Drug-Resistant Aspergillus flavus Is Highly Prevalent in the Environment of Vietnam: A New Challenge for the Management of Aspergillosis?

Tra My N. Duong 1,2, Phuong Tuyen Nguyen 2, Thanh Van Le 2, Huong Lan P. Nguyen 3, Bich Ngoc T. Nguyen 4,5, Bich Phuong T. Nguyen 6, Thu Anh Nguyen 1,6, Sharon C.-A. Chen 1,7, Vanessa R. Barrs 1,8, Catriona L. Halliday 1,7, Tania C. Sorrell 1,9, Jeremy N. Day 2,10 and Justin Beardsley 1,2,9,*

1 Marie Bashir Institute for Infectious Diseases and Biosecurity, The University of Sydney, Sydney 2145, Australia; mydnt@oucru.org (T.M.N.D.); thanh.nguyen@sydney.edu.au (T.A.N.); Sharon.Chen@health.nsw.gov.au (S.C.-A.C.); vanessa.barrs@cityu.edu.hk (V.R.B.); Catriona.Halliday@health.nsw.gov.au (C.L.H.); tania.sorrell@sydney.edu.au (T.C.S.)
2 Oxford University Clinical Research Unit, Ho Chi Minh City 70000, Vietnam; nguyenptuyen@gmail.com (P.T.N.); lethanhvan150895@gmail.com (T.V.L.); jday@oucru.org (J.N.D.)
3 Hospital for Tropical Diseases, Ho Chi Minh 70000, Vietnam; bshuonglan@gmail.com
4 National Lung Hospital, Hanoi 10000, Vietnam; ngocn4@hotmail.com
5 Tuberculosis and Lung Diseases Department, Hanoi Medical University, Hanoi 10000, Vietnam
6 Woolcock Institute of Medical Research, Hanoi 10000, Vietnam; phuong125a@gmail.com
7 Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research, NSW Health Pathology, Westmead Hospital, Sydney 2145, Australia
8 Department of Veterinary Clinical Sciences, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon Tong, Hong Kong, China
9 Westmead Institute for Medical Research, Westmead, Sydney 2145, Australia
10 Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7FZ, UK
* Correspondence: Justin.beardsley@sydney.edu.au; Tel.: +61-8627-3402

Received: 30 September 2020; Accepted: 12 November 2020; Published: 18 November 2020

Abstract: The burden of aspergillosis, especially Chronic Pulmonary Aspergillosis, is increasingly recognized, and the increasing presence ofazole-resistant environmental Aspergillus fumigatus has been highlighted as a health risk. However, a sizable minority of aspergillosis is caused by Aspergillus flavus, which is assumed to be sensitive to azoles but is infrequently included in surveillance. We conducted environmental sampling at 150 locations in a rural province of southern Vietnam. A. flavus isolates were identified morphologically, their identity was confirmed by sequencing of the beta-tubulin gene, and then they were tested for susceptibility to azoles and amphotericin B according to EUCAST methodologies. We found that over 85% of A. flavus isolates were resistant to at least one azole, and half of them were resistant to itraconazole. This unexpectedly high prevalence of resistance demands further investigation to determine whether it is linked to agricultural azole use, as has been described for A. fumigatus. Clinical correlation is required, so that guidelines can be adjusted to take this information into account.

Keywords: Aspergillus flavus; azole resistance; environmental

1. Introduction

Aspergillus are ubiquitous, globally distributed environmental saprophytes. People constantly inhale Aspergillus spores from the environment, although disease is relatively rare and requires either...
an unusually high load of spores or a weakened host. Infection, when it occurs, can result in a range of diseases; globally, the most common disease is chronic pulmonary aspergillosis (CPA), which affects more than three million people per year [1]. The mortality and morbidity of CPA can be mitigated by anti-fungal therapy. However, our ability to treat CPA and other Aspergillus infections is threatened by drug-resistant strains emerging under selective pressure from environmental contamination with anti-fungals used in agriculture [2].

Aspergillus fumigatus is the best studied Aspergillus species. In most environments, it is the species most readily isolated and causes 80–90% of human aspergillosis cases [3]. There is a relatively large body of evidence on its global environmental distribution, drug resistance, and mechanisms of resistance. Over the last decade, azole-resistance has emerged in A. fumigatus and has been well-described internationally, with prevalence rates of 2–14% [4–13] that increase to over 30% in selected environments with heavy azole contamination, including Vietnam [14,15]. Azole resistance is most frequently conferred by mutations in the cyp51a gene [16].

In contrast, less is known about Aspergillus species from section Flavi, the second leading human pathogenic Aspergillus, accounting for 15–20% of infections [17]. This section contains several species complexes including Aspergillus flavus, Aspergillus oryzae, Aspergillus tamarii, Aspergillus parasiticus, Petromyces alliaceus, Aspergillus nomius, Aspergillus qizutongi, Aspergillus beijingensis, and Aspergillus novoparasiticus [18]. The A. flavus complex contains the most important human pathogens, implicated in infections ranging from CPA to fungal keratitis. It is difficult to differentiate A. flavus sensu stricto from other species in the complex, so diagnostic laboratories generally report isolates as A. flavus species complex.

Evidence from Asia, the Middle East, and Africa identifies A. flavus as the predominant species in clinical isolates [17]. For example, in a study of CPA patients in Pakistan, A. flavus was the infecting organism in 44% of cases, compared to A. fumigatus, which was found in 33% of cases [19]. On environmental sampling, A. flavus is generally amongst the top three most frequently isolated Aspergillus species. Again, as might be expected from the human data, it is isolated more frequently than A. fumigatus in some settings [20]. A. flavus appears well adapted to hot humid conditions [20]. Although detailed surveillance data are lacking, this raises the possibility that A. flavus may play an outsized role in CPA in countries of Africa and Southeast Asia, which also have high burdens of susceptible people because of high tuberculosis (TB) incidence.

Unlike A. fumigatus, emergence of drug resistance in A. flavus has not previously been documented. Clinical breakpoints for A. flavus have been defined for itraconazole, with sensitivity to doses <1 mg/L indicating susceptibility, and to doses >2 mg/L indicating a resistant phenotype, using European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods [21]. Epidemiological cut-off values (ECV) are defined for posaconazole (0.5 mg/L), voriconazole (2 mg/L), and amphotericin B (4 mg/L) [22]. The reported prevalence of resistance to azoles in clinical isolates has been stable, generally in the range of 0–5% [23].

Due to southern Vietnam’s hot and humid climate, we hypothesized that A. flavus would be readily isolated from the environment. Furthermore, based on our experience with azole-resistant A. fumigatus in Vietnam and considering the significant environmental contamination from azoles, as a result of their poorly regulated agri-chemical use, we hypothesized that the prevalence of azole resistance in A. flavus would exceed the low levels reported elsewhere.
2. Materials and Methods

2.1. Environmental Sampling

From January to March 2019, samples were collected at 150 locations across Ca Mau—a rural province in southern Vietnam, representative of the five key land use types: national park \((n = 30)\), rice farm \((n = 30)\), fruit farm \((n = 15)\), shrimp farm \((n = 45)\), and urban residential area \((n = 30)\). At each site, we collected (1) air samples with an OxoidTM Air Sampler (100 litres/minute for 10 min), with airflow directed onto a dichloran rose–bengal chloramphenicol plate, (2) soil (at a depth of 10–15 cm), and (3) decomposing leaves (via a swab) or water (if the sampling site was in a body of 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 h.

2.2. Isolation and Identification of Aspergillus

Soil: 5 g of soil were suspended in 15 mL of sterile saline with 1% Tween 20 and vortexed thoroughly. The soil samples were heated at 75 °C for 30 min to optimize the yield of thermo-tolerant fungi, such as Aspergillus, as previously described [24,25]. The treated suspension was diluted 1:10, and 100 µL of each dilution was plated onto a maltose extract agar supplemented with 100 mg/L chloramphenicol (MEAC).

Decomposing leaves swab: the swab was soaked in 9 mL of sterile saline with 1% Tween 20, which was vortexed thoroughly, and removed before centrifuging. The resulting pellet was re-suspended in 200 µL of sterile distilled water and diluted serially 10-fold (up to \(10^{-2}\)); 100 µL of each dilution was plated onto MEAC.

Water: 5 mL of water was centrifuged at 10,000 rpm for 10 min to concentrate the fungal spores. The pellet was resuspended in 100 µL of sterile distilled water and plated on MEAC.

The plates were incubated at 37 °C for 2 to 4 days and inspected daily. From every sample plate, one colony representative of each morphotype consistent with Aspergillus section Flavi was selected for species-level identification by phenotype [26,27] and sequencing of the \(\beta\)-tubulin gene [28]. The recovery rates for \(A.\ flavus\) was calculated as the number of samples with at least one colony, divided by the total number of samples for that sample type, and reported as a percentage.

2.3. Antifungal Susceptibility Testing

All \(A.\ flavus\) sensu stricto isolates were tested for antifungal susceptibility using the EUCAST microdilution method (version E.DEF 9.3.2, April 2020) [29]. Itraconazole, posaconazole, voriconazole, and amphotericin B were chosen for clinical relevance. \(A.\ flavus\) ATCC 204304 and \(Candida\ krusei\) ATCC 6258 were included as quality control strains. Minimal inhibitory concentrations (MICs) for each strain were determined in triplicate. We reported \(A.\ flavus\) results as resistant/susceptible or non-wild type (NWT)/wild-type (WT) according to EUCAST antifungal breakpoints and ECVs (version 2.0, 2020) [22]. We reported resistance/NWT prevalence as percentages.

3. Results

3.1. Recovery Rate

The three most commonly isolated Aspergillus species from our 450 samples (at 150 sites) were \(A.\ niger\) (99 isolates, recovery rate 22%), \(A.\ flavus\) (64 isolates, 14%), and \(A.\ fumigatus\) (54 isolates, 12%).
3.2. Prevalence of Anti-Fungal Resistance

Thirty-five *A. flavus* isolates were confirmed as *sensu stricto* and underwent susceptibility testing. MIC data for the quality-control strains were consistently within the EUCAST defined ranges. The prevalence of resistant/non-wild-type MICs are presented in Table 1. Table 2 shows the prevalence of resistant/non-wild type phenotypes by land-use type. MIC ranges and geometric means are shown in Table 3 (alongside results from selected recent international surveys of *A. flavus*). Table 4 shows the detailed isolate-level MIC data. Two isolates were resistant/non-wild-type to all antifungals tested.

![Table 1](image)

**Table 1.** Prevalence of resistant/non-wild-type phenotype amongst *Aspergillus flavus sensu stricto* isolates from the Mekong Delta Region of Vietnam against commonly used anti-fungal agents.

| Resistant/Non-WT Pattern | ITC       | POS       | VRC      | AmB       |
|-------------------------|-----------|-----------|----------|-----------|
| Resistant/non-WT (n/N)   | 17/35     | 27/35     | 6/35     | 9/35      |
| Resistant/non-WT % (95% CI) | 48.6%     | 77.1%     | 17.1%    | 25.7%     |

**WT** = wild-type; **ITC** = itraconazole; **POS** = posaconazole; **VRC** = voriconazole; **AmB** = amphotericin B; **CI** = confidence interval.

![Table 2](image)

**Table 2.** Prevalence of resistant/non-wild type phenotype amongst *A. flavus sensu stricto* isolates from the Mekong Delta Region of Vietnam by land-use type.

| Land Use Type     | Azole-R Isolates/Total Isolates (%) | AmB-R Isolates/Total Isolates (%) |
|-------------------|-------------------------------------|-----------------------------------|
| National park     | 8/8 (100)                           | 3/8 (37.5)                        |
| Rice farm         | 0/3 (0)                             | 1/3 (33.3)                        |
| Fruit farm        | 1/1 (100)                           | 1/1 (100)                         |
| Aqua culture      | 12/13 (92.3)                        | 2/13 (15.4)                       |
| Urban             | 9/10 (90)                           | 2/10 (20)                         |
| All sites         | 30/35 (85.7)                        | 9/35 (5.7)                        |

**Azole-R** = resistance/non-WT MIC to any azole; **AmB** = resistant/non-WT; **MIC** = minimal inhibitory concentration to amphotericin B.

![Table 3](image)

**Table 3.** MIC ranges and geometric mean for *A. flavus sensu stricto* isolates from the Mekong Delta Region of Vietnam, compared to published MICs for environmental isolates from Brazil (*n* = 40), Iran (*n* = 79), India (*n* = 68), and Europe (*n* = 19).

| Country  | ITC        | POS        | VRC        | AmB        |
|----------|------------|------------|------------|------------|
|          | MIC Range  | MIC GM     | MIC Range  | MIC GM     | MIC Range  | MIC GM     | MIC Range  | MIC GM     |
| Vietnam  | 1–8        | 1.52       | 0.5–2      | 0.91       | 1–4        | 2.16       | 2–>16      | 4          |
| Brazil   | 0.5–5      | 1.41       | 0.03–0.25  | 0.188      | 0.5–2      | 1.017      | -          | -          |
| Iran     | 0.031–2    | 0.25       | 0.03–0.5   | 0.13       | 0.063–2    | 0.55       | 1–16       | 3.4        |
| India    | 0.03–0.125 | 0.06       | 0.015–0.06 | 0.022      | 0.15–1     | 0.5        | -          | -          |
| Europe * | 0.03–0.25  | -          | 0.06–0.125 | -          | 0.125–0.25 | -          | -          | -          |

**GM** = geometric mean. * These MICs were determined using Sensititre YeastOne (Thermo Fisher Scientific, Waltham, MA, USA). **Bold** = previously unpublished results from this project.
Table 4. Anti-fungal MIC values of *A. flavus sensu stricto* isolates from the Mekong Delta Region of Vietnam (*n* = 35).

| *A. flavus sensu stricto* Isolate ID | MIC (µg/mL) | ITC | POS | VRC | AmB |
|--------------------------------------|-------------|-----|-----|-----|-----|
| FL_1                                 | 1           | 1   | 2   | 4   |     |
| FL_2                                 | 8           | 2   | 4   | 8   |     |
| FL_3                                 | 2           | 1   | 2   | 2   |     |
| FL_4                                 | 1           | 1   | 2   | 4   |     |
| FL_5                                 | 2           | 1   | 2   | 4   |     |
| FL_6                                 | 1           | 1   | 2   | 4   |     |
| FL_7                                 | 1           | 1   | 2   | 4   |     |
| FL_8                                 | 1           | 0.5 | 2   | 4   |     |
| FL_9                                 | 2           | 1   | 2   | 8   |     |
| FL_10                                | 1           | 1   | 2   | 2   |     |
| FL_11                                | 1           | 1   | 2   | 4   |     |
| FL_12                                | 1           | 1   | 2   | 4   |     |
| FL_13                                | 1           | 1   | 1   | 4   |     |
| FL_14                                | 1           | 0.5 | 1   | >16 |     |
| FL_15                                | 4           | 2   | 4   | 4   |     |
| FL_16                                | 2           | 1   | 2   | 2   |     |
| FL_17                                | 1           | 1   | 2   | 4   |     |
| FL_18                                | 1           | 1   | 2   | 4   |     |
| FL_19                                | 1           | 0.5 | 2   | 16  |     |
| FL_20                                | 2           | 2   | 2   | 2   |     |
| FL_21                                | 2           | 1   | 2   | 4   |     |
| FL_22                                | 4           | 1   | 4   | 8   |     |
| FL_23                                | 1           | 1   | 2   | 8   |     |
| FL_24                                | 2           | 1   | 2   | 8   |     |
| FL_25                                | 2           | 1   | 2   | 4   |     |
| FL_26                                | 1           | 0.5 | 2   | 2   |     |
| FL_27                                | 2           | 0.5 | 2   | 2   |     |
| FL_28                                | 2           | 1   | 4   | 4   |     |
| FL_29                                | 1           | 1   | 2   | 2   |     |
| FL_30                                | 2           | 1   | 4   | 4   |     |
| FL_31                                | 2           | 1   | 2   | 4   |     |
| FL_32                                | 1           | 0.5 | 4   | 8   |     |
| FL_33                                | 2           | 0.5 | 2   | 8   |     |
| FL_34                                | 1           | 0.5 | 2   | 4   |     |
| FL_35                                | 2           | 1   | 2   | 2   |     |
4. Discussion

The anti-fungal MICs of our isolates are a cause of alarm. The geometric mean is above the non-wild-type ECV for voriconazole, posaconazole, and amphotericin B. For itraconazole, the only agent with defined clinical breakpoints, almost half of the isolates were frankly resistant.

The MICs of our isolates are significantly higher than those in recent global reports. Table 3 highlights the differences, contrasting our results with those of comparable recent studies from 2017 in Brazil [30], 2018 in Iran [31], and 2018 in India [32]. Although not directly comparable, since results were obtained using Sensititre YeastOne, 2017 data from Europe are also presented [33].

We have not yet investigated the mechanisms underlying the decreased susceptibilities observed. In A. flavus, resistance is often conferred by efflux pumps, which become upregulated on exposure to azoles [32]. Our isolates may have had such exposure in the environment, since they were collected in a region of intensive agriculture where agri-chemicals are poorly regulated, and azole residues can be detected in cultivated soils (our unpublished data). However, in contrast to A. fumigatus, a link between agricultural azole use and resistance has not yet been found for A. flavus. Interestingly, we did not observe a lower prevalence of resistance in a national park compared to cultivated or urban land. However, the sample size for each land-use type was too small to speculate meaningfully on the impact of land use. Further investigation is required. This is the first study of A. flavus in Southeast Asia, and we have discovered an apparent hot spot for resistance. Our study should be replicated in other locations throughout Vietnam and neighboring countries in order to determine the extent of the risk in our region.

As anticipated, due to the hot and humid climate of southern Vietnam, A. flavus was more readily isolated from the environment than A. fumigatus, indicating that people are exposed to these spores. No clinical surveillance of infecting Aspergillus species has been conducted in Vietnam, so it is currently not possible to estimate the health impact of the unprecedented rates of resistance we have identified. Alongside understanding the distribution, mechanisms, and drivers of resistance, investigating its clinical impact through detailed multi-center surveillance must be a priority.

Author Contributions: Conceptualization, T.M.N.D., J.B., H.L.P., and B.N.T.N.; methodology, T.M.N.D., T.V.L., and P.T.N.; investigation, T.M.N.D.; data curation, T.M.N.D.; writing—original draft preparation, J.B., T.M.N.D., and P.T.N.; writing—review and editing, all; supervision, T.C.S., V.R.B., S.C.-A.C., C.L.H., and J.N.D.; project administration, B.P.T.N. and T.A.N.; funding acquisition, J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Acknowledgments: We thank David Denning and LIFE (Leading International Fungal Education, http://www.life-worldwide.org/) for input on early sampling design, 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. We thank also the Sydney Institute of Agriculture, University of Sydney, who supported this project.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bongomin, F.; Gago, S.; Oladele, R.O.; Denning, D.W. Global and Multi-National Prevalence of Fungal Diseases—Estimate Precision. J. Fungi 2017, 3, 57. [CrossRef] [PubMed]
2. Verweij, P.E.; Snelders, E.; Kema, G.H.J.; Mellado, E.; Melchers, W.J.G. Azole resistance in Aspergillus fumigatus: a side-effect of environmental fungicide use? Lancet Infect. Dis. 2009, 9, 789–795. [CrossRef]
3. Latgé, J. Aspergillus fumigatus and Aspergillosis. Clin. Microbiol. Rev. 1999, 12, 310–350. [CrossRef]
4. Tangwattanachuleeporn, M.; Minarin, N.; Saichan, S.; Sermsri, P.; Mitkornburee, R.; Chindamporn, A.; Gross, U.; Bader, O. Prevalence of azole-resistant Aspergillus fumigatus in the environment of Thailand. Med. Mycol. 2016, 55. [CrossRef]
5. Chen, Y.; Dong, F.; Zhao, J.; Fan, H.; Qin, C.; Li, R.; Verweij, P.E.; Zheng, Y.; Han, L. High Azole Resistance in Aspergillus fumigatus Isolates from Strawberry Fields, China, 2018. Emerg. Infect. Dis. 2020, 26, 81–89. [CrossRef] [PubMed]

6. Chowdhary, A.; Sharma, C.; Boom, M.V.D.; Yntema, J.B.; Hagen, F.; Verweij, P.E.; Meis, J.F. Multi-azole-resistant Aspergillus fumigatus in the environment in Tanzania. J. Antimicrob. Chemother. 2014, 69, 2979–2983. [CrossRef] [PubMed]

7. Ahmad, S.; Khan, Z.; Hagen, F.; Meis, J.F. Occurrence of triazole-resistant Aspergillus fumigatus with TR34/L98H mutations in outdoor and hospital environment in Kuwait. Environ. Res. 2014, 133, 20–26. [CrossRef]

8. Badali, H.; Vaezi, A.; Haghani, I.; Yazdanparast, S.A.; Hedayati, M.T.; Weig, M.; Groß, U. Environmental Isolates of Azole-Resistant Aspergillus fumigatus in Germany. Antimicrob. Agents Chemother. 2015, 59, 4356–4359. [CrossRef] [PubMed]

9. Sewell, T.R.; Zhang, Y.; Brickin, A.P.; Shelton, J.M.G.; Rhodes, J.; Fisher, M.C. Elevated Prevalence of Azole-Resistant Aspergillus fumigatus in Urban versus Rural Environments in the United Kingdom. Antimicrob. Agents Chemother. 2019, 63. [CrossRef] [PubMed]

10. Van der Linden, J.W.M.; Snelders, E.; Kampinga, G.A.; Rijnders, B.J.A.; Mattsson, E.; De Leenheer, P.; Sorrell, T.; Coutte, J.-M. Azole-resistant Aspergillus fumigatus in the environment of northern Italy, May 2011 to June 2012. Eurosurveillance 2014, 19, 20747. [CrossRef]

11. Bader, O.; Tünnermann, J.; van den Hoogen, J.; Gross, U.; Groß, U.; Groß, U. Environmental Isolates of Azole-Resistant Aspergillus fumigatus in Germany. Antimicrob. Agents Chemother. 2015, 67, 362–366. [CrossRef] [PubMed]

12. Duong Nu, T.M.; Nguyen, P.T.; Nguyen, T.A.; Marks, G.; Fox, G.; Chen, S.; Barrs, V.R.; Halliday, C.; Sorrell, T.; Day, J.; et al. Azole-resistant Aspergillus fumigatus is highly prevalent in Vietnam Preliminary results from Ca Mau environmental sampling study. Poster presented. In Proceedings of the Global Health Security Conference, Sydney, Australia, 18–20 June 2019.

13. Krishnan, S.; Manavathu, E.K.; Chandrasekar, P.H. Aspergillus flavus: an emerging non-fumigatus Aspergillus species of significance. Mycoses 2009, 52, 206–222. [CrossRef] [PubMed]

14. Gonçalves, S.S.; Stchigel, A.M.; Cano, J.; Guarro, J.; Colombo, A.L. In Vitro Antifungal Susceptibility of Clinically Relevant Species Belonging to Aspergillus SectionFlav. Antimicrob. Agents Chemother. 2013, 57, 1944–1947. [CrossRef]

15. Iqbal, N.; Irfan, M.; Mushtaq, A.; Jabeen, K. Underlying Conditions and Clinical Spectrum of Chronic Pulmonary Aspergillosis (CPA): An Experience from a Tertiary Care Hospital in Karachi, Pakistan. J. Fungi 2020, 6, 41. [CrossRef]

16. Arendrup, M.C.; Cuenca-Estrella, M.; Lass-Fle‘rl, C.; Hope, W. EUCAST technical note on the EUCAST definitive document EDef 7.2: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST)*. Clin. Microbiol. Infect. 2012, 18, E246–E247. [CrossRef]
22. EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Overview of Antifungal ECOFFs and Clinical Breakpoints for Yeasts, Moulds and Dermatophytes Using the EUCAST E.Def 7.3, E.Def 9.3 and E.Def 11.0 Procedures. Version 2.0, 2020; 1–8. Available online: http://www.eucast.org (accessed on 15 September 2020).

23. Rudramurthy, S.M.; Paul, R.A.; Chakrabarti, A.; Mouton, J.W.; Meis, J.F. Invasive Aspergillosis by Aspergillus flavus: Epidemiology, Diagnosis, Antifungal Resistance, and Management. J. Fungi 2019, 5, 55. [CrossRef]

24. Talbot, J.J.; Subedi, S.; Halliday, C.; Hibbs, D.E.; Lai, F.; Lopez-Ruiz, F.J.; Harper, L.; Park, R.F.; Cuddy, W.S.; Biswas, C.; et al. Surveillance for azole resistance in clinical and environmental isolates of Aspergillus fumigatus in Australia and cyp51A homology modelling of azole-resistant isolates. J. Antimicrob. Chemother. 2018, 73, 2347–2351. [CrossRef] [PubMed]

25. Nováková, A.; Hubka, V.; Dudová, Z.; Matsuzawa, T.; Kubátová, A.; Yaguchi, T.; Kolarík, M. New species in Aspergillus section Fumigati from reclamation sites in Wyoming (U.S.A.) and revision of A. viridinutans complex. Fungal Divers. 2013, 64, 253–274. [CrossRef]

26. Samson, R.; Visagie, C.; Houbraken, J.; Hong, S.-B.; Hubka, V.; Klaassen, C.; Perrone, G.; Seifert, K.; Susca, A.; Tanney, J.; et al. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud. Mycol. 2014, 78, 141–173. [CrossRef] [PubMed]

27. Glass, N.L.; Donaldson, G.C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 1995, 61, 1323–1330. [CrossRef] [PubMed]

28. EUCAST Definitive Document E. Def 9.3.2: Method for the Determination of Broth Dilution Minimum Inhibitory Concentrations of Antifungal Agents for Conidia Forming Moulds. 2020; 1–23. Available online: http://www.eucast.org (accessed on 15 September 2020).

29. Denardi, L.B.; Dalla-Lana, B.H.; De Jesus, F.P.K.; Severo, C.B.; Santurio, J.M.; Zanette, R.A.; Alves, S.H. In vitro antifungal susceptibility of clinical and environmental isolates of Aspergillus fumigatus and Aspergillus flavus in Brazil. Braz. J. Infect. Dis. 2017, 22, 30–36. [CrossRef]

30. Armaki, M.T.; Hedayati, M.T.; Ansari, S.; Omran, S.M.; Saber, S.; Rafati, H.; Zoll, J.; Van Der Lee, H.A.; Melchers, W.J.G.; Verweij, P.E.; et al. Genetic Diversity and In Vitro Antifungal Susceptibility of 200 Clinical and Environmental Aspergillus flavus Isolates. Antimicrob. Agents Chemother. 2017, 61, e00004-17. [CrossRef]

31. Paul, R.A.; Rudramurthy, S.M.; Dhaliwal, M.; Singh, P.; Ghosh, A.K.; Kaur, H.; Varma, S.; Agarwal, R.; Chakrabarti, A. Magnitude of Voriconazole Resistance in Clinical and Environmental Isolates of Aspergillus flavus and Investigation into the Role of Multidrug Efflux Pumps. Antimicrob. Agents Chemother. 2018, 62. [CrossRef]

32. Mello, E.; Posterraro, B.; Vella, A.; De Carolis, E.; Torelli, R.; D’Inzeo, T.; Verweij, P.E.; Sanguinetti, M. Susceptibility Testing of Common and Uncommon Aspergillus Species against Posaconazole and Other Mold-Active Antifungal Azoles Using the Sensititre Method. Antimicrob. Agents Chemother. 2017, 61, e00168-17. [CrossRef]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).