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S. Imbert, A. C Normand, S. Cassaing, F. Gabriel, L. Kristensen, et al.. Multicentric Analysis of the Species Distribution and Antifungal Susceptibility of Cryptic Isolates from *Aspergillus* Section *Fumigati*. *Antimicrobial Agents and Chemotherapy*, 2020, 64 (12), 10.1128/AAC.01374-20 . hal-03238921

HAL Id: hal-03238921

<https://normandie-univ.hal.science/hal-03238921>

Submitted on 28 May 2021

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Multicentric Analysis of the Species Distribution and Antifungal Susceptibility of Cryptic Isolates from *Aspergillus* Section *Fumigati*

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ABSTRACT The antifungal susceptibility of *Aspergillus* cryptic species is poorly known. We assessed 51 isolates, belonging to seven *Fumigati* cryptic species, by the EUCAST reference method and the concentration gradient strip (CGS) method. Species-specific patterns were observed, with high MICs for azole drugs, except for *Aspergillus hiratsukae* and *Aspergillus tsurutae*, and high MICs for amphotericin B for *Aspergillus lentulus* and *Aspergillus udagawae*. Essential and categorical agreements between EUCAST and CGS results were between 53.3 and 93.3%.

KEYWORDS *Aspergillus* section *Fumigati*, cryptic species, MALDI-TOF mass spectrometry, azole resistance, *Aspergillus thermomutatus*, *Aspergillus lentulus*, antifungal susceptibility testing

Aspergillus section *Fumigati* currently includes 58 cryptic species beside *Aspergillus fumigatus*, the predominant and eponymous species of the section (1–3). The notion of cryptic species is not only of taxonomic interest, as a link between the species and the susceptibility to antifungal agents has been shown inside several sections (2, 4, 5). Moreover, these cryptic species are increasingly found in human samples (6–8), including invasive aspergillosis cases (9, 10). Nevertheless, knowledge about their antifungal susceptibility is both patchy and contradictory. Furthermore, data are mainly obtained by reference methods, and no comparisons to the widely used concentration gradient strip (CGS) method are available. Lack of data may be explained by the difficulty of collecting these species, mainly related to the issues involved in obtaining a correct identification at the species level (2, 11). The MSI application is an independent and freely accessible online database (<https://msi.happy-dev.fr>) that was built in collaboration with the Belgian Coordinated Collections of Micro-organisms/Institute of Hygiene and Epidemiology Mycology (BCCM/IHEM) and includes references for 159 different *Aspergillus* species (12). The MSI application is of great interest for discriminating cryptic species from the *sensu stricto* species inside a given section (13).

Citation Imbert S, Normand AC, Cassaing S, Gabriel F, Kristensen L, Bonnal C, Lachaud L, Costa D, Guitard J, Hasseine L, Palous M, Piarroux M, Hendrickx M, Piarroux R, Fekkar A. 2020. Multicentric analysis of the species distribution and antifungal susceptibility of cryptic isolates from *Aspergillus* section *Fumigati*. Antimicrob Agents Chemother 64:e01374-20. <https://doi.org/10.1128/AAC.01374-20>.

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Received 29 June 2020

Returned for modification 11 August 2020

Accepted 3 September 2020

Accepted manuscript posted online 8 September 2020

Published 17 November 2020

TABLE 1 *In vitro* antifungal susceptibility of 51 *Aspergillus* section *Fumigati* cryptic isolates, determined by the EUCAST method

Species and parameter	Data for ^a :				
	ITZ	VRZ	PSZ	ISA	AMB
<i>A. thermomutatus</i> (n = 18)					
MIC range (mg/liter)	>8	0.5 to 8	0.125 to 1	0.5 to 8	0.125 to 1
MIC GM (mg/liter)	>8	3.05	0.397	2.08	0.463
MIC ₅₀ (mg/liter)	>8	4	0.5	2	0.5
MIC ₉₀ (mg/liter)	>8	8	0.5	4	1
<i>A. hiratsukae</i> (n = 12)					
MIC range (mg/liter)	0.06 to 1	0.25 to 2	0.03 to 0.25	0.06 to 0.5	0.125 to 1
MIC GM (mg/liter)	0.234	0.794	0.131	0.333	0.397
MIC ₅₀ (mg/liter)	0.25	1	0.125	0.5	0.5
MIC ₉₀ (mg/liter)	1	1	0.25	0.5	1
<i>A. lentulus</i> (n = 9), MIC range (mg/liter)	0.5 to >8	2 to 8	0.25 to 0.5	0.5 to 4	1 to 8
<i>A. udagawae</i> (n = 4), MIC range (mg/liter)	2 to >8	2 to 4	0.25 to 0.5	2 to 4	1 to 8
<i>A. felis</i> (n = 4), MIC range (mg/liter)	>8	2 to 8	0.25 to 0.5	1 to 4	0.25 to 2
<i>A. fischeri</i> (n = 3), MIC range (mg/liter)	>8	2	0.25 to 0.5	2	1 to 2
<i>A. tsurutae</i> (n = 1), MIC (mg/liter)	0.5	1	0.125	0.5	0.25

^aGM, geometric mean; ITZ, itraconazole; VRZ, voriconazole; PSZ, posaconazole; ISA, isavuconazole; AMB, amphotericin B.

Our goal was to assess the antifungal susceptibility of cryptic species from section *Fumigati* by reference and commercial methods. The network of users of the MSI application (108 laboratories from 19 countries) allows the collection of a great number of cryptic isolates in a multicentric and prospective study.

(Part of this study was presented during the 8th Trends in Medical Mycology congress, Nice, France, 11 to 14 October 2019.)

MSI online identification data were analyzed prospectively over the course of a 27-month period (August 2017 to October 2019). During this period, nine regular MSI users were asked, when they identified a *Fumigati* cryptic species, to ship the isolate to our institution, La Pitié-Salpêtrière Hospital, a tertiary care center in Paris, France. The participating centers were university hospitals (median of 1,600 beds [range, 780 to 3,000 beds]) located in France (Paris area [*n* = 3], Bordeaux, Rouen, Montpellier, Nice, and Toulouse) and Denmark (Aarhus). All isolates were subjected to DNA sequencing-based identification (*benA* and *cmd* genes) (14) and antifungal susceptibility testing by the EUCAST broth microdilution (BMD) reference method (15). A selection of 30 susceptible or resistant isolates were also tested by the CGS method. MICs obtained with the CGS method were adjusted to the next upper values matching the 2-fold dilution BMD scheme. Essential agreement (EA), categorical agreement (CA), very major error (VME), and major error (ME) between CGS and BMD results were determined as described previously (16).

Over the study period, *Fumigati* cryptic species represented 364 isolates (4.6%) of the total of 7,918 *Fumigati* isolates (including 7,554 *A. fumigatus sensu stricto* isolates) identified by the MSI application. Interestingly, this cryptic species frequency within the *Fumigati* section is significantly higher than that recently published in the setting of a 14-year retrospective monocentric study, in which 14 cryptic isolates (1.4%) were recovered among 999 *Fumigati* clinical isolates (17). In this context, the MSI application appears to be a very interesting tool to give more insight into the study of rare isolates and cryptic species.

In the present study, we collected 51 *Fumigati* cryptic isolates, which represent, to the best of our knowledge, the largest collection of clinical isolates. Based on DNA sequencing identification, isolates were distributed into seven species, namely, *Aspergillus thermomutatus* (*n* = 18), *Aspergillus hiratsukae* (*n* = 12), *Aspergillus lentulus* (*n* = 9), *Aspergillus felis* (*n* = 4), *Aspergillus udagawae* (*n* = 4), *Aspergillus fischeri* (*n* = 3), and *Aspergillus tsurutae* (*n* = 1). With the exception of 2 environmental isolates, all isolates were recovered from clinical samples, mainly from the respiratory tract (47/49 isolates). Four isolates were related to invasive aspergillosis and 1 to a sinus fungal ball, while 42

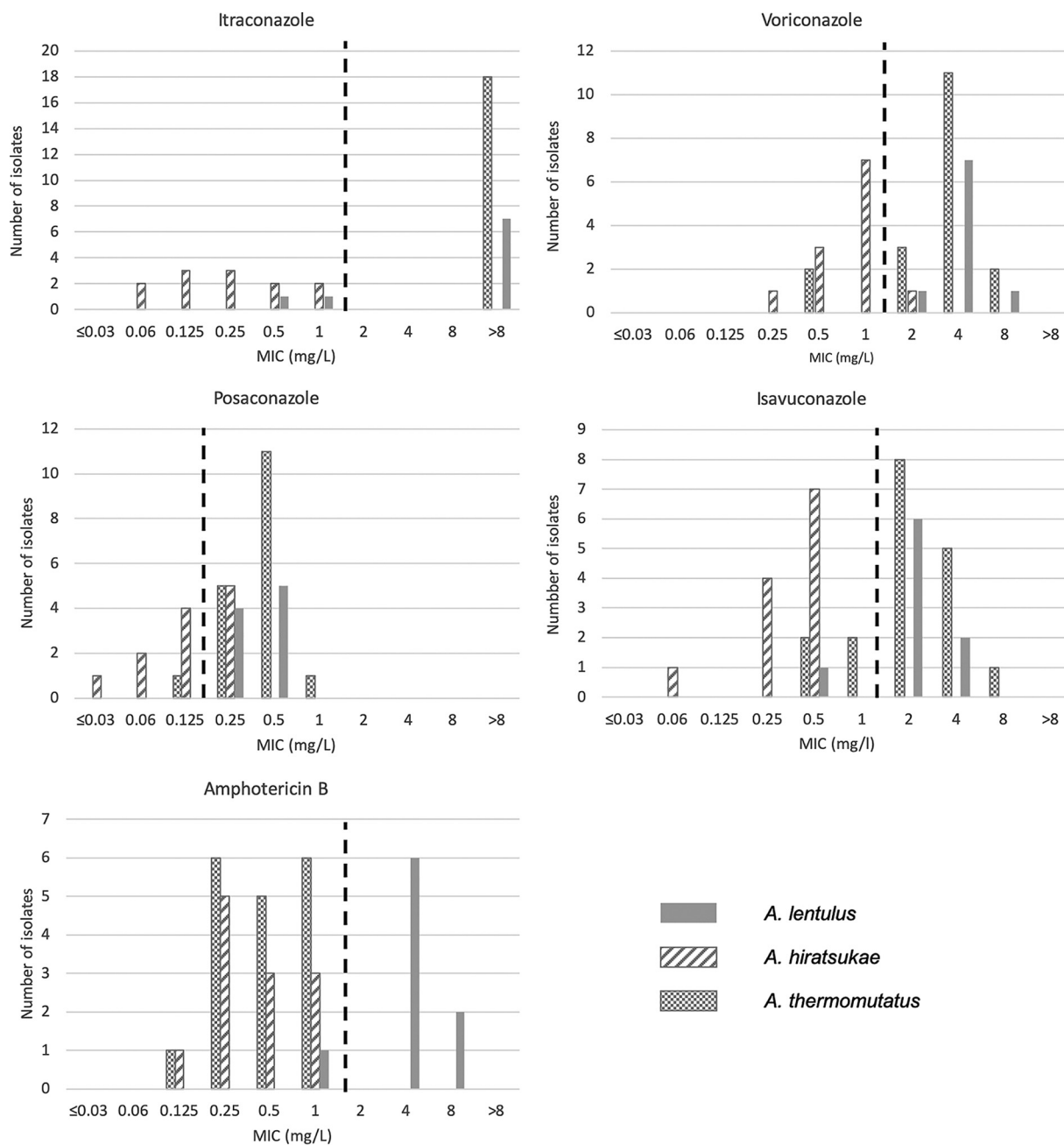


FIG 1 MIC distributions for the species *A. thermomutatus*, *A. hiratsukae*, and *A. lentulus*. Vertical dashed lines represent *Aspergillus fumigatus* clinical breakpoints.

were found as pulmonary colonizers in patients suffering from cystic fibrosis ($n = 24$) or other chronic respiratory diseases ($n = 18$). Thus, these findings highlight the importance of cryptic species in human diseases and confirm the need for accurate identification at the species level. Interestingly, the species *A. thermomutatus* was exclusively found in cystic fibrosis patients and originated mainly from two centers (Toulouse, 9 isolates [5 patients]; Aarhus, 6 isolates [3 patients]). This is consistent with previous reports in which this species (or its former teleomorph *Neosartorya pseudofischeri*) was found as a major colonizer in this disease (18, 19). However, this requires further investigations, which may be facilitated by the species' recent whole-genome sequencing (20).

MIC geometric means, MIC ranges, MIC₅₀ values, and MIC₉₀ values obtained by the

TABLE 2 Comparison between MIC values determined by the EUCAST and CGS methods for 30 *Aspergillus* section *Fumigati* cryptic isolates

Data for ^a :												
Parameter and species	Itraconazole			Voriconazole			Posaconazole			Isavuconazole		
	EUCAST, 48 h			EUCAST, 48 h			EUCAST, 48 h			EUCAST, 48 h		
	CGS, 48 h	CGS, 72 h	CGS, 48 h	CGS, 48 h	CGS, 72 h	CGS, 48 h	CGS, 48 h	CGS, 72 h	CGS, 48 h	CGS, 48 h	CGS, 72 h	CGS, 72 h
MIC (mg/liter)												
<i>A. Fischeri</i>	>8	4	4	2	0.5	1	0.25	0.5	0.5	2	1	2
<i>A. Fischeri</i>	>8	8	8	2	2	2	0.25	0.5	0.5	2	2	2
<i>A. hirsutulae</i>	0.125	0.008	0.03	1	0.03	0.06	0.06	0.008	0.008	0.25	0.03	0.03
<i>A. hirsutulae</i>	0.5	0.03	0.25	2	0.125	0.25	0.25	0.06	0.06	0.5	0.125	0.125
<i>A. hirsutulae</i>	0.25	0.125	0.5	1	0.25	0.5	0.125	0.06	0.125	0.25	0.25	0.25
<i>A. hirsutulae</i>	1	0.25	0.125	1	0.25	0.25	0.125	0.06	0.125	0.5	0.125	0.125
<i>A. hirsutulae</i>	0.125	0.25	0.25	1	0.5	0.5	0.125	0.06	0.125	0.5	0.125	0.5
<i>A. lentulus</i>	1	0.125	2	4	0.125	2	0.25	0.06	0.06	2	0.125	0.25
<i>A. lentulus</i>	>8	0.5	2	2	0.5	4	0.25	0.06	0.125	2	0.5	2
<i>A. lentulus</i>	0.5	1	32	4	2	32	0.25	0.125	0.25	2	0.5	4
<i>A. lentulus</i>	>8	1	32	4	2	32	0.5	0.25	0.5	2	32	32
<i>A. lentulus</i>	>8	1	8	8	2	8	0.5	0.125	0.25	0.5	1	8
<i>A. lentulus</i>	>8	2	4	4	4	32	0.25	0.125	0.5	2	4	4
<i>A. lentulus</i>	>8	4	32	4	32	32	0.5	0.5	0.5	4	8	8
<i>A. felis</i>	>8	1	2	8	2	32	0.5	0.125	0.25	4	0.5	1
<i>A. felis</i>	>8	4	4	4	2	32	0.5	0.25	0.25	1	4	16
<i>A. felis</i>	>8	8	32	8	8	32	0.5	0.5	0.5	4	32	32
<i>A. thermomutatus</i>	>8	0.5	0.5	4	0.5	2	0.25	0.125	0.25	2	0.5	1
<i>A. thermomutatus</i>	>8	1	1	0.5	0.5	2	0.25	0.125	0.25	0.5	0.5	1
<i>A. thermomutatus</i>	>8	1	2	4	1	4	0.5	0.125	0.5	1	4	1
<i>A. thermomutatus</i>	>8	1	2	8	1	2	1	0.125	0.25	4	0.5	1
<i>A. thermomutatus</i>	>8	2	2	4	0.25	0.25	0.5	0.03	0.03	2	0.25	1
<i>A. thermomutatus</i>	>8	0.25	4	2	0.5	16	0.125	0.125	0.25	2	0.125	1
<i>A. thermomutatus</i>	>8	1	4	4	1	8	0.5	0.125	0.5	2	1	2
<i>A. thermomutatus</i>	>8	1	16	4	1	16	0.5	0.25	1	2	4	0.125
<i>A. thermomutatus</i>	>8	1	16	4	2	8	0.5	0.5	0.5	4	4	0.125
<i>A. thermomutatus</i>	>8	1	2	2	0.5	8	0.25	0.06	0.25	2	0.125	0.5
<i>A. thermomutatus</i>	>8	8	8	4	4	4	0.5	0.5	0.5	4	0.5	1
<i>A. udagawae</i>	>8	0.5	4	4	2	8	0.5	0.125	0.25	2	1	2
<i>A. udagawae</i>	>8	1	4	4	2	16	0.5	0.125	0.5	4	2	32
EA within 2 dilutions (%)	NA	32.1	66.7	NA	78.6	70.0	NA	89.3	93.3	NA	73.3	53.3
Spearman's rank correlation coefficient (P)	NA	0.672	0.508	NA	0.679	0.59	NA	0.581	0.519	NA	0.408	0.817
ME (%)	NA	0.0	6.7	NA	17.9	10.0	NA	0.0	3.3	NA	6.7	33.3
VME (%)	NA	53.6	6.7	NA	21.4	3.3	NA	42.9	6.7	NA	26.7	0.0
CA (%)	NA	46.4	86.7	NA	60.7	86.7	NA	57.1	90.0	NA	66.7	66.7

^aNA, not applicable.

EUCAST BMD method are shown in Table 1. These results are in accordance with previous findings showing mainly higher MICs for cryptic species, compared with *A. fumigatus* (2, 4, 21, 22). The species *A. thermomutatus*, *A. lentulus*, *A. felis*, *A. udagawae*, and *A. fischeri* exhibited high MICs for all azole drugs, while *A. lentulus* and *A. udagawae* showed high MICs for amphotericin B. Of note, in the present study, including the largest series for *A. thermomutatus* ($n = 18$) and *A. hiratsukae* ($n = 12$), we found few MIC variations inside a given species (Fig. 1). This indicates that accurate identification at the species level could suggest the antifungal susceptibility profile. However, these findings must be strengthened through continued collection of isolates, especially for species represented by very few isolates in the present study.

Regarding the 30 isolates assessed by the CGS method, MICs were significantly correlated with those obtained by BMD (Spearman's rank correlation coefficients of >0.5 ; $P < 0.05$) (Table 2). We found moderate agreement between these two methods, with EA and CA values mainly under 90%, except for posaconazole. These results are quite different from those obtained recently with only *A. fumigatus sensu stricto* isolates, for which good agreement between the two methods was shown (23). Here, we included only *Fumigati* cryptic species characterized by low sporulation and growth rates (24). This could explain why results were particularly low after 48 h of incubation, with MICs for azole drugs being systematically lower with the CGS method ($P < 0.01$ by Wilcoxon test), as highlighted by high VME values (Table 2). When the incubation time was extended to 72 h for the CGS method (for BMD, growth was sufficient after 48 h), agreements were better for azoles but not for amphotericin B. Similar findings with the Sensititre YeastOne method were observed in a previous study focused on *Fumigati* cryptic species (21). Thus, regarding slow-growing *Fumigati* cryptic species and our results, we recommend that CGS MICs be determined after 72 h of incubation for azoles and 48 h for amphotericin B. Isavuconazole MIC determination by the CGS method is particularly open to question. The use of the *A. fumigatus sensu stricto* clinical breakpoints to categorize the isolates could also explain low CA values observed for agreement between the two methods. This supports the need to determine clinical breakpoints, or at least epidemiological cutoff values, to improve knowledge regarding these cryptic species.

ACKNOWLEDGMENTS

This study was supported by internal funding.
We declare no conflicts of interest.

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