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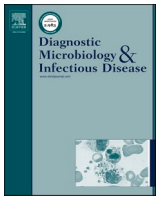

Antifungal susceptibility of a collection of *Aspergillus fumigatus* strains isolated from patients with invasive pulmonary aspergillosis and COVID-19 associated aspergillosis

Andrea Liberatore [a], Giulia Lombardi [a], Donatella Lombardo [a], Tiziana Lazzarotto [a,b], Claudio Foschi [a,b,*], Simone Ambretti [a,b]

[a] Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria of Bologna, Bologna, Italy
[b] Section of Microbiology, Department of Medical and Surgical Sciences, Alma Mater Studiorum - University of Bologna, Bologna, Italy

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ABSTRACT

We assessed the antifungal susceptibility of 86 *Aspergillus fumigatus* strains by a phenotypic test. Azole sensitivity was compared to a molecular test detecting cyp51A mutations. Azole resistance was quite limited, whereas strains from COVID-19 patients showed higher amphotericin B MICs. The molecular test showed a 100 %-agreement with the phenotypic assay.

1. Introduction

Invasive pulmonary aspergillosis (IPA) is a life-threatening fungal disease, mainly affecting immunocompromised patients [1]. Nevertheless, IPA can be diagnosed also in immunocompetent subjects, such as those with Sars-Cov-2 (COVID-19) infections (CAPA) [2].

Aspergillus fumigatus represents the most frequently isolated species, causing more than 70 % of IPA cases [3].

Currently, azoles such as voriconazole and isavuconazole, are the first line drugs for IPA treatment [4]. However, in the last decades the extensive use of azoles both in long-term treatments and in the agriculture field, has led to the emergence of azole resistance in *A. fumigatus* [5,6]. This resistance is primarily caused by alterations to the sterol biosynthesis pathway, resulting from point mutations of cyp51A and the insertion of tandem repeats (TR) in the promoter region of cyp51A (such as TR34/L98H and TR46/Y121F/T289A) [7,8].

In this scenario, the in-vitro evaluation of antifungal susceptibility of *A. fumigatus* strains is crucial to monitor azole resistance prevalence and to set up effective strategies for patient management. Considering that the reference broth microdilution method developed by EUCAST to assess antifungal susceptibility (available at: www.eucast.org) is time-consuming and labor-intensive, several commercial tests have been successfully introduced for antifungal-resistance surveillance [9].

Phenotypic methods can be hampered by slow growth rates and technical difficulties; thus, several fast and reliable molecular tools have been developed to detect resistance markers directly from clinical samples or from fungal colonies [10].

In this study we investigated the in-vitro susceptibility to antifungal drugs on a collection of 86 *A. fumigatus* strains isolated from patients with probable IPA or CAPA at IRCSS Policlinico S. Orsola in Bologna (Italy) between 2018 and 2023. Phenotypic results were compared with a genotypic test able to detect mutations in the cyp51A gene.

2. Materials and methods

2.1. Strain collection

Retrospectively, 86 strains of *Aspergillus fumigatus* were selected from a collection of moulds isolated during routine diagnostic procedures and stored at the Microbiology Unit of IRCCS Policlinico Sant'Orsola in Bologna (Italy). In this setting antifungal susceptibility testing of moulds is not routinely performed.

The fungal isolates were recovered from lower respiratory tract specimens of hospitalized patients with probable IPA (n = 65) or CAPA (n = 21) in the period 2018-2023 (43 strains in 2018-2020 and 43 in 2021-2023). The diagnosis of these conditions was performed following

* Corresponding author at: Section of Microbiology, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna; IRCCS Azienda Ospedaliero-Universitaria di Bologna, Via Massarenti 9, Bologna, Italy.
E-mail address: claudio.foschi2@unibo.it (C. Foschi).

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EORTC/MSGERC criteria [11].

Aspergillus species identification was achieved by macroscopic and microscopic evaluation of the fungal colonies and confirmed by MALDI-ToF mass spectrometry (Bruker Daltonics, Bremen, Germany). All the strains were frozen at -80°C and thawed at the time of analyses.

2.2. Antifungal susceptibility testing

Each *A. fumigatus* strain underwent an antifungal susceptibility testing, using 'MICRONAUT-AM' (Merlin, Bruker Daltonics), a commercial broth microdilution test [12].

Strains were cultured for 48h on Sabouraud Dextrose Agar at 32°C and afterwards, the antifungal susceptibility testing was performed according to the manufacturer's instructions. Obtained Minimum Inhibitory Concentration values (MICs), categorized following EUCAST guidelines, were correlated to the year of strain isolation (2018-2020 vs 2021-2023) and to the presence/absence of COVID-19.

2.3. Molecular detection of azole-resistance markers

A multiplex real-time PCR assay (Fungiplex *Aspergillus* Azole - R IVD PCR; Bruker Daltonics) was used to evaluate the presence of azole resistance markers (TR34 and TR46 mutations in the cyp51A gene) on all the isolates [13].

The extraction of nucleic acids from fungal colonies and the subsequent amplification were run on ELITeInGenius instrument (EliTechGroup, Puteaux, France).

2.4. Statistical analysis

An unpaired t test was used to evaluate statistically significant differences among experimental categories. A p value < 0.05 was considered as statistically significant.

3. Results

3.1. Prevalence of antifungal resistance and MIC distribution

Table 1 shows the resistance rate, the MIC_{50} and the MIC_{90} of all the antifungal drugs tested [14]. Out of the 86 *A. fumigatus* strains analysed, 2 of them (2.3 %) showed a resistance to an azole drug (1 to itraconazole, MIC = 2 mg/L and 1 to voriconazole, MIC = 8 mg/L), whereas 11 (12.8 %) were resistant to amphotericin B (MIC = 2 mg/L). The two strains resistant to azoles were susceptible to amphotericin B (MIC = 1 mg/L).

Table 1
Resistance rate, MIC_{50} and MIC_{90} for all the antifungal drugs tested on the collection of 86 *A. fumigatus* strains. The resistance rate is reported only for the antifungals for which EUCAST set clinical breakpoints (www.eucast.org). MIC_{50} is defined as the MIC value at which ≥50 % of the isolates are inhibited whereas MIC_{90} is defined as the MIC value at which ≥90 % of the strains are inhibited.

Antifungal drug	Resistance rate	MIC_{50}	MIC_{90}
Polyenes			
Amphotericin B	12.8 % (11/86)	1 mg/L	2 mg/L
Azoles			
Fluconazole	/	> 128 mg/L	> 128 mg/L
Voriconazole	1.2 % (1/86)	0.065 mg/L	0.5 mg/L
Posaconazole	0.0 % (0/86)	0.031 mg/L	0.065 mg/L
Itraconazole	1.2 % (1/86)	0.065 mg/L	0.25 mg/L
Echinocandins			
Micafungin	/	4 mg/L	>8 mg/L
Anidulafungin	/	1 mg/L	>8 mg/L
Caspofungin	/	4 mg/L	>8 mg/L
Others			
5- flucytosine	/	4 mg/L	8 mg/L

MICs for amphotericin B tended to decrease over time ($p = 0.09$); contrariwise, for all the azoles tested, higher MICs, even though not statistically significant, were observed in 2021-2023 compared to 2018-2020. Detailed results on MICs stratified by the years of isolation are displayed in Fig. 1.

Strains isolated from COVID-19 positive patients were characterized by higher MICs for amphotericin B than those isolated from negative subjects (1.2 ± 0.4 vs 1.0 ± 0.3, $p = 0.05$).

3.2. Presence of resistance markers

The molecular test for azole resistance markers showed a 100 %-agreement with the phenotypic assay. All the strains susceptible to azoles were negative for cyp51A mutations. The strain resistant to itraconazole was positive for TR34 mutation, while the strain resistant to voriconazole showed a TR46 mutation.

4. Discussion

We assessed the susceptibility to antifungal drugs on 86 *A. fumigatus* strains isolated from patients with IPA or CAPA in the period 2018-2023, comparing the sensitivity to azoles obtained by the broth microdilution assay with a genotypic method able to detect cyp51A gene mutations.

The distribution of MICs found in our setting was very similar to the one described recently by Franconi *et al.*, analyzing data over an 8-year period in Italy [15]. Indeed, the authors found resistance rates for *A. fumigatus* strains (amphotericin B: 10.9 %, posaconazole: 1 %, itraconazole: 0.2 %, voriconazole: 0.6 %) almost comparable to the ones found in our experience (respectively: 12.8 %, 0.0 %, 1.2 % and 1.2 %).

In agreement with other studies, we observed that about 90 % of *A. fumigatus* strains was characterized by a MIC for amphotericin B of 1 mg/L, with resistant strains showing MICs rarely exceeding 2 mg/L [2,16]. Additionally, in line with other national and international reports, we confirmed the very low resistance rates to azoles [2,3,17,18].

We noticed a slight increasing trend of azoles MICs in parallel with a decreasing level of amphotericin B MICs, in agreement with the recent observations of Jean and colleagues [18].

Interestingly, strains isolated from COVID-19 positive patients were characterized by higher MICs for amphotericin B than those isolated from negative subjects. Even though the reasons behind this observation are not clear, we can speculate that the use of liposomal amphotericin B to reduce the incidence of CAPA in mechanically ventilated COVID-19 patients, could be involved in this phenomenon [19,20].

When comparing the phenotypic susceptibility test with a PCR able to detect mutations associated to azole-resistance (Fungiplex Aspergillus Azole–R), we observed an excellent agreement between the two methods, with no discordant results. We expanded the knowledge about the use of this molecular method directly on fungal colonies [13,21]. Thanks to its rapidity (about 2h) and its outstanding performance, this test could represent a substantial tool for a better clinical management.

We are fully aware of some limitations of this study. At first, as previously reported [22], the phenotypic test used for antifungal susceptibility (MICRONAUT-AM) showed several categorical discordances for azole antifungals when compared to EUCAST reference method. Thus, further studies are needed to confirm its accuracy for the determination of azoles MICs for *A. fumigatus*. Moreover, additional studies are needed to expand the panel of *Aspergillus* strains analyzed, as well as to assess the effectiveness of azole resistance molecular testing directly on clinical specimens.

In conclusion, even though in our setting the rate of azole-resistance in *A. fumigatus* remains quite limited, it is crucial to continue monitoring the trend of this resistance for proper clinical management and implementation of effective antimicrobial stewardship programs. In this context, the use of PCR-based resistance testing may help to limit the clinical impact of azole resistance, thanks to its low turn-around-time and excellent performances.

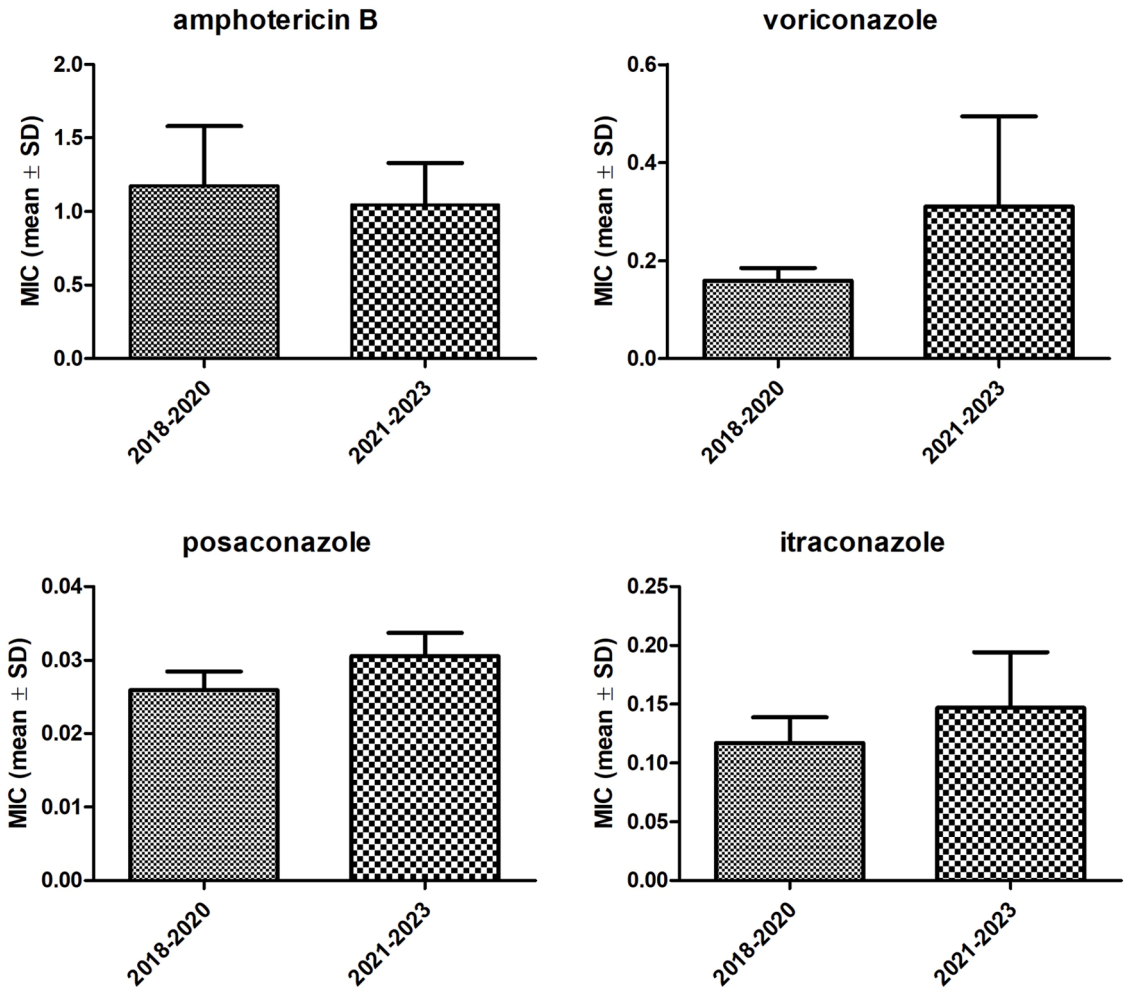

Fig. 1. Distribution of MIC values for amphotericin B and azoles stratified by the period of *A. fumigatus* strain isolation. Data are expressed as mean MIC values ± SD. The period 2018-2020 was compared to the period 2021-2023. Amphotericin B MICs: 1.17 ± 0.4 in 2018-2020 vs 1.04 ± 0.2 in 2021-2023, p = 0.09. Voriconazole: 0.31 ± 1.2 vs 0.15 ± 0.17, p = 0.41. Posaconazole: 0.03 ± 0.02 vs 0.02 ± 0.01, p = 0.26. Itraconazole: 0.14 ± 0.3 vs 0.11 ± 0.1, p = 0.57.

Ethical approval

The study was conducted according to the regulations of the Hospital Ethical Committee and to the 1964 Helsinki Declaration and its later amendments. All the samples were kept anonymous throughout the duration of the study.

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CRediT authorship contribution statement

Andrea Liberatore: Investigation, Formal analysis, Data curation. **Giulia Lombardi:** Data curation, Conceptualization. **Donatella Lombardo:** Data curation, Conceptualization. **Tiziana Lazzarotto:** Validation, Supervision, Resources, Conceptualization. **Claudio Foschi:** Writing – original draft, Project administration, Methodology, Data curation, Conceptualization. **Simone Ambretti:** Validation, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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