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Invasive aspergillosis due to *Aspergillus* cryptic species: a prospective multicenter study

Running title: Invasive aspergillosis due to cryptic species

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TRANSPARENCY DECLARATION

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Keywords: Invasive aspergillosis, cryptic species, antifungal resistance, *Aspergillus sublatus*, *Aspergillus calidoustus*, EUCAST, molecular epidemiology, azole prophylaxis

ABSTRACT (243 words)

Objectives: *Aspergillus* cryptic species are increasingly recognized causes of *Aspergillus* diseases, including life-threatening invasive aspergillosis (IA). However, as their accurate identification remains challenging in a routine practice, few is known from a clinical and epidemiological perspective. Recently, the MSI application has emerged as a powerful tool for the detection and identification of *Aspergillus* cryptic species. We aimed to use to the network of users of the MSI application to conduct a multicenter prospective screening of *Aspergillus* cryptic species related IA and analyze their epidemiological, clinical and mycological characteristics.

Methods: Over a 27-month period, the clinical involvement of 369 *Aspergillus* cryptic isolates, from 13 French and Danish MSI application users, was prospectively analyzed. Species identification was confirmed by DNA-sequencing and antifungal susceptibility testing was performed using EUCAST reference method. Fifty-one *A. fumigatus sensu stricto* invasive cases were also analyzed.

Results: Fifteen cryptic isolates were responsible of IA. Eight species were involved, including 5 cases related to the species *A. sublatus*. These species showed high rate of *in vitro* low susceptibility to antifungal drugs. In comparison with *A. fumigatus sensu stricto* invasive cases, pre-exposure to azole drugs was significantly associated with cryptic IA ($p = 0.02$).

Discussion: This study brings new insights in cryptic species related IA and underlines the importance to identify accurately at the species level these *Aspergillus* isolates. The increasing use

of antifungal drugs might lead in the future to an epidemiologic shift with an emergence of resistant isolates involved in IA.

INTRODUCTION (1763 words)

Aspergillus spp. are the most frequent filamentous fungi involved in human pathology. They are responsible for a wide spectrum of diseases, including invasive aspergillosis (IA), a life-threatening disease with an overall 3-month mortality up to 50%.^{1,2} Historically, few *Aspergillus* species were involved in human aspergillosis, the most frequent species being *A. fumigatus*, then *A. niger*, *A. flavus*, *A. nidulans* and *A. terreus*.³⁻⁶ However, in recent years, *Aspergillus* species taxonomy has been changed in depth by the introduction of the new concept of cryptic species. This latter encompasses species non-distinguishable by morphological characteristics which are gathered into a single species complex called “section”. The most recent taxonomy update reports 436 *Aspergillus* species distributed into 27 sections.⁷ The presence of *Aspergillus* cryptic species in clinical samples is increasingly reported, changing so aspergillosis epidemiology.⁸ Currently, more than 100 species have been reported in clinical samples, including 30 species responsible for IA.⁹ Data are scarce but retrospective multicenter studies have shown that *Aspergillus* cryptic species were involved between 10% to 30% of invasive cases.¹⁰⁻¹²

Beyond the taxonomic interest, the notion of cryptic species is of great interest given the link between a given species and its susceptibility to antifungal drugs as it has been shown for several sections.^{13,14} Unlike aspergillosis due to *A. fumigatus sensu stricto*, cryptic species related infections are less detailed from a clinical and epidemiological perspective. Lack of data may be related to difficulties in routinely obtaining accurate identification. Indeed, identification based on morphology, which is the easiest to implement and the most commonly shared in microbiology laboratories, is inadequate to distinguish different cryptic species within a section. Sequence-based identification, which represents the gold standard method,⁷ cannot be universally applied in routine settings. Finally, identification by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, although suitable for identification of *Aspergillus* spp., directly relies on the quality and completeness of the database used.¹⁵ Commercial databases dedicated to fungi often fail to provide relevant mold identification as they usually include very few cryptic species.¹⁵ The mass spectrometry identification (MSI) application is an independent and freely accessible online database (<https://msi.happy-dev.fr>), dedicated to the identification of various parasitic and fungal pathogenic agents, from their mass spectra acquired by Bruker® MALDI-TOF Microflex^{16,17}. The fungal database was built in collaboration with the BCCM/IHEM (Belgian

Coordinated Collections of Micro-organisms/Institute of Hygiene and Epidemiology Mycology) and is, to our knowledge, the most extensive for filamentous fungi. Recently we demonstrated that this MSI application was a powerful tool for the detection and identification of *Aspergillus* cryptic species.¹⁸

The aim of this study was to take advantage of the network of users of the MSI application for prospective screening of cryptic species related IA and to analyze their epidemiological, clinical and mycological characteristics.

METHODS

Over a 27-month period (August 2017 – October 2019), a standardized clinical report form was prospectively fulfilled by 13 French and Danish MSI application users each time they identified an *Aspergillus* cryptic species. Isolates and forms were sent anonymously to the La Pitié-Salpêtrière Hospital, Paris, France for data management, according to French legislation on human research. If applicable, cases were classified as proven or probable IA according to the criteria of the European Organization of Research and Treatment of Cancer (EORTC) / Mycoses Study Group Education and Research Consortium (MSGERC),¹⁹ or putative IA according to the AspICU criteria for intensive care unit patients.²⁰ These latter encompass a positive culture of the respiratory tract with a positive direct examination, associated with clinical and imaging criteria, in the absence of EORTC/MSGERC risk factors.

Species identification was confirmed by sequencing a part of the beta-tubulin and calmodulin genes. Primer pairs Bt2a/Bt2b for beta-tubulin and CL1/CL2A or cmd5/cmd6 for calmodulin, were used as previously described.²¹ The resulting DNA sequences were analyzed using ChromasPro v1.7.1 (Technelysium Pty Ltd) and most were deposited on Genbank (accession numbers MK297710 - MK297892 and MK297532 - MK297699). All isolates responsible of IA were assessed for antifungal susceptibility testing (AFST) by broth microdilution (BMD) reference method, according to EUCAST guidelines, vE.DEF 9.3.1 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9.3.1_Mould_testing_definitive_revised_2020.pdf). Five antifungal drugs were assessed with final drug concentration ranges of 0.016 to 8 mg/L for posaconazole and itraconazole and 0.03 to 16 mg/L for voriconazole, isavuconazole and amphotericin B. One among three quality control strains (*C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *A. fumigatus* ATCC 204305) was

included in each plate alternatively. All isolates recovered over the study period and belonging to cryptic species involved in IA, were also assessed for AFST.

We also sought to compare cryptic IA data with those of *A. fumigatus sensu stricto* related IA. For this purpose, we analyzed all IA cases for which an *A. fumigatus sensu stricto* culture had been obtained from 2 centers, resulting in a total of 51 "control" cases over the same study period.

RESULTS

Clinical involvement was recovered for 369 cryptic isolates belonging to 67 species from 13 sections. The *Nidulantes* section was the most represented (119 isolates, 16 species) followed by the section *Nigri* (53 isolates, 7 species), *Fumigati* (49 isolates, 7 species) and *Circumdati* (48 isolates, 8 isolates).

Fifteen isolates (4.1%), from four centers, were responsible of IA (2 proven, 11 probable and 2 putative). The *Nidulantes* section was the most frequent (6 cases), followed by the sections *Usti* and *Fumigati* (three cases each). The remaining isolates belonged to the *Nigri* (n=2) and the *Flavi* (n=1) sections (Table 1). Beside these invasive isolates, 17 were involved in chronic pulmonary aspergillosis, 225 in bronchial colonization, 53 in superficial infection (onychomycosis and otomycosis) and 59 were considered as non-clinically relevant.

As shown in Table 1, the main underlying condition for cryptic IA was solid organ transplantation (n=6, 40%), then hematological malignancy (n=5, 33.3%) and acute respiratory distress syndrome in intensive care unit's patients (n=3, 20%). This latter included two patients with severe influenza infection requiring extra-corporeal membrane oxygenation. A majority of patients (n=13, 86.7%) were receiving immunosuppressive therapy, including 10 (66.7%) with corticosteroids. Five patients were neutropenic (neutrophil count < 500/mm³) at time of IA diagnosis. An ongoing antimould active treatment initiated before the diagnosis of IA was reported in five patients (four with an azole drug and one with an echinocandin). Interestingly, two *A. calidoustus* cases occurred in patients previously treated by voriconazole against a probable *A. fumigatus* IA. All patients received a targeted antifungal therapy, including 6 (40%) with multiple antifungal drugs. Thirteen patients (86.7%) received at least one line of azole-based therapy. Overall three-month mortality was 60% (9/15), including four of the five patients infected by *A. sublatus*.

All IA isolates, except the 5 *A. latus* isolates, exhibited high Minimal Inhibitory Concentrations (MICs) to at least one drug. *A. alliaceus* and *A. tubingensis* showed very high MICs to a unique drug (amphotericin B and itraconazole respectively) (Table 2). *A. calidoustus* showed *pan*-azole high MICs. Species from the *Fumigati* section and *A. sydowii* showed multidrug resistance with high MICs to azole drugs and amphotericin B, and high MICs to itraconazole, posaconazole and amphotericin B respectively. These species-specific antifungal susceptibility patterns, were confirmed on a greater number of isolates, with ranges, MIC50 and MIC90 in accordance to IA isolates MICs (Table 2).

As shown in Table 3, no significant differences in patient characteristics, clinical presentation or 3-month outcome were observed between cryptic IA and the 51 *A. fumigatus sensu stricto* related-IA. The only parameter which was associated with the occurrence of cryptic IA was an ongoing azole drug treatment at the time of IA diagnosis (p=0.02).

DISCUSSION

Invasive aspergillosis due to cryptic species remains rare disease. In this multicentric prospective study, the MSI application user network allowed us to collect clinical and microbiological data for 369 cryptic *Aspergillus* isolates, from 13 centers in France and Denmark. To note, over the study period (27 months), 4602 *Aspergillus* cryptic isolates were identified on the application, over the 15305 *Aspergillus* total isolates (30.1%), by 112 centers in 19 countries.

Among the 369 cryptic isolates included in the study, only 15 were linked to invasive diseases. They belong to eight distinct cryptic species distributed into five sections. The species *A. sublatus* was the most abundant (five cases) of this case series, although few cases are reported in literature.²² However, *A. sublatus* could be more frequent as previously expected inside the *Nidulantes* section. Data from the MSI application routine utilization reinforce this observation, since *A. sublatus* accounts for as many identifications as *A. nidulans* (data not shown). The same phenomenon has already been described for the species *A. tubingensis* and *A. calidoustus* in the *Nigri* and *Usti* sections respectively.^{23,24} Consequently, the classification of species into cryptic or non-cryptic is likely to evolve over time, depending on subsequent epidemiological studies. In contrast, cryptic species from the sections *Fumigati* and *Flavi* remain less frequent than the *sensu stricto* species *A. fumigatus* and *A. flavus* respectively. In this study we report a species diversity inside the section *Fumigati*, with three distinct species involved in IA. This is consistent with

previous reports where not less than 10 cryptic species from the *Fumigati* section have been described in IA.

One of the pitfalls to a better understanding of cryptic species related IA is that only cases with a positive culture can be considered, which, given the modest sensitivity of the culture for diagnosis, limits the number of cases considered. For the two centers with exhaustive data, cryptic IA account for 21% and 18.8% of the total number of IA with a positive culture, which is consistent with previous reports.^{11,12}

Most importantly, pre-exposure to azole drugs was significantly associated with occurrence of IA due to cryptic species in comparison to those related to *A. fumigatus sensu stricto*. This might be explained by the high rate of azole decreased susceptibility observed in cryptic species. Indeed, while the *sensu stricto* species *A. fumigatus* is naturally *pan*-susceptible to antifungal drugs, *Fumigati* cryptic species exhibit decreased susceptibility to azole drugs and/or amphotericin B, which is consistent with previous results.^{13,14} *Pan*-azole resistance is the rule for *A. calidoustus* isolates, as previously reported for the whole *Usti* section.²⁴ Finally, *A. sydowii* exhibits high MICs to azole drugs and amphotericin B. However, despite this decreased susceptibility, no other significant differences with *A. fumigatus* cases were observed, including 3-month outcome. Nevertheless, the increasing use of antifungal drugs might lead in the future to an epidemiologic shift with an emergence of resistant isolates in clinical practice, as already suggested with the use of azole prophylaxis.²⁵

In conclusion, this study brings new insights in cryptic species related IA and underlines the importance to identify accurately at the species level these *Aspergillus* isolates, given their intrinsic low susceptibility to antifungal drugs.

AUTHOR'S CONTRIBUTIONS

SI designed the study, performed experiments, centralized data and wrote the manuscript, SC, CB, FG, DC, MB, LL, LH, LK, JG, CS, HR, SB and MH participated in data collection, ACN performed experiments, RP participated in the design of the study, AF designed the study and participated in the writing.

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Table 1: patient characteristics, clinical presentation and outcome for the 15 cases of invasive aspergillosis related to *Aspergillus cryptic* species

Patients	Sex	Age (years)	Underlying diseases	Neutropenia (<500/ μ L)	Immunosuppressive therapy	Antifungal pre-exposure (dosage)	Invasive aspergillosis classification	Radiological findings	Section	Species	Galactomanann index		Targeted antifungal therapy (dosage)	3-month outcome
											BAL	Serum		
Patient 01	F	50	Solid organ transplant (lung)	No	Corticosteroids + calcineurin inhibitor + mycophenolate	No	Probable (lung)	Bilateral ground glass opacities	<i>Usti</i>	<i>A. calidoustus</i>	NA [#]	0.52	Voriconazole (NA [#]) then posaconazole (NA [#]) + aerosolized amphotericin B	Alive
Patient 02	F	44	Solid organ transplant (lung)	No	Corticosteroids + calcineurin inhibitor + mycophenolate	No	Probable (lung)	Lung consolidations	<i>Fumigati</i>	<i>A. fischeri</i>	0.11	0.04	Voriconazole (NA [#]) then isavuconazole (NA [#])	Death
Patient 03	M	66	Hematological malignancy (acute leukemia)	Yes	Corticosteroids + methotrexate	Posaconazole (300 mg/d, 2 weeks)	Probable (lung)	Ground glass opacities and lung consolidation	<i>Nidulantes</i>	<i>A. sydowii</i>	> 12	2.36	Caspofungin + liposomal amphotericin B (3mg/kg/d) then voriconazole IV	Alive
Patient 04	F	58	Auto-immune disease (antiphospholipids syndrom)	No	Corticosteroids (+ bolus)	No	Probable (lung)	Alveolar consolidations	<i>Nidulantes</i>	<i>A. sublatus</i>	0.84	8.78	Voriconazole (400 mg x 2/d)	Alive

Patient 05	M	67	Hematological malignancy (acute leukemia)	Yes	No	No	Probable (lung)	Multiple lung consolidations	<i>Fumigati</i>	<i>A. udagawae</i>	2.92	0.29	Voriconazole (200 mg x 2/d)	Death
Patient 06	M	52	Hematological malignancy (lymphoproliferative disease)	Yes	Brentuximab	No	Probable (lung)	NA	<i>Nidulantes</i>	<i>A. sublatus</i>	1	0.375	Liposomal amphotericin B (3 mg/kg/d) then voriconazole (200 mg x 2/j)	Death
Patient 07	M	28	Solid organ transplant (liver)	No	Calcineurin inhibitor	No	Probable (lung)	Lung consolidations and bilateral ground glass opacities	<i>Nidulantes</i>	<i>A. sublatus</i>	4.06	3.6	Voriconazole (NA [#])	Death
Patient 08	M	64	Solid organ transplant (heart)	No	Corticosteroids + calcineurin inhibitor + mycophenolate	Voriconazole (200 mg x 2/d, 3 months)	Probable (lung)	Inverted halo opacity	<i>Usti</i>	<i>A. calidoustus</i>	0.08	0.64	Voriconazole (200 mg x 2/d)	Alive
Patient 09	F	58	ICU ARDS + ECMO (severe influenza infection)	No	Corticosteroids	No	Putative (lung)	Bilateral lung opacities	<i>Nidulantes</i>	<i>A. sublatus</i>	> 8	> 8	Amphotericin B deoxycholate (NA [#])	Death
Patient 10	M	54	ICU ARDS + ECMO (severe influenza infection)	No	Corticosteroids (+ bolus)	No	Proven (lung)	Bilateral lung consolidations and maxillary sinusitis	<i>Nigri</i>	<i>A. tubingensis</i>	> 8	6.9	Voriconazole (200 mg x 2/d)	Death
Patient 11	M	74	Hematological malignancy (myelodysplastic syndrom)	Yes	Corticosteroids	No	Probable (lung)	Lung nodules and consolidation	<i>Nidulantes</i>	<i>A. sublatus</i>	0.46	0.088	Voriconazole (200 mg x 2/d) then liposomal	Death

													amphotericin B (3mg/kg/d)	
Patient 12	M	70	ICU ARDS	No	No	Caspofungin (50 mg/d, 2 months)	Proven (lung)	Alveolar consolidations and ground glass opacities	<i>Fumigati</i>	<i>A. lentulus</i>	NA [#]	3.2	Liposomal amphotericin B (3mg/kg/d) + caspofungin	Death
Patient 13	M	67	Solid organ transplant (lung)	No	Corticosteroids (+ bolus) + calcineurin inhibitor	No	Probable (lung)	Lung consolidations	<i>Nigri</i>	<i>A. tubingensis</i>	NA [#]	NA [#]	Voriconazole (250 mg x 2/d) + aerosolized amphotericin B	Alive
Patient 14	M	58	Solid organ transplant (heart)	No	Corticosteroids + calcineurin inhibitor	Voriconazole (200 mg x 2/d, 3 months)	Proven (bone)	Vertebral osteolysis	<i>Usti</i>	<i>A. calidoustus</i>	NA [#]	0.83	Voriconazole (NA [#])	Death
Patient 15	M	65	Hematological malignancy (acute leukemia)	Yes	Induction chemotherapy	Posaconazole (300 mg/d, 3 weeks)	Probable (lung)	Bilateral lung nodules	<i>Flavi</i>	<i>A. alliaceus</i>	NA [#]	0.56	Voriconazole (200 mg x 2/d)	Alive

Table 2: antifungal susceptibility testing for cryptic species involved in invasive aspergillosis

Section	Species		MIC				
			MIC50 / MIC90 (Range); mg/L				
			Itraconazole	Voriconazole	Posaconazole	Isavuconazole	Amphotericin B
<i>Flavi</i>	<i>A. alliaceus</i>	Invasive isolate (n=1)	0.125	0.25	0.125	0.25	>16
		All isolates (n=12)	0.125 / 0.125 (0.06 - 0.125)	0.25 / 0.5 (0.25 - 0.5)	0.06 / 0.125 (0.015 - 0.25)	0.5 / 1 (0.25 - 1)	> 16 / > 16 (4 - > 16)
<i>Fumigati</i>	<i>A. fischeri</i>	Invasive isolate (n=1)	> 8	2	0.5	2	1
		All isolates (n=3)	> 8	2	0.25 - 0.5	2	1 - 2
	<i>A. udagawae</i>	Invasive isolate (n=1)	2	2	0.25	2	4
		All isolates (n=4)	2 - > 8	2 - 4	0.25 - 0.5	2 - 4	1 - 8
	<i>A. lentulus</i>	Invasive isolate (n=1)	0.5	4	0.25	2	1
		All isolates (n=9)	0.5 - > 8	2 - 8	0.25 - 0.5	0.5 - 4	1 - 8
<i>Nidulantes</i>	<i>A. sydowii</i>	Invasive isolate (n=1)	> 8	1	1	1	> 16
		All isolates (n=34)	> 8 / > 8 (0.25 - > 8)	2 / 2 (0.25 - 4)	0.5 / 1 (0.25 - 1)	1 / 2 (0.25 - 2)	4 / > 16 (1 - > 16)
	<i>A. latus</i>	Invasive isolates (n=5)	0.25 - 0.5	0.25 - 0.5	0.125 - 0.5	0.125 - 0.25	0.5
		All isolates (n=30)	0.5 / 0.5 (0.25 - 1)	0.5 / 0.5 (0.25 - 1)	0.25 / 0.5 (0.125 - 0.5)	0.25 / 0.5 (0.125 - 0.5)	0.5 / 0.5 (0.25 - 1)
<i>Nigri</i>	<i>A. tubingensis</i>	Invasive isolates (n=2)	> 8	2	0.25	2 - 4	0.5
		All isolates (n=10)	> 8 / > 8 (0.5 - > 8)	2 / 2 (1 - 2)	0.25 / 0.25 (0.125 - 0.5)	4 / 4 (1 - 4)	0.5 / 0.5 (0.125 - 0.5)
<i>Usti</i>	<i>A. calidoustus</i>	Invasive isolates (n=3)	> 8	8 - 16	> 8	4	1
		All isolates (n=18)	> 8 / > 8 (> 8)	8 / 16 (4 - 16)	> 8 / > 8 (> 8)	4 / 4 (2 - > 8)	1 / 2 (0.25 - 2)

MIC: Minimal Inhibitory Concentration. MIC50 and MIC90 are shown when more than 10 isolates for the same species were assessed during the study period.

Table 3: characteristics comparison between *Aspergillus* cryptic species and *A. fumigatus sensu stricto* invasive aspergillosis

	Cryptic species (n=15)	<i>A. fumigatus</i> (n=51)	p value
Demographic characteristics			
Median age (years)	58 (28 - 74)	57 (23 -84)	0.52
Sex ratio (M/F)	11/4	38/13	1
Underlying diseases			
Hematological malignancy	5 (33%)	14 (27.5%)	0.75
Hematopoietic stem cell transplant	0 (0%)	4 (7.8%)	0.57
Solid organ transplantation	6 (40%)	20 (39.2%)	1
Acute respiratory distress syndrom	3 (20%)	7 (13.7%)	0.68
Other	1 (6.7%)	6 (11.8%)	1
Underlying Immunosuppressive condition			
Neutropenia (absolute neutrophils count < 500/ μ L)	5 (33%)	12 (23.5%)	0.5
Immunosuppressive drug	13 (86.7%)	39 (76.5%)	0.49
Including corticosteroids	10 (66.7%)	29 (58.9%)	0.56
Antifungal drug pre-exposure			
	5 (33%)	13 (25.5%)	0.53
Including azole drugs	4 (26.7%)	2 (3.9%)	0.02
Targeted antifungal therapy			
	15 (100%)	49 (96.1%)	1
Including azole drugs	13 (86.7%)	43 (84.3%)	1
Combination therapy	6 (40%)	13 (25.5%)	0.33
Invasive aspergillosis classification			
Proven	2	6	1
Probable	11	50	0.73
Putative	2	5	0.65
Galactomannan index positivity			
Serum (≥ 0.5)	10/14 (71.4%)	26/48 (54.2%)	0.36
Broncho-alveolar lavage (≥ 1)	6/10 (60%)	26/45 (57.8%)	1
Three-month all cause mortality			
	9 (60%)	31 (61%)	1

*Student t-test, Chi square test or Fischer exact test were used when suitable