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Abstract

Black aspergilli of the section *Nigri* are rarely differentiated at the species level when originating from human specimens. We wondered whether some cryptic species could be more frequently observed in some clinical entities. We analyzed the 198 black isolates consecutively collected from the external ear canal (EEC; n = 66), respiratory specimens (n = 99), and environment (n = 33). DNA was extracted and species identification was performed upon the partial calmodulin gene. We identified by decreasing frequency: *Aspergillus welwitschiae* (35.3%), *Aspergillus tubingensis* (34.3%), *Aspergillus niger* (17.2%), *Aspergillus luchuensis* (4%), *Aspergillus* aff. *welwitschiae* (3%), *Aspergillus neoniger* (2%), *Aspergillus piperis* (1.5%), *Aspergillus japonicus* (1.0%), *Aspergillus vadensis* (0.5%), and two *Aspergillus* tubingensis clade (1%). The distribution of the three main cryptic species was different between EEC and respiratory samples ($P < 0.001$) but not different between respiratory and environment samples ($P = 0.264$). *Aspergillus welwitschiae* was more often associated with EEC (54.5%), whereas *A. tubingensis* and *A. niger* were predominant in respiratory samples (39.4 and 26.3%, respectively). Among the 99 respiratory isolates, only 10 were deemed responsible for probable invasive aspergillosis, of which six were mixed with other pathogenic moulds. This study shows the interest to pursue the identification of clinical isolates in the *Aspergillus* section *Nigri* to unravel some specific associations with clinical entities. The association of *A. welwitschiae* with otomycosis suggests a better fitness to infect/colonize the ear canal. Also, members of the *Aspergillus* section *Nigri* alone are rarely responsible for invasive aspergillosis.

Lay summary

We analyzed 198 black aspergilli isolates collected from different samples type to determine their species identification. We observe a different distribution of species between ear canal and respiratory samples ($P < 0.001$), suggesting a better fitness of *A. welwitschiae* to infect the ear canal.

Key words: Black aspergilli, *Aspergillus welwitschiae*, *Aspergillus tubingensis*, *Aspergillus niger*, otomycosis, invasive aspergillosis.

Introduction

Black aspergilli of the section *Nigri* are well known in biotechnology[1] and for involvement in plant diseases[2], but are rarely reported in human invasive fungal infections. Indeed, the section *Nigri* is by far outnumbered by *Aspergillus fumigatus* in invasive pulmonary aspergillosis.[3,4] When isolated in medical laboratories from non-sterile sites, such as respiratory specimens, the black aspergilli are often disregarded as a contaminant.[5,6] One notable exception is the external ear fungal infection, where black aspergilli are frequently reported in different geographical areas, such as Germany[7], Hungary[8], Turkey[9], Iran[10,11], Egypt[12], Nigeria[13], India[14], Japan[15], or China.[16] In both respiratory and external ear canal (EEC) specimens, the black aspergilli are rarely identified at the species level since no association with a specific clinical entity has been reported, in contrast with agriculture where the identification to the cryptic species is essential for deciphering between the different plant diseases, considering species-specific mycotoxin production.[2,17]

Aspergillus section *Nigri* is indeed a taxonomical group containing several phenotypically close species hardly differentiated using standard laboratory procedures. The taxonomy of the black aspergilli has recently been rearranged into six different series[18] and 32 distinct current taxa. [19,20] The assignation of black aspergilli to the species level is now possible by sequencing partial regions of the beta-tubulin or the calmodulin genes.[18,21,22] This later locus is recommended considering the possibility of tubulin paralogs within the *Nigri* section[18] and has shown higher average bootstrap support compared to the beta-tubulin phylogeny.[21] We were interested in identifying cryptic species among the isolates recovered from clinical and environmental specimens in our laboratory and in determining whether given species correlated with specific clinical entities.

Patients and methods

Collection of isolates and antifungal susceptibility testing

We retrospectively studied all the isolates consecutively recovered from EEC and respiratory specimens between October 2010 and December 2018 in our laboratory. Saint-Louis Hospital is a 650-bed university hospital with major clinical activities in hematology and associated with Lariboisière Hospital, which is a reference center for otorhinolaryngology. The EEC isolates were obtained from patients with external otomycosis and the respiratory isolates from patients with various backgrounds investigated for pulmonary symptoms. The patients were classified according to the revised European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) definition[23] by a multidisciplinary hospital group as already described.[24] Information regarding previous therapies including topical treatment for patients with otomycosis was not available. We added isolates collected from systematic air sampling as a survey in hematology wards of our hospital.

The clinical samples were cultured on Sabouraud-chloramphenicol-gentamicin agar medium (Bio-Rad, Marnes-la-Coquette, France) and incubated at 30°C for a maximum of 3 weeks. Air samples were cultured on MALT extract agar plates (VWR, Fontenay-sous-Bois, France) and incubated at 30°C for a maximum of 10 days. Isolates were phenotypically identified as *Aspergillus* section *Nigri* based on macroscopic and microscopic criteria. For each positive culture, spores from several colonies were stored at − 20°C using specific cryotubes (PRO-LAB Diagnostics, Richmond Hill, Canada).

The susceptibility profile was determined only when medically indicated, i.e. for the isolates deemed responsible for invasive aspergillosis (IA) or for those serially recovered from patients with otomycosis with reoccurrence of the same symptoms.

Antifungal susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution method following the procedure E. DEF 7.3.2 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9.3.2_Mould_testing_definitive_revised_2020.pdf) with some modifications.[25] All drugs were purchased from Alsachim (Strasbourg, France).[26]

Molecular investigations

After thawing, five to ten cryopreserved beads were seeded on a Sabouraud-chloramphenicol-gentamicin agar plate. DNA was extracted from approximately 1 cm^2 of mycelium from a 5-day-old culture at 30°C. The mycelium was suspended in 1 ml of ATL lysing solution (Qiagen, Germantown, MD, USA) in a tube containing 500 µl of ceramic beads (MAGNA Lyser, Roche Diagnostics, Mannheim, Germany), and disrupted using a Precellys apparatus (Bertin technologies, Montigny-le-Bretonneux France). Then, 400 µl of the supernatant was used to purify DNA using the MagNA Pure LC DNA Isolation Kit-Large Volume (Roche Diagnostics) with a MagNaPure apparatus according to the manufacturer's instructions. The extracted DNA was stored at − 20°C until use. The calmodulin gene was amplified using the primers cmd5 (5′-CCGAGTACAAGGAGGCCTTC-3′) and cmd6 (5′-CCGATAGAGGTCATAACGTGG-3′)[27] and the sequencing performed as already published.[28]

Strain identification was achieved by sequence similarity search (BLASTn) against curated fungal reference databases available at the online MycoBank database (http://www.mycobank.org/). Multiple sequence alignments were carried out using the MAFFT algorithm with default settings in Geneious v. R9 software (Auckland, New Zealand). After alignment, sequences were trimmed to perform phylogenetic tree construction on the size of a standardized sequence (457 bp).

Table 1. Repartition of the different species identified among 198 isolates according to sampling origin.

Total number of isolates n = 198	External otitis isolates n = 66	Respiratory isolates n = 99	Environmental isolates n = 33	*P* values
Aspergillus welwitschiae (70; 35.3%),	36/66 (54.5%)	25/99 (25.2%)	9/33 (27.3%)	$P < 10^{-3}$ $P = 0.0005$
Aspergillus tubingensis (68; 34.3%)	18/66 (27.3%)	39/99 (39.4%)	11/33 (33.3%)	$P < 10^{-3}$ $P = 0.0005$
Aspergillus niger (34; 17.2%),	5/66 (7.6%)	26/99 (26.3%)	3/33 (9.1%)	$P < 10^{-3}$ $P = 0.0005$
Aspergillus luchuensis (8; 4%),	1/66	1/99	6/33	
Aspergillus aff *welwitschiae* (6; 3%)	3/66	2/99	1/33	
Aspergillus neoniger (4; 2%)	2/66	1/99	1/33	
Aspergillus piperis (3; 1.5%)	1/66	1/99	1/33	
Aspergillus japonicus (2; 1.0%),	0/66	2/99	0/33	
Aspergillus *vadensis* (1; 0.5%)	0/66	1/99	0/33	
Aspergillus spp. Section *Nigri* tubingensis clade (2, 1%)	0/66	1/99	1/33	

Phylogenetic analysis was performed by maximum-likelihood with MEGA10.X.[29] The best substitution model, according to the Bayesian information criterion, was the Kimura-2 parameter with gamma-distributed evolutionary rates. Support for internal branches was assessed with 500 bootstrap replicates. A branch present in greater than 70% of bootstrap replicate is deemed robust[30] A graphic representation of the phylogenetic tree was created using the Interactive Tree Of Life (iTOL) webserver (https://itol.embl.de/).[31] In addition to the clinical isolates, calmodulin gene sequences corresponding to *Aspergillus* section *Nigri* type strains were downloaded from GenBank and incorporated into the phylogenetic analysis (Supplementary Table 1).

Ethical consideration

The present study is a non-interventional study without any change in the usual diagnostic procedures. The analysis relied on existing data from previously performed tests according to physicians' prescriptions. According to the French Health Public Law (CSP Art L1121-1.1), such protocols do not require approval by an ethics committee and are exempt from the otherwise mandatory informed consent requirements.

Results

Identification and distribution of cryptic species of Aspergillus section *Nigri*

A total of 207 isolates were sequenced: 73 from 66 patients with external otitis samples; 101 from 99 patients investigated for respiratory symptoms; and from 33 isolates from the indoor-hospital environment. For patients with serial samples, only the first one was considered. Thus, 198 isolates (66 EEC isolates, 99 respiratory isolates, and 33 air-sampled isolates) were considered for the comparison of species distribution according to the sample site (Table 1).

Based on the BLAST analysis of the calmodulin sequences, we identified a total of nine species. The percentage of similarity to assign the sequence to a reference name was ≥ 99.1% for all but for two sequences, one with 98.8% similarity with *Aspergillus piperis* CBS 112811, and one with 96.7% similarity with *Aspergillus vadensis* CBS 113365, both placed in the tubingensis-clade. Three species, *Aspergillus welwitschiae* (70/198; 35.3%), *Aspergillus tubingensis* (68/198; 34.3%), and *Aspergillus niger* (34/198; 17.2%) accounted for 87% (n = 172) of the total (Table 1).

The phylogenetic tree inferred from the calmodulin gene sequences is presented in Figure 1. The maximum likelihood analyses distinguished three clades: the japonicus-clade (two isolates), the niger-clade (110 isolates), and the tubingensis-clade (86 isolates). These three clades were well supported with bootstrap values of 100, 99, and 96, respectively. The niger-clade comprised three species grouped in *A. welwitschiae* (n = 70) (bootstrap value = 87), *A. niger* (n = 34) (bootstrap value = 99), and *A.* aff. *welwitschiae* (n = 6) (bootstrap value = 85). The tubingensis-clade comprised five species: *A. tubingensis* (68 isolates) grouped together with a low support (bootstrap value = 67), and within the second branch, clinical strains grouped together according to the identified species: *A. luchuensis* (eight isolates) (bootstrap value = 84), *A. neoniger* (four isolates) (bootstrap value = 90), *A. piperis* three isolates) (bootstrap value = 74), and *A. vadensis* (one isolate) (bootstrap value = 90).

When considering the two main clades tubingensis and niger, there was no difference in the repartition between EEC and respiratory isolates ($P = 0.12$) or between respiratory and environment isolates ($P = 0.13$). However, at the species level, when

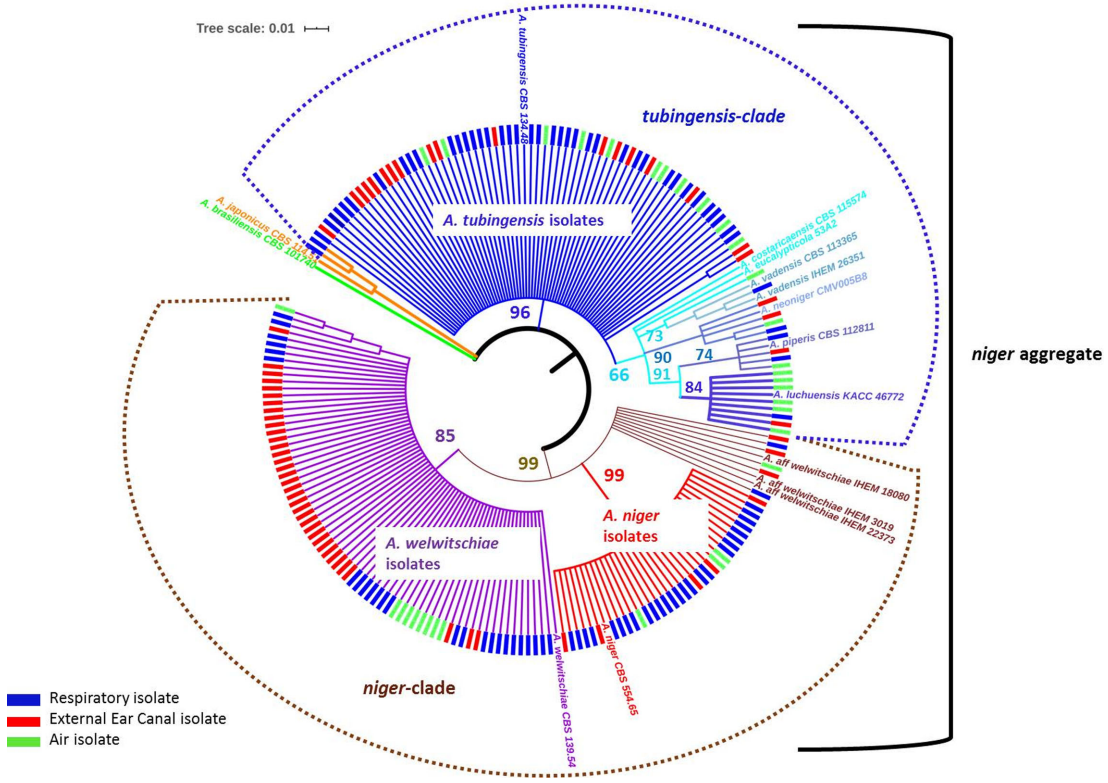

Figure 1. Maximum likelihood tree based on partial calmodulin sequences for 198 strains of *Aspergillus* section *Nigri* according to their anatomical site of isolation. Leaf labels highlighted in blue represent respiratory samples, leaf labels highlighted in red external ear canal samples, and leaf labels highlighted in green air samples. Dark blue branches correspond to *A. tubingensis* isolates, red branches to *A. niger* isolates, purple branches to *A. welwitschiae* isolates, and orange branches to *A. japonicus* isolates. Bootstrap values (in percentage) are presented closed to their dedicated branches.

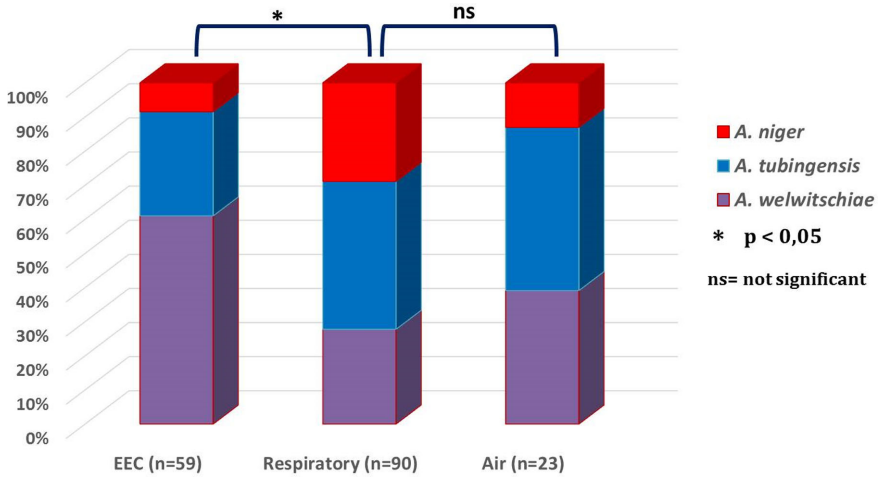

Figure 2. Repartition of the three main species (*Aspergillus welwitschiae, Aspergillus tubingensis,* and *Aspergillus niger*) according to the site of sampling.

considering only the three main species, *A. welwitschiae*, *A. tubingensis*, and *A. niger*, the repartition was significantly different between EEC and respiratory samples (Pearson's Chi-squared test; $P < 0.001$), but not between respiratory and air samples ($P = 0.264$). The three main species repartition according to the site of sampling is presented in Figure 2. Twenty-six isolates (13%) belonging to less frequent or less well-defined species were sampled from air (n = 10/26, 38.5%), respiratory tract (n = 9/26, 34.6%), and EEC (7/26; 26.9%). Of note, six of the eight *Aspergillus luchuensis* isolates were from air samples.

The calmodulin sequences of 196 black aspergilli isolates from this study, with a defined species, were deposited in GenBank and assigned to the accession numbers MW340535–MW340610 for the *A. welwitschiae*, MW340611-

MW340678 for *A. tubingensis*, MW340679-MW340712 for *A. niger*, MW340713-MW340720 for *A. luchuensis*, MW345843 and - MW345846 for *A. japonicus*, MW345839, MW345840, MW345842 and MW345847 for *A. neoniger*, MW345841, MW345844 and MW345848 for *A. piperis*, and MW345845 for *A. vadensis*. GenBank accession numbers assigned to the two black aspergilli isolates without a defined species, belonging to the tubingensis-clade were MW509615 and MW509616.

Clinical data

The 66 patients (31 men, 35 women, mean age 44.4 ± 15.7 years) with external otomycosis had *A. welwitschiae* identified, *i.e.*, 54.5% of EEC samples. Four patients (two men and two women) had recurrent infections and were sampled two to four times from 1 month to 3 years apart. These seven recurrent isolates were all identified as *A. welwitschiae* as the initial isolate with no change in antifungal susceptibility pattern (not shown).

Ninety-nine respiratory specimens from as many patients (50 males, 49 females, mean age 60.4 ± 14.4 years) consisted in broncho-alveolar lavage fluids (n = 8), bronchial aspiration (n = 51), and sputum (n = 40). Underlying disease conditions included 44 chronic pulmonary diseases (12 chronic obstructive pulmonary diseases, five asthma, six fibrosis, eight inflammatory diseases, nine others, and four not specified), 30 hematological malignancies, four common variable immunodeficiency, 16 solid tumors, and five other diseases. There was no significant difference of the cryptic species distribution among the respiratory specimens according to underlying disease and the type of specimens (not shown). Among the 30 patients with hematological malignancies, ten were classified as having probable IA according to the revised EORTC/MSG consensus definitions (Table 2).

Among the ten patients with probable IA, *A. welwitschiae* (n = 3), *A. tubingensis* (n = 5), and *A. niger* (n = 2) were identified (Table 2). Six patients had other molds in the same or concomitant respiratory specimens (*Aspergillus hiratsukae* and *Rhizomucor miehei* in one patient each, *A. fumigatus* in four patients). In four cases (three *A. tubingensis* and one *A. welwitschiae*), the black *Aspergillus* was the only species cultured. Of note, three of the four patients were alive at 3 months after diagnosis. The ten isolates responsible for probable IA had MICs to antifungals in the range of what was observed in the French National Reference Centre for Invasive Mycoses and Antifungals with no significant difference between the cryptic species (Supplementary Table 2).

Among the 89 patients considered as colonized, two had a second isolation of black aspergilli on a distant sample. For one patient, the first sampling yielded *A. welwitschiae* and the second 2 years later, *A. tubingensis*. For the second patient, respiratory specimens yielded *A. tubingensis* and *A. niger* 5 months later.

Discussion

To our knowledge, the present study is the first one analyzing a comprehensive collection of black aspergilli over a given period of time in a single university hospital without selection of a particular site of isolation.

Accurate identification of black Aspergilli isolates remains challenging, and we faced uncertainty for 8 strains over the 198. Nevertheless, recent studies in the field, allowed us to compare our strains with new identified species. Our six strains denominated as *Aspergillus aff welwitschiae*, according to the terminology proposed by D'hooge et al in 2019,[32] presented 98–99.4% identity with the deposited calmodulin sequence (Genbank accession MN583580) of the new species, *Aspergillus vinaceus* sp. nov. recently described.[33]

We mainly identified three cryptic species (*A. niger*, *A. tubingensis*, and *A. welwitschiae*) among the black aspergilli isolates studied. The main observation was that cryptic species were not distributed evenly between EEC and respiratory specimens. We here show that, inside the niger-clade, the two cryptic species *A. welwitschiae* and *A. niger* were associated with different clinical features when comparing EEC and respiratory samples. Indeed, *A. welwitschiae* was recovered in more than half (54.5%) of the EEC samples and only a fourth (25.2%) of the respiratory specimens, while *A. niger* accounted for 26.3% of the respiratory isolates compared to only 7.6% if the EEC isolates.

The higher prevalence of *A. welwitschiae* in EEC has already been reported in otomycosis cases.[8] Very recently, a trend was also noticed in a study including five French hospitals having analyzed selected isolates with more *A. welwitschiae* in-ear samples than *A. tubingensis*.[34] Having worked with isolates of the same geographical origin could have introduced biases since the environment is the source of the black aspergilli. However, we think on the contrary that such sampling alleviates the potential bias due to differences in the environment. Indeed, if the local environment was the major factor to explain the higher frequency of *A. welwitschiae* in EEC, we would have probably observed the same repartition as the air samples, which was not the case in contrast to respiratory samples. To explain the higher prevalence of *A. welwitschiae* in EEC, a first hypothesis could be a better adaptation in the external ear environment featured by the presence of cerumen, a complex composite of different fatty molecules.[35] The differences could also be explained by mycotoxins production, as reported in plant diseases.[17] Ochratoxin toxicity is mediated through oxidative stress, but nothing is known about trans-cutaneous absorption and local toxicity.[36] The molecular mechanism of fumonisins toxicity is the inhibition of ceramide synthase resulting in modifications of sphingolipids rheostat, which might be more relevant given the fatty content of the cerumen.[37]

Table 2. Demographic, clinical, and mycological data, antifungal treatment, and outcome of the 10 patients with probable invasive aspergillosis due to black aspergilli.

Patient number sex/age (years)	Underlying conditions	Date of diagnosis	Respiratory specimen	Mycologic findings	*Nigri* cryptic species cultured	Associated mold	Previous antifungal therapy	Antifungal treatment	Outcome
F/17	AML	11/07/2014	Sputum	Negative DE and serum GM	*A. niger*	*Neosartorya hiratsukae*	Fluconazole	VRZ	Alive at 3 months
F/75	Lymphoma under ibrutinib	09/09/15	Bronchial aspirate	Positive DE and negative serum GM	*A. welwitschiae*	*A. fumigatus*	No	VRZ	Death at 93 days
M/71	MDS	17/10/2016	Sputum	Negative DE and serum GM	*A. niger*	*Rhizomucor miehei*	No	LAMB	Death at day 8
M/27	AML, allo-HSCT (14/09/2017)	2/10/2017	Sputum	Positive DE and negative serum GM	*A. tubingensis*	None	No	LAMB and VRZ, then VRZ alone	Alive at 6 months
F/78	MDS	19/04/2017	BAL fluid	Positive DE serum GM and BAL GM	*A. tubingensis*	*A. fumigatus*	No	VRZ	Alive at 6 months
M/68	MDS	22/05/2014	Sputum	Negative DE and serum GM	*A. tubingensis*	*A. fumigatus*	VRZ	VRZ	Alive at 5 months
F/71	AML	28/07/14	Bronchial aspirate	Negative DE and serum GM	*A. tubingensis*	None	No	VRZ	Alive at 3 months
F/39	MDS, Fanconi anemia, allo-HSCT (5/12/2013), hepatic GVHD treated with etanercept	20/12/13	BAL fluid	Negative DE and BAL GM	*A. tubingensis*	None	Caspofungin	LAMB	Death at day 40
F/66	MDS, allo-HSCT (10/10/17)	30/10/18	Sputum	Negative DE and serum GM	*A. welwitschiae*	*A. fumigatus*	VRZ	Isavuconazole	Alive at 7 months
M/65	Lymphoma under rituximab	26/05/2017	Sputum	Positive DE and negative serum GM	*A. welwitschiae*	None	No	VRZ	Alive at 4 months

[a]AML, acute myeloid leukaemia; MDS: myelodysplastic syndrome; allo-HSCT: allogeneic hematopoietic stem cell transplantation (date of allo-HSCT); BAL: bronchoalveolar lavage; DE: direct examination, GM: galactomannan antigen; VRZ, voriconazole; PSZ, posaconazole; LAMB: liposomal amphoterin B.

However, although more frequent, *A. welwitschiae* is not the only cryptic species identified in EEC. Because of the retrospective design of the study, no systematic and homogeneous report of the clinical symptoms was available, and we are thus unable to draw a correlation between a cryptic species and a more specified clinical setting than external otomycosis.

Another possibility to explain the higher prevalence of *A. welwitschiae* in EEC might be a higher resistance to antifungal drugs of *A. welwitschiae* compared to other cryptic species, knowing that patients can use over-the-counter topical treatment containing antifungal drugs for long periods of time. Some authors report lower MICs to azole drugs for *A. welwitschiae* than for *A. tubingensis* or *A. niger*.[34,38,39] In contrast, a recent study reports a decreased antifungal susceptibility to azoles in clinical isolates of the tubingensis-clade.[32] Additionally, when the iterative isolates from a treated patient were tested, we did not observe an increase in MICs to antifungals. Moreover, most of the over-the-counter preparations used contain nystatin, a polyene

antifungal, and no difference in the MICs to amphotericin B, another polyene, of the cryptic species has been reported. Thus, the hypothesis of a higher resistance of *A. welwitschiae* to antifungals seems unlikely to explain a higher frequency in EEC.

Another observation is the similarity of repartition of the cryptic species between the respiratory isolates and the air isolates. This questions the causality link of any molds other than *A. fumigatus* recovered from respiratory specimens in the symptoms observed.[40] This can simply reflect the inefficiency of the pulmonary tract in eliminating them and not an infectious process. Thus, when black aspergilli were isolated in respiratory specimens in chronic pulmonary conditions, none were considered and treated, in contrast to the experience of Takeda et al.[41] Moreover, the mold recovery highly depends on the methods used, including the type of specimens, volume seeded, temperature, and length of incubation, which is far from being harmonized/consensual.[42] Repeating sampling over several days can also increase the probability of isolating additional molds. For instance, two patients in the present study had serial samples with black aspergilli, which were not of the same cryptic species. Therefore, in optimizing the cultural conditions and multiplying the samples, one increases the probability to cultivate environmental molds.

Defining probable IA due to cryptic species of black aspergilli is difficult. The consensus criteria for IA do not distinguish between the *Aspergillus* species[23] even in the 2020 updated definitions.[43] Here, only four patients had a probable IA due to black aspergilli alone according to the EORTC/MSG definitions. Similarly, Vermeulen et al. also reported few IAs with only 16 patients over 7 years.[5] Of these 16 patients, only six had hematological conditions, three of them had mixed infections with Mucorales or *Scedosporium apiospermum* and died, while two out the three with pure black aspergilli cultures survived at 6 weeks.[5] The good prognosis of these two patients is similar to that of the three patients in our series with IA due to black aspergilli alone. When looking at the cryptic species, we observed three *A. tubingensis* and one *A. welwitschiae*. By sequencing the beta-tubulin gene, Balajee et al. identified six *A. tubingensis* and 13 *A. niger* among the 19 black aspergilli of the TRANSNET collection.[4] The low frequency of IA where black aspergilli are reported as the sole pathogen involved preclude further analysis on the role of specific cryptic species.

In conclusion, our observation underlined for the first time the higher frequency of the cryptic species *A. welwitschiae* as responsible for otomycosis in a French area, which should warrant additional research to explain why the EEC is more suitable for this species. However, our results need to be confirmed in other settings. In Korea, when comparing environmental isolates and clinical isolates from patients with hematologic malignancies, *A. tubingensis* was more often isolated from environmental samples and *A. welwitschiae* from clinical samples, which is in contrast to our findings with respiratory isolates.[38] Thus, our work underlines the need for pursuing the identification process of clinical isolates to unravel some particularities of cryptic species of the black aspergilli, which can be currently achieved more rapidly using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry than using sequencing.[32]

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

All authors declare that they have no conflict of interests.

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