Distribution of *Aspergillus* species and prevalence of azole resistance in respiratory samples from Swiss Tertiary Care Hospitals

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Abstract

Among 400 *Aspergillus* spp. from respiratory samples in Switzerland, *A. fumigatus* was the most frequent species. Non-*fumigatus Aspergillus* spp. were more prevalent among solid-organ transplant recipients and after azole exposure. Azole-resistance was detected in four *A. fumigatus* isolates, three of them with the "environmental" mutation TR_{34}/L98H in the *cyp51A* gene.

Keywords: *Aspergillus fumigatus*; Non-*fumigatus Aspergillus* spp.; Azole-resistance; *Cyp51A* gene; TR_{34}/L98H mutation.

Introduction

Aspergillus spp. cause a broad spectrum of diseases in humans, including life-threatening invasive aspergillosis (IA) in immunocompromised hosts. Infections are most frequently caused by *Aspergillus fumigatus*. Nevertheless, non-*fumigatus Aspergillus* spp. are increasingly reported as common etiologic agents in some geographic regions [1]. Antifungal triazoles are the drugs of choice for prophylaxis and treatment of IA. However, over the last two decades azole resistance among *A. fumigatus* has emerged worldwide and has been associated with a high mortality rate in immunocompromised hosts, raising significant public health concerns [2].

Azole resistance is generally driven by mutations in the *cyp51A* gene, which encodes the azole's target in the ergosterol biosynthetic pathway: the enzyme lanosterol 14-α demethylase. Two different routes of azole resistance in *A. fumigatus* have been reported in humans. In patients under long-term azole therapy, wild-type isolates may develop resistance by various mutations in hotspot regions of *cyp51A* gene [2]. Alternatively, azole-naïve patients can be infected by isolates that have already acquired resistance in the environment, as a probable consequence of the widespread use of fungicides in agriculture. Typical mutations found in isolates that have developed azole resistance in the environment include TR_{34}/L98H and TR_{46}/Y121F/T289A [2].

The prevalence of azole resistance among *A. fumigatus* is very variable according to the geographical areas [3], which warrants the need for local and national epidemiological surveys. The aims of this study were to assess species distribution, clinical setting, prevalence, and molecular mechanism of azole resistance among consecutively collected *Aspergillus* isolates from respiratory samples of patients being treated in hospitals within the Fungal Infection Network of Switzerland

(FUNGINOS).

Methods

Study design

This was a multi-centre, prospective cohort study. Consecutive patients with *Aspergillus* spp. isolated in upper and lower respiratory samples were included between January 2018 and April 2019 in all five Swiss university hospitals and two large teaching hospitals collaborating to FUNGINOS. The study was approved by all local ethics committees (Project-ID 2017-00984) and registered at ClinicalTrials.gov (NCT03443336). A general informed consent for research purposes was available for all patients included. Individual patient informed consent was not required.

Data collection and definition

Data on gender, age, underlying diseases, risk factors for invasive mold infections, mold-active azole exposure in the three months prior to *Aspergillus* spp. detection, antifungal treatment received, and 3-month outcome were collected. IA and influenza-associated pulmonary aspergillosis (IAPA) were classified according to the EORTC/MSG criteria and the consensus case definition proposed in 2020, respectively [4, 5]. In the case of multiple *Aspergillus* isolates from the same patient, the following inclusion criteria were applied: different species were always included; same species isolated at different time points were included if there was a relevant clinical change (i.e. different clinical interpretation – colonization vs. infection -, azole exposure, etc.) or an interval time ≥ 3 months between isolation.

Microbiological workup

All *Aspergillus* isolates were cultured according to routine mycological procedures and identified at the section or species level at the different participating centres according to their local routine procedures (i.e. phenotypic identification, MALDI-TOF and/or sequencing). Antifungal susceptibility testing was performed by broth microdilution using a commercial kit (Sensititre YeastOne®, ThermoScientific) [6] at Geneva University Hospital (isolates from this centre) and Lausanne University hospital (all other isolates). The results were reported as minimum inhibitory concentration $(MIC)_{50}$/MIC_{90} and MIC ranges. *Aspergillus* isolates with a MIC above the usual epidemiological cut-off values (ECV) for at least one of the mold-active triazoles (voriconazole, posaconazole, itraconazole) were submitted to partial sequencing of the beta-tubulin (*BenA*) and calmodulin (*CaM*) genes for identification at the species level [7, 8]. *Aspergillus fumigatus sensu stricto* isolates were subsequently submitted to sequencing of the entire *cyp51A* gene and promoter region for detection of mutations.

Statistical analysis

Continuous variables were expressed as median and interquartile range (IQR) and categorical variables as frequencies and percentages. Characteristics of patients with *A. fumigatus* vs. non-*fumigatus Aspergillus* spp. were compared using the Fisher's exact test for categorical and Wilcoxon rank sum test for continuous variables. Two-sided P-values of less than 0.05 were considered significant. R statistics version 3.6.3 was used for statistical analysis.

Results

During the 16-month study period, 400 *Aspergillus* spp. isolates from 365 patients were included. They were obtained from sputum (n=237/400, 59.3%), tracheobronchial aspirate (94, 23.5%), bronchoalveolar lavage (38, 9.5%), upper respiratory samples such as throat swabs or sinus samples (18, 4.5%) and bronchial/lung biopsy (13, 3.3%). The most frequent species (at section/complex level) was *A. fumigatus* complex (355, 88.8%), followed by *A. niger* complex (20, 5.0%), and *A. flavus* complex (12; 3.0%). Clinical information was available for 342 of the 365 (93.7%) included patients. The demographic and clinical characteristics are summarized in **Table 1**. Median age was 60 (IQR 31-73) years and 51.8% were male. At the time of sampling, 58.8% of the patients were hospitalized (n=201/342), 27.9% of them (n=56/201) in an intensive care unit (ICU). Overall, 13 patients (3.8%) had received a prior mold-active azole therapy. The most prevalent underlying diseases were chronic lung diseases (n=170/342, 49.7 %), cystic fibrosis (97, 28.4%), and solid or hematological malignancies (50, 14.6%). Forty patients (40, 11.7%) were diagnosed with IA (11 proven, 29 probable) and 37 received mold-active therapy. Three-month mortality rate among patients with IA was 27.5% (n=11). The remaining 302 patients (88.3%) were considered to be colonized. Species distribution and prevalence of azole resistant strains were similar between the groups of infected and colonized patients (supplementary Table S1).

In 23 patients (6.7%), the isolation of *Aspergillus* spp. occurred in the context of an ongoing infection with influenza: four of 23 (17.4%) were diagnosed with IAPA and were treated with mold-active therapies. Among patients with cystic fibrosis, all isolates except one (n=96/97, 99.0%) were interpreted as being colonizers.

Forty-five of 400 *Aspergillus* isolates were non-*fumigatus Aspergillus* species (11.3%). They were detected more often among solid organ transplant recipients and patients who had received prior azole therapy within the last three months (**Table 1**).

Antifungal susceptibility testing results are summarized in Table S2, showing geometric means (GMs), MIC causing inhibition of 50% and 90% of the isolates tested (MIC_{50} and MIC_{90}, respectively), and MIC ranges of all antifungals tested.

Five strains (n=5/400, 1.3%) showed a high MIC for at least one of the mold-active triazoles: four *A. fumigatus* and one *A. calidoustus* strains. The main clinical and microbiological features associated with the detection of these resistant isolates are summarized in the supplementary Table S3. The sequencing analysis of the four *A. fumigatus* isolates revealed the presence of the mutation TR_{34}/L98H, typically found in environmental isolates, in three strains and the point mutation M220K in one. One of the TR_{34}/L98H mutant strains was detected in an onco-hematological patient, who was subsequently diagnosed with a disseminated invasive aspergillosis with osteovertebral, pulmonary and cerebral involvement. Three mutant strains derived from a single centre. The prevalence of azole resistant *A. fumigatus* was 1.1% (4/355). The overall prevalence of azole resistant strains was 1.3% considering all *Aspergillus* isolates (5/400) and 1.4% at patient level (5/365).

Discussion

This FUNGINOS study provides a representative survey of species distribution and susceptibility to triazoles of *Aspergillus* spp. isolated from respiratory tract samples in Switzerland.

In accordance with previous reports, *A. fumigatus* was the most frequently isolated species. We found *A. niger* as the most common non-*fumigatus Aspergillus* spp.. By

contrast, data from the United States, Brazil and other European countries describe *A. flavus* as the most common non-*fumigatus Aspergillus* in medical centers [9-11].

Our study demonstrates a low prevalence (1.1%) of azole resistant *A. fumigatus* in clinical samples in Switzerland. The similar species and resistance distribution observed in our cohort between infected and colonized patients, supports using the global dataset of *Aspergillus* isolates as a clinically meaningful estimation of azole resistance. The mutation TR_{34}/L98H, typically found in environmental isolates, is the most prevalent resistance mechanism detected in our setting. The presence of this mutation in Switzerland was first reported in 2018, initially from environmental samples of *A. fumigatus* and, subsequently, in clinical samples from two patients suffering from cystic fibrosis from a single centre [12]. Our data are consistent with that reported in some of the neighbouring countries, where the prevalence of azole resistant *Aspergillus* spp. ranges between 1.3 and 3.5% [3]. In contrast, azole resistance is an increasing concern in some other European countries, most notably in the Netherlands where the rate of azole resistance is 11% among *A. fumigatus* isolates. Resistance rates significantly increased in recent years (from 8 to 15% in the period 2013-2018), and the mutations TR_{34}/L98H and TR_{46}/Y121F/T289A account for most cases [13].

The reason for these differences in species distribution and antifungal susceptibilities may be attributed to geographical and ecological diversity, frequency of underlying diseases and the use of broad-spectrum antifungal agents in hospitals and/or agriculture [10, 11].

This study has several limitations. First, it was not designed to screen for the presence of resistant isolates in the environment and, therefore, does not allow tracing the source of the azole resistant strains found in clinical samples. Second,

the lack of dedicated methods for selective isolation of azole resistant *Aspergillus* isolates in routine laboratory practices may underestimate the actual prevalence of resistance. Third, we used a commercial broth microdilution assay (Sensititre YeastOne®) rather than the EUCAST reference method for the antifungal susceptibility testing. This may result in differences in MIC results and potentially reduce the ability to detect resistant strains. Nevertheless prior studies found good essential agreements with the reference methods [6, 14] and, therefore, we consider it unlikely that this affects the validity of our results.

In conclusion, azole resistance among *Aspergillus* spp. has emerged worldwide and represents a serious public health concern. The prevalence in Switzerland is currently low, therefore the use of triazoles as first line for the empirical management of invasive infections seems to be appropriate. However, periodical surveillance studies are of paramount importance in order to inform the local epidemiology and to monitor the development and spread of mutant strains. Concurrently, the assessment of environmental samples is equally important for early identification of hotspots, which may serve as reservoirs of azole resistant *Aspergillus* spp.

Patient consent statement

A general informed consent for research purposes was available for all patients included. Individual patient informed consent was not required. The study was approved by all local ethics committees (Project-ID 2017-00984) and registered at ClinicalTrials.gov (NCT03443336).

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Potential conflicts of interest

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Table 1. Demographic and clinical characteristics of the patients stratified by fungal pathogen and clinical manifestation.

	All patients (n=342)	Invasive aspergillosis (n=40)	*A. fumigatus* (n=306) *	Non-*fumigatus Aspergillus* [&] (n=29) *	P-value °
Age, median (IQR), years	60 (31-73)	62 (51-64)	60 (30-73)	57 (45-69)	>0.9
Male, n (%)	177 (51.8)	26 (65.0)	158 (51.6)	15 (51.7)	> 0.9
Prior azole therapy [§], n (%)	13 (3.8)	8 (20.0)	8 (2.6)	4 (13.8)	**0.02**
Underlying diseases, n (%)					
COPD/lung diseases	170 (49.7)	13 (32.5)	150 (49.0)	14 (48.3)	0.3
Cystic fibrosis	97 (28.4)	1 (2.5)	90 (29.4)	5 (17.2)	0.2
Malignancies	50 (14.6)	11 (27.5)	46 (15.0)	3 (10.3)	0.7
Solid tumors	39 (11.4)	3 (7.5)	36 (11.8)	3 (10.3)	> 0.9
Hematologic malignancies	11 (3.2)	8 (20.0)	10 (3.3)	0 (0.0)	> 0.9
Transplantation	31 (9.1) [#]	16 (40.0) [#]	23 (7.5)	7 (24.1)	**0.02**
SOT	25 (7.3) [#]	11 (27.5) [#]	17 (5.6)	7 (24.1)	**0.006**
HCT	7 (2.0) [#]	6 (15.0) [#]	6 (2.0)	0 (0.0)	> 0.9
Diabetes	38 (11.1)	5 (12.5)	33 (10.8)	5 (17.2)	0.5
Active influenza infection	23 (6.7)	4 (10.0)	19 (6.2)	4 (13.8)	0.2
Autoimmune diseases	18 (5.3)	1 (2.5)	13 (4.2)	4 (13.8)	0.1
Chronic renal failure	44 (12.9)	8 (20.0)	41 (13.4)	3 (10.3)	0.8
Hospital admission, n (%)	201 (58.8)	39 (97.5)	183 (59.8)	14 (48.3)	0.4
ICU admission, n (%)	56 (16.4)	13 (32.5)	49 (16.0)	6 (20.7)	0.3
Antifungal therapy, n (%)	44 (12.9)	37 (92.5)	37 (12.1)	5 (17.2)	0.6
Mortality, n (%)	38 (11.1)	11 (27.5)	37 (12.1)	1 (3.4)	0.3

IQR: interquartile range; COPD: chronic obstructive pulmonary disease; SOT: solid organ transplant; HCT: hematopoietic cell transplantation; ICU: intensive care unit.

* Seven patients had both *A. fumigatus* and non-*fumigatus* in respiratory samples and they were excluded from this analysis.

[§] Mold-active triazoles received in the three months prior to *Aspergillus* spp. detection.

[&] Non-*fumigatus Aspergillus* species: *A. niger* (n=14), *A. flavus* (n=6), *A. nidulans* (n=3), *A. terreus* (n=2), *A. amstelodami* (n=1), *A. versicolor* (n=1), *A. nomius* (n=1), *A. welwitschiae* (n=1).

° P-values refer to the comparison between patients with *A. fumigatus* and those with non-*fumigatus Aspergillus* spp.

[#] One patient had in his past medical history both SOT and HCT.