in soils. Of 119 ASF isolates, 55% were resistant (or non-wild type) to itraconazole, 65% to posaconazole and 50% to voriconazole. Azole resistance was more frequent in *A. fumigatus sensu stricto* isolates (95%) than other ASF species (32%). Resistant isolates and agricultural azole residues were over-represented in samples from cultivated land. *cyp51A* gene sequence analysis showed 38/56 resistant *A. fumigatus sensu stricto* isolates carried known resistance mutations, with TR_{34}/L98H most frequent (34/38).

Introduction

Aspergillus causes serious disease in humans, ranging from allergic exacerbations of asthma to acute fulminating invasive infections in immunocompromised hosts. Globally, the most common form of disease is chronic pulmonary aspergillosis (CPA), which affects over 3 million people each year (Bongomin *et al.*, 2017). CPA results in progressive, debilitating lung disease, especially in tuberculosis (TB) survivors, illustrating the often neglected burden of disease related to *Aspergillus* (Brown *et al.*, 2012; Bongomin *et al.*, 2017). Over the last decade, our ability to treat CPA and other forms of aspergillosis has come under threat from the emergence of drug-resistant strains, often attributed to the use of anti-fungals in agriculture (Verweij *et al.*, 2009; Berger *et al.*, 2017; Beer *et al.*, 2018).

Although there are hundreds of species of *Aspergillus*, only a handful cause disease in humans, predominantly within *Aspergillus* section *Fumigati* (ASF). ASF is comprised of several different complexes including the *Aspergillus fumigatus* complex (containing species morphologically identical to *A. fumigatus sensu stricto*) (Samson *et al.*, 2007; Peterson, 2008). Although *A. fumigatus sensu stricto* is the leading human pathogen, infections caused by other species within ASF are also described. Because it can be difficult to distinguish these species morphologically from *A. fumigatus sensu stricto*, many diagnostic laboratories identify them only as *A. fumigatus* complex (Serrano *et al.*, 2011). We report the prevalence of resistance amongst all isolates from ASF in light of its clinical importance, and specifically for *A. fumigatus sensu stricto* in which, as described below, resistance is emerging globally in association with agricultural azole-use. Throughout this paper we will use the following terms: ASF for *Aspergillus* section *Fumigati*, *A. fumigatus* complex for the species complex, *A. fumigatus* for *A. fumigatus sensu stricto* and ARAF for azole-resistant *A. fumigatus sensu stricto.*

There are only three classes of antifungal drugs available to treat *Aspergillus* infections: azoles, echinocandins, and amphotericin B. Because azoles are potent, relatively inexpensive, and have low toxicity, they are the mainstay of antifungal therapy. In many settings, they are the only therapy available (Maertens, 2004; Patterson *et al.*, 2016). However, infections caused by ARAF are increasingly reported worldwide (Pfaller, 2012). This resistance can arise from long-term azole therapy (Camps *et al.*, 2012) or, more commonly, from direct acquisition of a resistant strain from the environment (Snelders *et al.*, 2008). Regardless of the mechanism, resistance has important implications for human health, carrying an excess mortality of up to 21% (van der Linden *et al.*, 2015; Lestrade *et al.*, 2018).

Resistance in environmental *Aspergillus* is driven by off-target selection pressures from the widespread use of azole fungicides in agriculture (Berger *et al.*, 2017); this causative link has been well-demonstrated via studies in azole-contaminated environments, such as market-gardens and sawmills (Jeanvoine *et al.*, 2017; Rocchi *et al.*, 2018). The principal resistance mechanisms associated with environmental azole use are alterations in the *cyp51A* gene encoding lanosterol 14α-demethylase (target of azole antifungals), with TR_{34}/L98H and TR_{46}/Y121F/T289A genotypes dominating. Importantly, these genotypes were recently reported to be responsible for >90% of ARAF clinical infections in The Netherlands (Snelders *et al.*, 2008; van der Linden *et al.*, 2011) and emerging data are confirming that resistant strains from the environment are closely reflected in the infections of local populations (Rhodes *et al.*, 2021).

Before 1999, the prevalence of ARAF was negligible (Howard *et al.*, 2009a; van Paassen *et al.*, 2016). However, recent environmental studies from Australia, Belgium, Denmark, Germany, India, Iran, Italy, Kuwait, Switzerland, Tanzania, Thailand, The Netherlands, Taiwan and Portugal have shown that ARAF isolates are globally distributed, with the prevalence varying from 2-14% (van der Linden *et al.*, 2011; Badali *et al.*, 2013; Ahmad *et al.*, 2014; Anuradha *et al.*, 2014; Chowdhary *et al.*, 2014; Prigitano *et al.*, 2014; Bader *et al.*, 2015; Tangwattanachuleeporn *et al.*, 2017; Sewell *et al.*, 2019; Chen *et al.*, 2020) (Table 1).

The emergence of ARAF is a global health problem (Snelders *et al.*, 2008; Chowdhary and Meis, 2018) which poses particular threats to many low and middle income countries such as Vietnam, where populations are at high risk of CPA due to underlying lung damage (e.g from prior TB or smoking-related chronic obstructive pulmonary disease (COPD)) (WHO Western Pacific Region: Viet Nam - statistics summary (2002 - present); Denning *et al.*, 2011; Beardsley *et al.*, 2015) and the capacity to test for susceptibility is lacking. Vietnam sits at a vulnerable nexus of

high TB and COPD incidence (WHO Western Pacific Region: Viet Nam - statistics summary (2002 - present); Beardsley *et al.*, 2015), intensive farming with widespread agricultural azole use (Pham *et al.*, 2013), and limited fungal diagnostic laboratory capacity; together making azole resistance both likely and clinically important. In this paper we describe the problem of drug-resistant *Aspergillus* in the environment of Vietnam's Mekong Delta region, addressing our hypothesis that agriculturally driven-ARAF is common in Vietnam, and that its prevalence is linked to land usage. This study is the most comprehensive investigation of its type in Southeast Asia.

Table 1. Global prevalence of azole resistance for environmental *Aspergillus fumigatus* complex isolates.

Country	Source of sample	Prevalence of azole resistance	Year	Reference
United Kingdom	soil	12/178 (6.7)	2019	Sewell *et al*. (Sewell *et al.*, 2019)
India	soil	44/630 (7.0)	2014	Chowdhary *et al*. (Chowdhary *et al.*, 2014)
Iran	soil	5/41 (12.2)	2013	Badali *et al*. (Badali *et al.*, 2013)
Kuwait	air, water, floor swab	8/115 (7.0)	2014	Ahmad *et al*. (Ahmad *et al.*, 2014)
China	soil	21/206 (10.2)	2019	Chen *et al*. (Chen *et al.*, 2020)
Thailand	soil	10/99 (10.1)	2017	Tangwattanachuleeporn *et al*. (Tangwattanachuleeporn *et al.*, 2017)
Tanzania	soil, woody debris	115/108 (3.9)	2014	Anuradha *et al*. (Anuradha *et al.*, 2014)
Australia	soil, air	4/185 (2.2)	2018	Talbot *et al*. (Talbot *et al.*, 2018)
Taiwan	soil, air	34/451 (7.5)	2018	Wang *et al.* (Wang *et al.*, 2018)
Portugal	air, floor swab	8/99 (8.1)	2021	Gonçalves *et al.* (Gonçalves *et al.*, 2020)

Results

Isolation of environmental *Aspergillus*

Our sampling yielded 533 *Aspergillus* spp. isolates: 157 (29.5%) were recovered from air, 271 (50.8%) from soil, and 105 (19.7%) from decayed leaves / water (Table S3). Of these isolates, 324 were from ASF, including 62 *A. fumigatus sensu stricto*. Many of the 262 isolates of non-*fumigatus*

ASF species demonstrated overlapping morphology and comprised ten different species (Table S1).

The recovery rate was 309/450 samples (68.7%) for any *Aspergillus* spp., 257/450 (57.1%) for ASF species, and 54/450 (12%) for *A. fumigatus*. The recovery rate by sample type is presented in Table S2.

Detection of azole-resistant *A. fumigatus* and non-*fumigatus* ASF isolates

Antifungal susceptibility testing was performed on 62 *A. fumigatus* and 57 randomly selected non-*fumigatus* ASF isolates (total 119). 59/62 (95.2%, 95% CI 86.5-99%) *A. fumigatus* isolates were resistant to at least one azole (Table 2), compared with 18/57 (31.6%, 19.1-45.2%) non-*fumigatus* ASF isolates (P<0.0001, by Chi-squared) (Table S4). Resistance to amphotericin B was detected in 6/62 (9.7%, 3.6-19.9%) *A. fumigatus* isolates, compared with 9/57 (15.8%, 7.5-27.9%) isolates of non-*fumigatus* ASF isolates. Five *A. fumigatus*, one *A. spathulatus*, and one *A. laciniosus* isolates were resistant to all three azoles and amphotericin B. Full MIC results are shown in Table S5.

The logistic regression model showed the following odds ratios (95% CI, P-value) for detection of itraconazole resistance / non wild-type (NWT) MIC for all ASF isolates (per land use type, compared to national park): fruit farms 7.16 (1.53-43.36, P=0.018), rice farms 5.70 (1.80-19.71, P=0.004), shrimp farms 3.15 (1.01-10.40, P=0.052), and urban residential 9.31 (2.52-41.71, P=0.002) (Table S6). The prevalence of resistance for both *A. fumigatus* and non-*fumigatus* ASF isolates by land-use type are shown in Figure 3, and their spatial distribution by land type is shown in Figure 1 and Figure S1, respectively. The results specifically for *A. fumigatus* resistance are presented in Table 2 and show that the lowest prevalence of resistance was detected in national

park land for all azoles tested, however the confidence limits for the difference crossed the null and the logistic regression failed to converge in the model restricted to *A. fumigatus* isolates.

Figure 1. Environmental sampling sites in Ca Mau province, Vietnam, showing where resistant *Aspergillus fumigatus sensu stricto* isolates and agricultural azoles were detected. Inset, Vietnam with Ca Mau province highlighted in red. Land use, by ward, colour coded. Black dot = sampling site; red dot = azole-resistant *Aspergillus fumigatus sensu stricto* detected; black cross = residual agricultural azole detected.

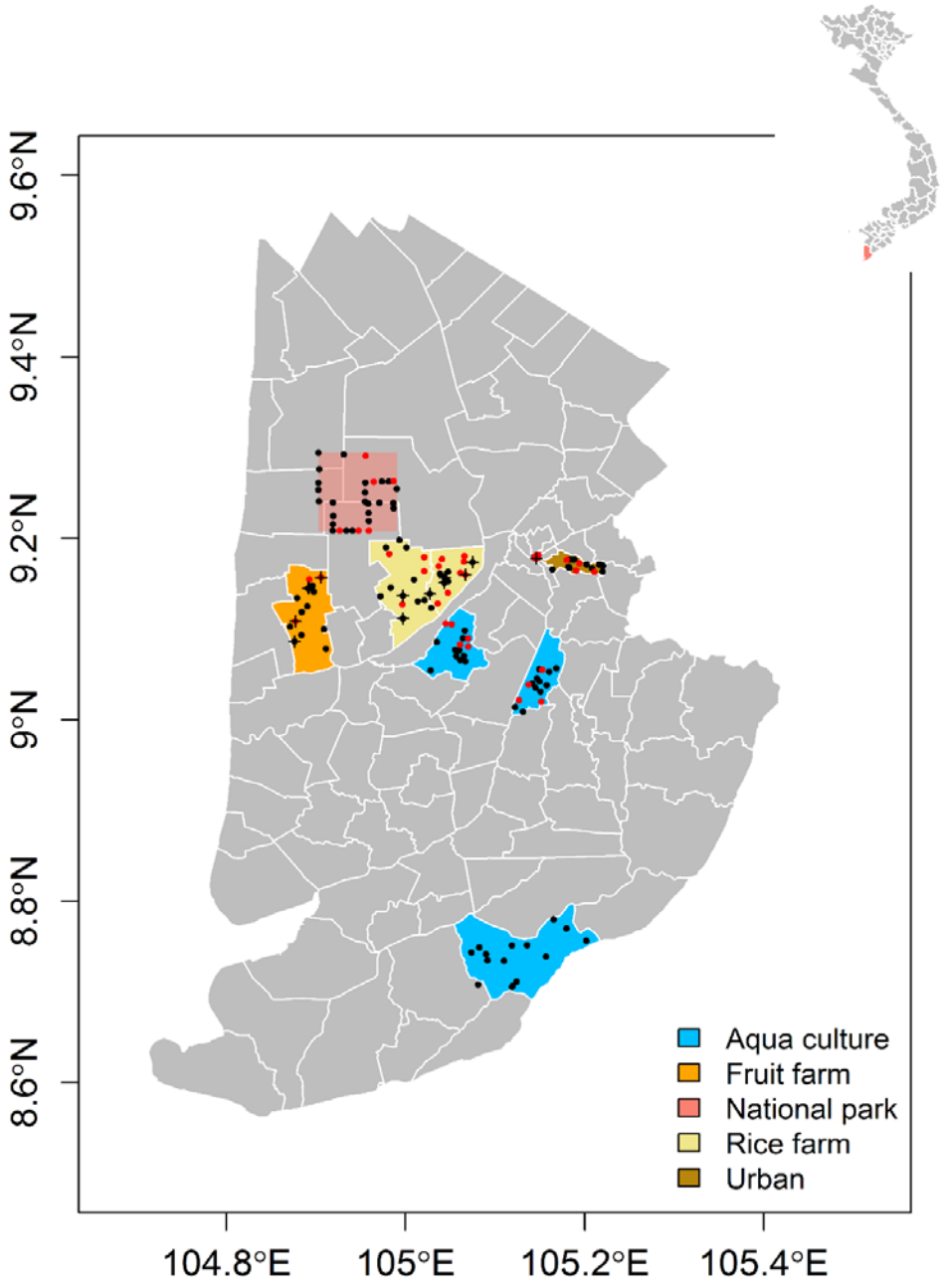

Table 2. Prevalence of antifungal resistance / non-wild type MICs in *Aspergillus fumigatus sensu stricto* (*n* = 62) and all *Aspergillus* section *Fumigati* complex isolates (*n* = 119) by different land use types within Ca Mau province, Vietnam.

	Antifungal-resistant isolates / total isolates (%)				
	ITC-R	**POS-R**	**VRC-R**	**IPV-R**	**AMB-R**
Part I: *Aspergillus fumigatus sensu stricto* isolates (*n* = 62)					
National park	8/10 (80)	9/10 (90)	7/10 (70)	7/10 (70)	3/10 (30)
Rice farm	15/16 (94)	15/16 (94)	12/16 (75)	12/16 (75)	2/16 (12.5)
Fruit farm	4/4 (100)	4/4 (100)	3/4 (75)	3/4 (75)	1/4 (25)
Shrimp farm	13/14 (92.9)	13/14 (92.9)	11/14 (78.6)	11/14 (78.6)	0/14 (0)
Urban	17/18 (94.4)	18/18 (100)	15/18 (83.3)	15/18 (83.3)	0/18 (0)
All sites	57/62 (91.9)	59/62 (95.2)	48/62 (77.4)	48/62 (77.4)	6/62 (9.7)
Part II: Non-*fumigatus sensu stricto* isolates from *Aspergillus* section *Fumigati* (n = 57)					
National park	0/14 (0.0)	0/14 (0.0)	0/14 (0.0)	0/14 (0.0)	0/14 (0.0)
Rice farm	2/16 (12.5)	7/16 (43.8)	3/16 (18.8)	2/16 (12.5)	4/16 (25)
Fruit farm	1/9 (11.1)	5/9 (55.6)	3/9 (33.3)	1/9 (11.1)	2/9 (22.2)
Shrimp farm	3/12 (25)	4/12 (33.3)	3/12 (25)	3/12 (25)	2/12 (16.7)
Urban	2/6 (33.3)	2/6 (33.3)	2/6 (33.3)	2/6 (33.3)	1/6 (16.7)
All sites	8/57 (14)	18/57 (31.6)	11/57 (19.3)	8/57 (14)	9/57 (15.8)
Part III: All *Aspergillus* section *Fumigati* species combined (*n* = 119)					
National park	8/24 (33.3)	9/24 (37.5)	7/24 (29.2)	7/24 (29.2)	3/24 (12.5)
Rice farm	17/32 (53.1)	22/32 (68.8)	15/32 (46.9)	14/32 (43.8)	6/32 (18.8)
Fruit farm	5/13 (38.5)	9/13 (69.2)	6/13 (46.2)	4/13 (30.8)	3/13 (23.1)
Shrimp farm	16/26 (61.5)	17/26 (65.4)	14/26 (53.8)	14/26 (53.8)	2/26 (7.7)
Urban	19/24 (79.2)	20/24 (83.3)	17/24 (70.8)	17/24 (70.8)	1/24 (4.2)
All sites	65/119 (54.6)	77/119 (64.7)	59/119 (49.6)	56/119 (47)	15/119 (12.6)

ITC-R, itraconazole resistant; POS-R, posaconazole resistant; VRC-R, voriconazole resisant; IPV-R, resistant to itraconazole, posaconazole and voriconazole; AMB-R, amphotericin resistant.

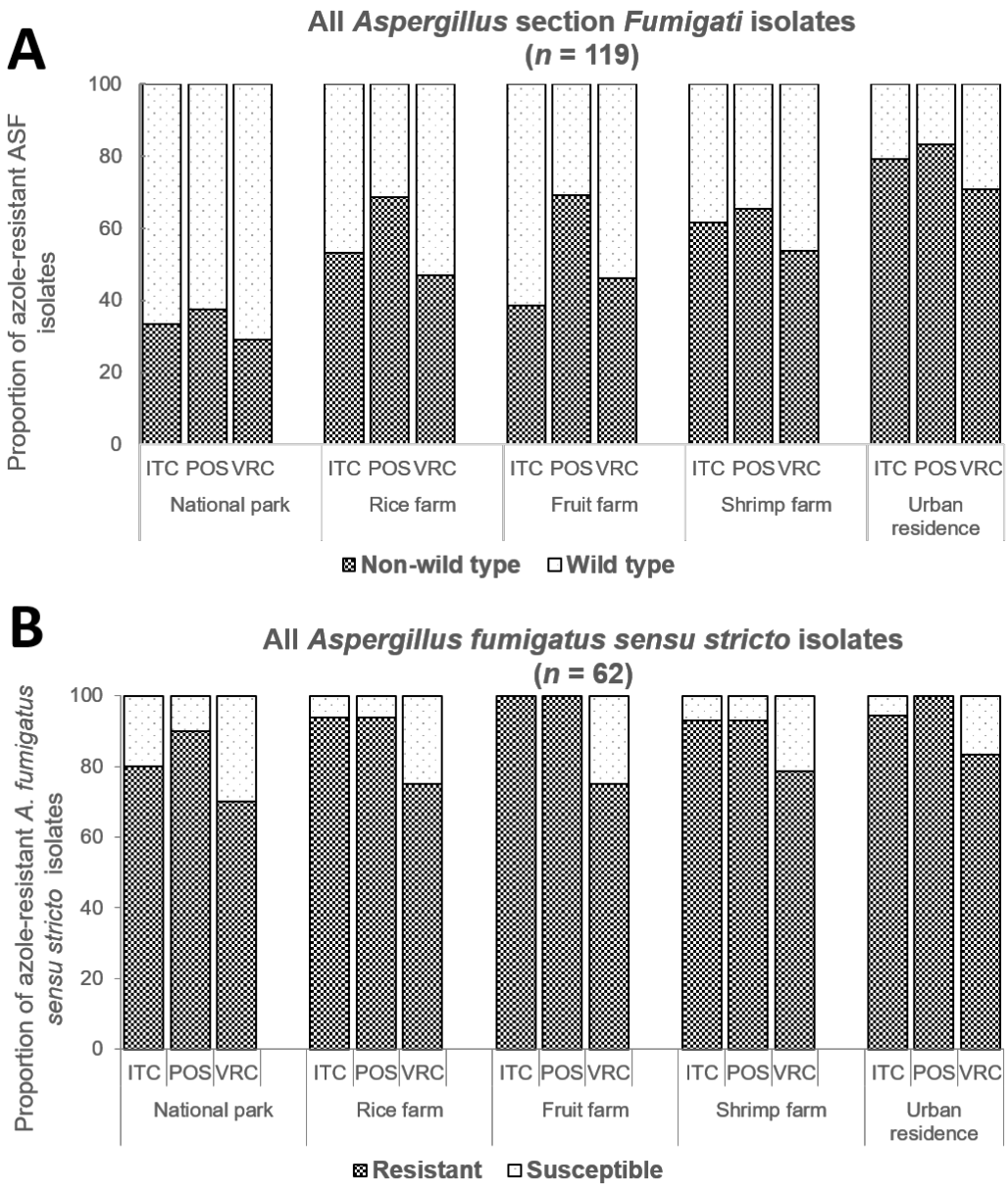

Figure 2. Proportion of *Aspergillus* section *Fumigati* isolates resistant or non-wild-type to itraconazole, posaconazole and voriconazole by land use type. Antifungal susceptibility of all tested *Aspergillus* section *Fumigati* (A) and all *Aspergillus fumigatus sensu stricto* (B). ITC, itraconazole; POS, posaconazole; VRC, voriconazole.

Detection of residual agricultural azoles in soil samples

Of the five agricultural azoles, difenoconazole (0.022-0.187 mg/kg), propiconazole (0.019-0.112 mg/kg), and tebuconazole (0.013-0.102 mg/kg) were detected at 13/145 (9%) locations including 7 rice farms, 5 fruit farms and 1 urban residential area. Bromuconazole and epoxiconazole were not detected at any sampling sites. Distribution varied by land use type, with detection most frequent in soil from cultivated land. Azole residues were undetectable in national park sites, and rarely detected from urban sites (Table 3). In our logistic regression model, detection of azole residues was not predictive of resistant *Aspergillus* strains being isolated (OR 0.48, 95%CI 0.12-2.12, *P*=0.307), and it did not improve the fit of the model (Table S5).

Table 3. Azole fungicide residues detected in soil samples from different land use types within Ca Mau province, Vietnam.

Country	No. soil samples collected	Azole-detected sites / total sample sites (%) [1]					
		DFC	TBC	PPC	BMC	EPC	Total combined
National park	30	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)
Rice farm	30	7/30 (23.3)	2/30 (6.7)	5/30 (16.7)	0/30 (0)	0/30 (0)	7/30 (23.3)
Fruit farm	15	4/15 (26.7)	1/15 (6.7)	4/15 (26.7)	0/15 (0)	0/15 (0)	5/15 (33.3)
Shrimp farm	45	0/45 (0)	0/45 (0)	0/45 (0)	0/45 (0)	0/45 (0)	0/45 (0)
Urban	25	1/25 (3.3)	1/25 (3.3)	1/25 (3.3)	0/25 (0)	0/25 (0)	1/25 (4.0)
All sites	145	12/145 (8.3)	4/145 (2.8)	2/145 (1.4)	0/145 (0)	0/145 (0)	13/145 (9.0)

[1] Detection limit for all 5 fungicides was 0.003 mg/kg. DFC, Difenoconazole; TBC, tebuconazole; PPC, propiconazole; BMC, bromuconazole; EPC, epoxiconazole.

Molecular mechanism of azole resistance in *A. fumigatus sensu stricto*

We sequenced the entire *cyp51A* gene of 56 ARAF isolates (amplification of the CYP3 fragment failed for three isolates) and identified four mutations previously associated with resistance: TR_{34}/L98H (34/56, 60.7%, 46.8-73.5%), N248K (2/56, 3.6%, 0.4-12.3%), TR_{34}/L98H/S297T/F495I (1/56, 1.8%, 0.4-9.6%), and F46Y/M172V/N248T/D255E/E427K (1/56, 1.8%, 0.4-9.6%). Thirty-three of 34 isolates carrying TR_{34}/L98H demonstrated pan-azole

resistance to itraconazole, posaconazole, and voriconazole. Eighteen of the ARAF isolates (18/56, 32.1%, 20.3-46%) did not exhibit any mutations in the *cyp51A* gene (Table 4).

Table 4. Antifungal susceptibility phenotypes by different *cyp51A* mutations, for all identified azole-resistant *Aspergillus fumigatus sensu stricto* isolates (*n* = 56).

***cyp51A* mutation**	**Number of antifungal-resistant isolates**						**Frequency (%) 95% CI**
	IPVA-R [1]	**IPV-R [2]**	**IPA-R [3]**	**IP-R [4]**	**P-R [5]**	**Total combined**	
TR_{34}/L98H	3	30	0	1	0	34	34/56 (60.7) 46.8 - 73.5
TR_{34}/L98H/S297T/F495I	0	1	0	0	0	1	1/56 (1.8) 0.04 - 9.6
F46Y/M172V/N248T/D255E/E427K	0	1	0	0	0	1	1/56 (1.8) 0.04 - 9.6
N248K	0	1	0	1	0	2	2/56 (3.6) 0.44 - 12.3
NONE	1	8	1	6	2	18	18/56 (32.1) 20.3 - 46

[1] IPVA-R: resistant to itraconazole, posaconazole, voriconazole and amphotericin B; [2] IPV-R: resistant to itraconazole, posaconazole and voriconazole; [3] IPA-R: resistant to itraconazole, posaconazole and amphotericin B; [4] IP-R: resistant to itraconazole and posaconazole; [5] P-R: resistant to posaconazole.

Discussion

The emergence of ARAF is an increasing global health problem (Snelders *et al.*, 2008; Chowdhary and Meis, 2018), which poses particular threats in Vietnam given a high prevalence of risk factors for aspergillosis, a lack of surveillance data for ARAF, and limited diagnostic laboratory capacity. We systematically sampled sites representing five major land use types, and successfully recovered a large number of environmental ASF isolates. We found an alarmingly high prevalence of azole resistance in *A. fumigatus*: 95.2% of isolates were resistant to at least one azole, with the lower 95% CI of 86.5% well above rates previously described anywhere in the world (Table 1) (van der Linden *et al.*, 2011; Badali *et al.*, 2013; Ahmad *et al.*, 2014; Anuradha *et*

al., 2014; Chowdhary *et al.*, 2014; Prigitano *et al.*, 2014; Bader *et al.*, 2015; Tangwattanachuleeporn *et al.*, 2017; Sewell *et al.*, 2019; Chen *et al.*, 2020). *A. fumigatus* had a higher prevalence of resistance than the other species within ASF, in marked contrast to published international experience, where acquired resistance in *A. fumigatus* is rare but increasing, and it is the non-*fumigatus* ASF species which are sometimes noted for their innate azole resistance (Van Der Linden *et al.*, 2011). Although our focus is azole-resistance, we note that over 10% of isolates were non-susceptible to Amphotericin B.

We postulate that the high prevalence of ARAF isolates in Vietnam is due to poorly regulated, widespread use of fungicides in agriculture. In support, resistant / non-WT MICs were more common in isolates from cultivated soils than those from national park land, and azole residues were principally detected in cultivated soil samples. However, our overall detection of azoles was low, and our survey may have been underpowered to demonstrate a link between contamination and resistance. Although azole residues can persist for several weeks in soil in temperate settings (Tomlin, 2003), it is uncertain how long they persist in tropical soils with regular flooding (from both rain and agricultural practice), and detection likely underestimates usage. In fact, given the known high consumption of agricultural azoles in Vietnam (Vietnamese Pesticide Assocation, 2015), confirmed in conversations between authors and local farmers, failure to detect azole residues is unlikely to indicate a lack of azole use.

Interestingly, we found a high prevalence of resistance in densely populated urban areas, where soil azole residues were seldom detected. Although seemingly paradoxical, this may be explained by resistance being ‘transported’ into urban areas by wind dispersal of conidia from neighbouring farming areas or in association with farm produce. Produce from farming areas is brought into the city in large quantities for sale in markets and by the roadside. There is significant

waste associated with the sale of fruit and vegetables, and this azole-contaminated composting waste is an ideal environment for proliferation of resistant *Aspergillus,* as recognized in a Dutch study of environmental 'hot-spots' (Schoustra *et al.*, 2019). Exploratory data supporting this suggestion showed that although 20/24 (83.3%) of urban ASF isolates had non-wild type azole MICs, these were observed more frequently in air samples (12/12, 100%) than soil samples 2/6 (33.3%) (Table S7). A recent report from the UK also showed high prevalence of ARAF in urban (13.8%) *versus* rural environments (1.1%) (Sewell *et al.*, 2019). Azole residues were not measured, but the authors linked the high prevalence of resistance to azole use in flower cultivation. This explanation could also apply in Vietnam since azoles are likely used in flowerpots, hanging baskets, and roadside plantings, although we did not sample these in this study. Regardless of how the spores get into urban settings, our results show that urban populations in Vietnam are exposed to high environmental burdens of air-borne azole-resistant *Aspergillus*. The co-location of large numbers of people, including many TB survivors prone to *Aspergillus* infection, and resistant *Aspergillus* species is concerning for public health in the region.

In our study, the TR_{34}/L98H mechanism of resistance dominated as it has in other regions (Mellado *et al.*, 2007; Snelders *et al.*, 2008; Howard *et al.*, 2009b; Lockhart *et al.*, 2011; Mortensen *et al.*, 2011; Burgel *et al.*, 2012; Chowdhary, Kathuria, Randhawa, *et al.*, 2012; Chowdhary, Kathuria, Xu, *et al.*, 2012; Badali *et al.*, 2013; Vermeulen *et al.*, 2013; Seyedmousavi *et al.*, 2013). The presence of TR_{34}/L98H in Vietnam confirms that this mechanism is global, and raises questions about how spread occurs: airborne migration of resistant TR_{34}/L98H-bearing spores, transport of contaminated goods and produce, or spontaneous local development under selective pressure?

In the context of Vietnam with limited laboratory capacity to diagnose fungal infections or test for antifungal susceptibility, it is extremely challenging for clinicians to confirm the causative fungal pathogens and to select appropriate antifungal therapy. International treatment guidelines for CPA recommend empiric treatment with azole antifungals - our findings highlight the urgency of investigating clinical isolates for resistance, as existing empiric azoles may not be suitable for patients in Vietnam.

Our study has several limitations. First, it was conducted in only one province of Vietnam and hence may not be generalizable to the rest of the country. However, in a small pilot study of 10 air samples in Ho Chi Minh City, ~300 km northeast of Ca Mau, we isolated 14 *A. fumigatus* of which seven were azole-resistant and carried the TR_{34}/L98H genotype. Second, it would have been useful to collect azole usage information in Ca Mau to supplement the data on contamination of soils by azole residues. Due to resources, we have thus far only investigated resistance mutations of *cyp51A* – further molecular investigations would likely have identified other mechanisms. Finally, although international studies indicate that the prevalence of resistance in human pathogens reflects that in the environment (Rhodes *et al.*, 2021), it is imperative that our environmental findings are now correlated with clinical isolates.

In conclusion, the present survey is the most comprehensive study on the prevalence of environmental ARAF in Southeast Asia to date, and raises significant 'red flags' for the region. The study should be urgently replicated in countries surrounding the Mekong Basin, especially since spores are readily transmissible by air and resistance could spread between regions and countries. Surveillance of clinical isolates is also urgently needed. The high resistance rates in this region have potentially serious implications for existing *Aspergillus* treatment guidelines, where empiric azole therapy may not be fit for purpose.

Experimental Procedures

Environmental sampling

From January - March 2019, we collected 450 samples of air, soil and decomposing leaves (or water if sampling site was in a body of water) from 150 different locations across Ca Mau, a large rural province in southern Vietnam's Mekong delta region (population 1.4 million). Vietnam is administratively divided into provinces, districts, and wards and we sampled wards representative of the five key land use types for the province: national park (n = 30), rice farm (n = 30), fruit farm (n = 15), shrimp farm (n = 45) and urban residential area (n = 30) (Figure 1). A team of five local field workers assigned each ward a predominant land use by consensus, and we then randomly generated geographic coordinates for sampling sites across relevant wards. Field workers used mobile global positioning system (GPS) devices to locate sites (to within 10 meters or within a 200 meters radius of the GPS location, if the precise location was inaccessible). At each site, we collected air samples with an Oxoid^{TM} Air Sampler (100 liters/minute for 10 minutes) with airflow directed onto 1) a dichloran rose bengal chloramphenicol (DRBC) plate, and 2) a DRBC plate supplemented with 4 mg/L itraconazole. We also collected two ground samples including soil (at a depth of 7-10 cm), decomposing leaves (via a swab) or water. All samples were individually sealed in zip-lock bags transported in a cool box with ice-packs to the Oxford University Clinical Research Unit in Ho Chi Minh City (HCMC) within 24 hours.

Isolation and identification of *A. fumigatus* isolates

Soil: five grams of soil were suspended in 15 mL of sterile saline with 0.1% Tween 20, and vortexed thoroughly. Soil samples were heated at 75 °C for 30 minutes to optimize *A. fumigatus*

yields, as previously described (Nováková *et al.*, 2014; Talbot *et al.*, 2018); 100 µL aliquots were plated onto a maltose extract agar supplemented with 100 mg/L chloramphenicol (MEAC), and a MEAC plate supplemented with 4 mg/L itraconazole (MEAC/ITC). Throughout, we used MEAC/ITC plates to enhance detection of azole resistant isolates, in case overall rates of resistance were low, but used isolates from MEAC plates for all primary analyses.

Decomposing leaves swab / water: swabs of decomposing leaves were soaked in 9 mL of sterile saline with 0.1% Tween 20, vortexed thoroughly, and removed before centrifuging. For water samples, ten mL was centrifuged at 10,000 rpm for 10 minutes to concentrate particulate matter including fungal spores. The resulting pellet was re-suspended in 200 µL of sterile distilled water and diluted ten-fold; 100 µL aliquots were plated onto MEAC and MEAC/ITC.

We incubated all plates (including the air sample plates) at 37^{o}C for 1 to 3 days and inspected daily. Mould colonies of different morphotype were distinguished and counted manually. We collected one colony representative of each *Aspergillus* morphotype and any indeterminate colony morphotypes from every plate. These were sub-cultured onto new plates and incubated for 5 days until pure cultures were obtained.

Isolates were identified based on morphology (Samson *et al.*, 2007, 2014) and β-tubulin sequencing using the primer pair Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995). The nucleotide sequences obtained were compared with those already deposited in the data bank of the National Center for Biotechnology and Information (NCBI), using BLAST search tool (Altschul *et al.*, 1990). The identification of the species was determined based on the best score.

We calculated recovery rates (%) for *A. fumigatus* and other ASF species from MEAC plates, by counting the number of samples with at least one colony and dividing that figure by total number of samples for that sample type.

Antifungal susceptibility testing

We tested the susceptibility of all *A. fumigatus sensu stricto* and randomly selected non-*fumigatus* ASF species to itraconazole, posaconazole, voriconazole, and amphotericin B using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) microdilution method (version E.DEF 9.3.2 April 2020). *A. fumigatus* ATCC 204305 and *Candida krusei* ATCC 6258 were included as quality control (QC) strains in every run of the assay. Results for QC strains were consistently within the acceptable EUCAST defined ranges for those testing agents. Purity of cell suspension of isolates and controls was checked. Minimal inhibitory concentrations (MICs) for each strain were determined in triplicate. We report *A. fumigatus* results as resistant / susceptible according to EUCAST Breakpoint Tables version 10.0, 2020 (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs for antifungal agents, version 10.0, 2020), whilst other species from ASF are reported as wild-type (WT) / non-wild type (NWT) (The European Committee on Antimicrobial Susceptibility Testing. Overview of antifungal ECOFFs and clinical breakpoints for yeasts and moulds using the EUCAST E.Def 7.3 and E.Def 9.3 procedures. Version 1.0, 2020). We reported resistance / NWT rates as percentage (95% confidence interval).

Detection of residual fungicide in soil samples

Residual concentrations of the five agricultural azoles most widely used in Vietnam (difenoconazole, propiconazole, tebuconazole, bromuconazole, epoxiconazole (Vietnamese

Pesticide Assocation, 2015)) were measured in soil samples. Azoles were extracted using the QuEChERS method (Anastassiades *et al.*, 2003), and the concentration determined by liquid chromatography coupled with tandem mass spectrometry (6410 Triple quad LC-MS/MS model, Agilent, USA). The minimum limits of detection and quantification for all tested compounds were 0.003 and 0.01 mg/kg, respectively.

DNA extraction for sequence analysis

Genomic DNA was extracted using a modified MasterPure^{TM} yeast DNA purification (Lucigen Corporation, Cambridge, UK) protocol, which included an additional two-step bead-beating treatment to enhance DNA yield (Abdolrasouli *et al.*, 2015). Cultured *A. fumigatus* conidia suspended in lysis solution were subjected to bead beating with 1.0 mm zirconia/silica beads (BioSpec) for 2×45 seconds using a vortex mixer and placed on ice for 2 minutes before repeating. DNA was precipitated with isopropanol and quantified using a NanoDrop (Thermo Fisher Scientific Corporation, MA, USA).

Sequencing of *cyp51A* gene

For all ARAF, we amplified the entire *cyp51A* region (GenBank accession no. AF338659) and its promoter by PCR in four fragments (promotor region: TR34-F 5'-TAATCGCAGCACCACTTCAG-3' and TR34-R 5'-GCCTAGGACAAGGACGAATG-3'; CDS fragment 1: *CYP*1-L 5'-CACCCTCCCTGTGTCTCCT-3' and *CYP*1-R 5'-AGCCTTGAAAGTTCGGTGAA-3'; CDS fragment 2: *CYP*2-L 5'-CATGTGCCACTTATTGAGAAGG-3' and *CYP*2-R 5'- CCTTGCGCATGATAGAGTGA-3'; CDS fragment 3: *CYP*3-L 5'-TTCCTCCGCTCCAGTACAAG-3' and *CYP3*-R 5'-CCTTTGAAGTCCTCGATGGT-3') (Chen *et al.*, 2005). Each fragment was sequenced from both 5’ and 3’ ends (Apical Scientific Sdn. Bhd, Malaysia). Assembled sequences were aligned against

the wild-type (GenBank accession no. AF338659) to inspect for nucleotide changes using the Fungal Resistance Database (FunResDb) (Weber *et al.*, 2018).

Statisitcal methods

Confidence intervals for proportions were calculated using the Clopper-Pearson exact method. We calculated odds ratios for isolates resistant to itraconazole (the most clinically important anti-fungal agent in Vietnam) in rice, shrimp and fruit farm samples (i.e. cultivated land), and rural residential areas in comparison with national park land samples using logistic regression with and without correction for detection of agricultural azoles in soil (glm function in R version 3.6.3 (R Core Team (2014), 2014).

Acknowledgments and Conflict of Interest

We thank Prof David Denning and LIFE (Leading International Fungal Education, http://www.life-worldwide.org/) for input on early sampling design. Prof Nguyen Viet Nhung and National Lung Hospital, Hanoi, Vietnam, for providing ethical approval for the study. Staff at Woolcock Vietnam, for their assistance in performing field work. CSDP (Centre for Social Disease Prevention), Ca Mau, Vietnam, for the local approval of environmental sampling. None of the authors have any relevant conflicts of interest to declare.

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List of Tables and Figures

Table 1. Global prevalence of azole resistance for environmental *Aspergillus fumigatus* complex isolates.

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Table 3. Azole fungicide residues detected in soil samples from different land use types within Ca Mau province, Vietnam.

Table 4. Antifungal susceptibility phenotypes by different *cyp51A* mutations, for all identified azole-resistant *Aspergillus fumigatus sensu stricto* isolates (*n* = 56).

Figure 1. Environmental sampling sites in Ca Mau province, Vietnam, showing where resistant *Aspergillus fumigatus sensu stricto* isolates and agricultural azoles were detected. Inset, Vietnam with Ca Mau province highlighted in red. Land use, by ward, colour coded. Black dot = sampling site; red dot = azole-resistant *Aspergillus fumigatus sensu stricto* detected; black cross = residual agricultural azole detected.

Figure 2. EUCAST MIC distributions of triazoles against *Aspergillus* section *Fumigati* isolates by land use type. Antifungal susceptibility of all tested *Aspergillus* section *Fumigati* (A) and all *Aspergillus fumigatus sensu stricto* (B). ITC, itraconazole; POS, posaconazole; VRC, voriconazole.