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Original article

Aspergillosis by cryptic *Aspergillus* species: A case series and review of the literature

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ABSTRACT

Background: The cryptic *Aspegillus* species are rare, these microorganisms are usually more resistant to common antifungal therapies. Therefore, a correct identification is important when evaluating the impact of such species in aspergillosis.
Aims: We aimed to describe the frequency, clinical and microbiological characteristics, and the outcomes of those cases of aspergillosis caused by cryptic species in a tertiary hospital.
Methods: We retrospectively identified all microbiologically documented cases of aspergillosis between January 2013 and December 2018. Definitive species identification of clinically significant isolates was achieved via sequencing methods. The polymerase chain reaction (PCR) products were sequenced, and the results obtained were compared to sequences deposited in GenBank. Antifungal susceptibility testing was performed using the Sensititre® YeastOne® panel.
Results: A total of 679 *Aspergillus* isolates were recovered from 489 patients, of which 109 were clinically relevant. Ten (9.2%) isolates were identified as cryptic species: *Aspergillus arcoverdensis* (2), *Aspergillus lentulus* (2), *Aspergillus ellipticus* (2), *Aspergillus alliaceus* (1), *Aspergillus nomius* (1), *Aspergillus tubingensis* (1) and *Aspergillus montevidensis* (1). Most patients already suffered some type of immunosuppression. Half of these patients had required intensive care before the infection showed up, and most of them had a pulmonary infection. Mortality at the 100-day follow-up was 40%. Antifungal susceptibility testing was performed on three of the isolates (*A. arcoverdensis*, *A. tubingensis* and *A. nomius*), which showed high minimum inhibitory concentrations (MIC) for azoles and amphotericin B.
Conclusions: The frequency of cryptic species in our centre was 9.2%. Most patients had some degree of immunosuppression, and the mortality rate was 40%.

Aspergilosis por especies crípticas de *Aspergillus*: serie de casos y revisión de la literatura

Palabras clave:
Especies crípticas
Aspergillus
Infección fúngica de brecha
Hongos multirresistentes

RESUMEN

Antecedentes: Las especies crípticas dentro del género *Aspergillus* son poco habituales, pero suelen mostrar una mayor resistencia al tratamiento antifúngico convencional. Por tanto, una correcta identificación de la especie es necesaria para evaluar el impacto de estas especies crípticas en el desarrollo de la aspergilosis.

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Objetivos: El objetivo de este estudio fue describir las características clínicas, epidemiológicas y microbiológicas, así como la evolución clínica, de los casos de aspergilosis por especies crípticas en un hospital de tercer nivel.
Métodos: Se analizaron de forma retrospectiva todos los casos documentados de aspergilosis con identificación microbiológica entre enero de 2013 y diciembre de 2018. La identificación definitiva de los aislamientos clínicos se realizó mediante métodos de secuenciación. Los productos de amplificación obtenidos por la reacción en cadena de la polimerasa (PCR) fueron secuenciados, y los resultados se analizaron utilizando la base de datos del GenBank. Para el análisis de susceptibilidad a los antifúngicos de los aislamientos identificados se utilizó el panel Sensititre® YeastOne®.
Resultados: Se identificaron un total de 679 aislamientos de *Aspergillus* de 489 pacientes, de los cuales un total de 109 eran clínicamente relevantes. Diez (9,2%) de los aislamientos correspondían a especies crípticas: *Aspergillus arcoverdensis* (2), *Aspergillus lentulus* (2), *Aspergillus ellipticus* (2), *Aspergillus alliaceus* (1), *Aspergillus nomius* (1), *Aspergillus tubingensis* (1) y *Aspergillus montevidensis* (1). La mayoría de los pacientes tenían algún tipo de inmunosupresión previa. La mitad de estos pacientes habían requerido de cuidados intensivos antes de la infección, y la mayoría sufría una infección pulmonar. La mortalidad a los 100 días de seguimiento fue del 40%. Se realizaron pruebas de sensibilidad a los antifúngicos en 3 de los aislamientos *(A. arcoverdensis*, *A. tubingensis* y *A. nomius)*, que mostraron valores de concentración mínima inhibitoria (CMI) más altos para los azoles y la anfotericina B.
Conclusiones: La frecuencia de especies crípticas en nuestro centro fue del 9,2%. La mayoría de los pacientes tenía algún grado de inmunosupresión y la tasa de mortalidad fue del 40%.

Aspergillus genus comprises the most common moulds causing invasive human infections. These infections are usually severe and have become a leading cause of morbidity and mortality, especially amongst immunocompromised patients.[25]

The number of patients at risk of these infections is on the rise. Patients with solid organ transplants and haematological malignancies are experiencing higher life expectancy due to advances in care and standard of living, being a growing risk group. Additionally, many patients receiving prolonged antifungal prophylaxis face developing a breakthrough fungal infection due to resistant fungi.[15]

Aspergillus fumigatus is the most frequent agent in human aspergillosis, followed by *Aspergillus flavus* and *Aspergillus terreus*.[1] However, a multicentre study carried out in Spain (FILPOP study) showed that cryptic species retrieved from clinical samples accounted for 14.5%; in general, these microorganisms were more resistant to the commonly used antifungal agents.[2] Therefore, the identification of the species involved is extremely important, so as to evaluate the impact of cryptic species in invasive aspergillosis and determine whether the current fungal treatment approach (prophylactic and empiric) is appropriate against infections caused by these species.[2]

The objective of this study was to describe the frequency, clinical and microbiologic characteristics, and outcomes of those cases of aspergillosis caused by cryptic species in a 700-bed tertiary hospital between January 2013 and December 2018.

Methods

Patients and data collection

All microbiologically documented cases of aspergillosis between January 2013 and December 2018 were included in the study. The information about patients' demographics, clinical data, treatment provided, and outcomes were obtained from their medical records.

Samples were processed following routine procedures in our laboratory. When a culture tested positive for a filamentous fungus, morphological species identification procedure was performed. Micro- and macroscopic characteristics were considered when identifying the fungus in question into different *Aspergillus* complexes or species (see Table 1).

Definitive species identification of clinically significant isolates was achieved via sequencing methods. The target regions amplified were that of the Internal Transcribed Spacer 2 (ITS2)—a common DNA spacer in all fungi that allows for *Aspergillus* identification at a complex level—and a portion of the β-tubulin encoding gene, which promotes better discrimination among *Aspergillus* species.[5] DNA was extracted from the colony grown in different culture media.

DNA extraction was performed using the EZ1 automatic system® (Qiagen, USA) after mechanic fragmentation of the colony/sample with glass beads. Amplification was done—with some slight modifications—following the method described by Turenne.[22] The SmartCycler® thermocycler (Bio-Rad, USA) was used, and the results were viewed as a peak in the melting curve. Afterwards, polymerase chain reaction (PCR) amplification products were sequenced using AppliedBiosystem® (AppliedBiosystem® genetic kit, USA); the sequences were compared to reference sequences in the GenBank database (www.ncbi.nml.nih.gov/GenBank) and the in-house database of the Mycology Reference Laboratory in Spain.

Antifungal susceptibility testing of the isolates was not routinely performed, being carried out only in three cryptic isolates via the Sensititre® YeastOne® Panel (Thermo Scientific™, UK).

Literature review

The literature review consisted of a search on Medline via PubMed using several keywords' combinations, such as "cryptic species", "invasive aspergillosis", "human pathogens", "identification", "epidemiology", "management", and "treatment". We selected and reviewed all articles published until December 2019 that included the cryptic species identified in our study.

Ethics statement

No ethical approval was required.

Results

Throughout the study period, a total of 679 *Aspergillus* isolates were recovered from 489 patients. Only 109 cases were clinically relevant, whilst 321 were considered colonisers or contaminants by the clinician in charge. Fifty-nine

Table 1
Clinical data from patients with aspergillosis caused by cryptic *Aspergillus* species.

Patient	Age/gender	Underlying condition	Prior ICU stay (30-day)	Site of infection	Sample	Morphological identification	Genetic identification	Treatment	Outcome
1	79/M	Non-Hodgkin Lymphoma	Yes	Lungs	BAL	*Aspergillus* spp.	*A. alliaceus*	Voriconazole: First dose 400 mg/12 h maintenance dose 200 mg/12 h for 4 weeks	Recovery
2	61/M	Heart transplantation	No	Lungs	BAL	*Aspergillus* spp.	*A. montevidensis*	Voriconazole: First dose 400 mg/12 h Maintenance dose 300 mg/12 h for 6 months[c]	Recovery
3	63/M	HIV	No	Lungs	BAL	*A. fumigatus*	*A. arcoverdensis*	Voriconazole: 400 mg/12 h for 6 months [d]	Recovery
4	66/M	Cirrhosis (Child–Pugh C)	Yes	Lungs	BAL	*Aspergillus* spp.	*A. lentulus*	Anidulafungin: First dose 200 mg Maintenance dose 100 mg + liposomal amphotericin B 3 mg/kg (200 mg)	Death
5	48/M	HIV	Yes	Liver	Hepatic biopsy	*A. niger*	*A. tubingensis*	No antifungal treatment	Death
6	65/M	Pulmonary cavity	No	Lungs	Pulmonary biopsy	*A. fumigatus*	*A. ellipticus*	Lobectomy + Voriconazole: First dose 400 mg/12 h Maintenance dose 200 mg/12 h for 90 days	Recovery
7	62/M	Accidental inoculation [a]	No	Central nervous system, pulmonary	Cerebrospinal fluid, BAS	Negative culture [b]	*A. ellipticus*	Posaconazole 200 mg/6 h + terbinafine 250 mg/12 h + micafungin 150 mg/24 h	Death
8	51/M	Thymoma	Yes	Pleural	Pleural liquid (pleural fluid aspiration)	*A. fumigatus*	*A. lentulus*	Liposomal amphotericin B administered through a thoracic drain 25 mg/24 h for 3 weeks + voriconazole: First dose 400 mg/12 h Maintenance dose 200 mg/12 h for 4 weeks	Recovery
9	68/M	HIV	No	Lungs	BAL	*A. fumigatus*	*A. arcoverdensis*	Anidulafungin: First dose 200 mg Maintenance dose 100 mg/24 h + liposomal amphotericin B administered through a thoracic drain 25 mg/24 h for 3 weeks	Recovery
10	39/M	Waldenstrom's disease	Yes	Lungs	BAS	*Aspergillus* spp.	*A. nomius*	Anidulafungin: First dose 200 mg Maintenance dose 100 mg/24 + isavuconazole 200 mg/8 h (6 doses) + nebulised liposomal amphotericin B 25 mg/24 h	Death

[a] Suspected direct inoculation after local corticoid injection.
[b] Negative culture. Microbiological diagnosis was achieved by direct DNA amplification from the clinical sample.
[c] In this patient voriconazole concentration was measured in order to control the dose.
[d] In this patient a high dose of voriconazole was maintained due to the interaction with the antiretroviral therapy. M: male; BAL: bronchoalveolar lavage; HIV: human immunodeficiency virus; BAS: bronchial aspirate; ICU: intensive care unit.

patients could not be accounted in the follow-up period, making any clinical assessment related to their isolates impossible.

A. fumigatus was the most frequently isolated species (506 isolates), followed by *A. flavus* (78 isolates), *Aspergillus niger* (16 isolates) and *A. terreus* (60 isolates). We found nineteen *Aspergillus* isolates that were informed as *Aspergillus* spp. and definitive species identification was not achieved (17 colonisers/contaminants). Of the 109 clinically relevant isolated strains, *A. fumigatus* (76 isolates) was the most frequent, followed by *A. flavus* (10 isolates), cryptic species (10 isolates), *A. niger* (6 isolates) and *A. terreus* (5 isolates). Isolates belonging to cryptic species represented 9.2% of this cohort, and final identification included the following: *Aspergillus* section *Fumigati* was represented by two *Aspergillus arcoverdensis* (deposited in GenBank under accession numbers MN078293 and MN078294); two *Aspergillus lentulus* (GenBank, accession numbers MN078297 and MN078298) and two *Aspergillus ellipticus*. *Aspergillus* section *Flavus* included one *Aspergillus alliaceus* (GenBank, accession number MN078295) and one *Aspergillus nomius* (GenBank, accession number MN204617); *Aspergillus* section *Nigri* included one *Aspergillus tubingensis* (GenBank, accession number MN078296). One *Aspergillus montevidensis*—previously known as *Aspergillus amstelodami* (GenBank, accession number MN078299)—was also isolated (Table 1). The samples were predominantly recovered from respiratory tract, except in two cases in which a liver biopsy and cerebrospinal fluid were obtained. All the samples yielded a positive culture, except for that of the cerebrospinal fluid. The diagnosis in that patient was achieved by performing a direct PCR (ITS2 region and β-tubulin encoding gene) on the sample.

Table 2
Summary of the literature found regarding *Aspergillus* cryptic species.

Identification	Brief description	Reference
A. alliaceus	Aspergilli from the section *Flavi*, well known as human pathogen, capable of causing different type of infections, from onychomycosis to invasive aspergillosis.	Ozhak-Baysan B et al., 2010; Bongomin F et al., 2018
A. montevidensis	Previously known as *A. amstelodami* (*Eurotium amstelodami*, *Aspergillus hollandicus*). This is a rare Aspergilli related with a farmer's lung disease and invasive rhinosinusitis infection.	Hubka V et al., 2013; Roussel S et al., 2010; Shivaprakash MR et al., 2008
A. arcoverdensis	To our knowledge we describe the first isolates causing invasive infection, in contrast with the isolation of the species in soil in 2015.	Matsuzawa T et al., 2015
A. lentulus	Aspergilli from the section *Fumigati.* One of the first cryptic species described. Currently known as a cause of invasive aspergillosis.	Balajee SA et al., 2005; Yoshida H et al., 2015; de Azevedo Bastos VR et al., 2015
A. tubingensis	Aspergilli from the section *Nigri* capable of producing different pathologies, from oral cavity infection to invasive pulmonary infection.	Dóczi I et al., 2009; Bathoorn E et al., 2013; Deepa A et al., 2014; Vermeulen E et al.,2014; Li Y et al., 2015
A. ellipticus	Before our study, this species has not been related with invasive infection.	Staab JF et al., 2009
A. nomius	Aspergilli from the section *Flavi*, well known as human pathogen, capable of causing different type of infections, from onychomycosis to invasive aspergillosis.	Negri CE et al., 2014; Bongomin F et al., 2018

Table 3
MIC (mg/ml) values of three isolates belonging to *Aspergillus* cryptic species.

Antifungal	*Aspergillus arcoverdensis*	EUCAST breakpoints[a]	*Aspergillus tubingensis*	EUCAST breakpoints[a]	*Aspergillus nomius*	EUCAST breakpoints[a]
Posaconazole	0.5	≤0.125 (S)	0.5		0.5	
Voriconale	1		1		2	
Itraconazole	0.5	≤1(S)	2		1	≤1 (S)
Amphotericin B	1		2	≤1 (S)	2	

The values obtained were compared with EUCAST breakpoints. In the case of *A. arcoverdensis*, if breakpoints for *A. fumigatus* are used, this isolate would be considered resistant to posaconazole. For *A. flavus* (*A. nomius* section *Flavi*) EUCAST has breakpoints for isavuconazole and itraconazole only, being our isolate susceptible to itraconazole. Finally, for *A. niger* (*A. tubingensis* section *Nigri*), EUCAST has breakpoints for amphotericin B only and, according to them, our isolate was resistant. S: Susceptible

[a] The European Committee on Antimicrobial Susceptibility Testing. Breakpoints table of MICs for antifungal agents, version 10.0, 2020. http://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/.

Table 1 shows the clinical data of those patients with aspergillosis caused by cryptic species. All patients were males, with a median age of 62.5 years (IQR 12.2). Most patients (80%) had immunosuppressive baseline diseases: haematological malignancies (two patients), solid organ transplantation (one patient), HIV infection with a low CD_4 count (<200 cells/ml) (two patients), solid neoplasm, and cirrhosis (one patient each). Additionally, two (20%) patients had received corticosteroids, and five (50%) had required admission to intensive care unit (ICU) in the previous 30 days. Overall, five (50%) patients presented a positive galactomannan (value > 0.5) result in serum or bronchoalveolar lavage, with mean values of 1.12 (SD 0.33) and 2.25 (SD 1.95) in serum and bronchoalveolar lavage, respectively. Excluding one patient, whose fungal infection was diagnosed *post mortem* by molecular tests, all patients received systemic antifungal treatment. Four (40%) patients received antifungal combination therapy. Mortality at 100-day follow-up was 40%. Table 2 shows literature research related to the cryptic species identified in our study.

Antifungal susceptibility testing was performed in three isolates: *A. arcoverdensis* (patient 3), *A. tubingensis* (patient 5) and A. nomius (patient 10). Susceptibility testing was not performed on the remaining isolates belonging to cryptic species, as they could not be recovered. Overall, minimum inhibitory concentration (MIC) values were higher for azoles (range 0.5–2 μg/ml) and amphotericin B (range 1–2 μg/ml). Table 3 lists the MIC values obtained.

Discussion

During the study, we identified 10 cases of aspergillosis caused by cryptic *Aspergillus* species, accounting for 9.2% of clinically relevant isolates. This incidence is similar to the 12% rate identified for cryptic species in another Spanish study evaluating all clinical strains of filamentous fungi isolated from deep tissue and respiratory samples.[1]

Aspergilli from the section *Fumigati* are well-known human pathogens, with *A. lentulus* being one of the first cryptic species detailed.[6] Following its description, many cases of invasive aspergillosis caused by this pathogen have been reported.[4,6] Most of them cause pulmonary infections, as observed in our study. Interestingly, we report a case of pleural aspergillosis, which is extremely rare.[12] We believe that *A. lentulus* colonisation of the pleural cavity was, in this case, secondary to a thymoma surgery or, most likely, a bronchopleural fistula. Additionally, we describe two cases of pulmonary infection due to *A. arcoverdensis* in patients with HIV infection and low CD_4 counts. This cryptic *Aspergillus* species was first isolated in a clinical sample in 2015 by Matsuzawa et al.,[16] but to our best knowledge, our isolates are the first described in an invasive infection. Similarly, *A. ellipticus*—from the complex *Niger,* a variety of *A. fumigatus* rather than a cryptic species—has not been previously associated with infection.[16] In our cohort, *A. ellipticus* caused both a pulmonary and a central nervous system infection. Species identification was confirmed after recovering the isolates from cerebrospinal fluid and bronchial aspirate.

A. alliaceus and *A. nomius,* from section *Flavi,* can cause a variety of clinical manifestations, ranging from chronic otitis to invasive aspergillosis.[10,12,16] In our study, these species caused possible pulmonary infections. *A. tubingensis*, a pathogen from section *Nigri* (black aspergilli), is the causative agent behind many infections in which *A. niger* was the species informed.[8,17] *A. tubingensis* has been involved in different pathologies, including osteomyelitis,[7] keratitis,[11] and pulmonary infections.[23]

Finally, we also report a case of pulmonary infection due to *A. montevidensis* (*Eurotium amstelodami*, *Aspergillus hollandicus*), which does not belong to any of the previous sections. This rare fungus has been associated with the development of farmer's lung disease.[20] It has rarely been described as an invasive and causative agent of rhinosinusitis.[21]

As expected, most patients in our cohort had immunosuppression. It is important to establish that half of the patients required ICU admission in the 30 days before being diagnosed wit a fungal infection. Although invasive aspergillosis has been classically linked to haematological malignancies and stem cell transplantations, a new risk factor like ICU stay is increasingly being reported.[14,15] This remark is significant, as mortality in our cohort was extremely high.

Three cryptic strains were subjected to antifungal susceptibility testing. Data from different studies suggest that many of the cryptic species isolated could be more resistant to common antifungal drugs.[24,27] This fact could be relevant since recent studies have shown that patients with triazole-resistant invasive aspergillosis have higher mortality compared with triazole-susceptible cases.[1,13] In our study, MICs were relatively high for azoles and amphotericin B. However, not standardised clinical breakpoints are available for these species. *A. tubingensis* MICs were in accordance with results found by Alastruey-Izquierdo et al.[2,3] The other two isolates tested showed similar MICs with other studies. However, in the case of *A. nomius,* susceptibility data is scarce, given that it has seldom or never been reported as human pathogen. *A. arcoverdensis* isolate was slightly more susceptible to itraconazole and amphotericin B, similarly to the data obtained with *A. lentulus,* that typically shows reduced susceptibility to amphotericin B and azoles.[9,26]

This study had some limitations. We could not calculate the frequency of cryptic species isolated from all clinical samples. Many of the isolates considered as colonisation/contamination were not identified at a species level with molecular methods. However, we were able to calculate the incidence of cryptic species amongst species causing aspergillosis. Another limitation of this study is that antifungal susceptibility testing could only be performed on three of ten isolates identified as cryptic species. Lastly, not all the patients met the criteria for invasive aspergillosis, according to the most frequently used definitions.[18,19] However, these criteria are essentially focused on onco-haematological patients, and all our cases were considered clinically relevant after a thorough clinical evaluation.

In conclusion, the frequency in our centre of *Aspergillus* isolates belonging to cryptic species was 9.2%. Most patients had some type of immunosuppression and/or had required ICU admission. The overall mortality related to aspergillosis by cryptic species was 40%. Identifying cryptic species may prove significant when it comes to selecting the appropriate antifungal treatment and reinforcing the knowledge about these new species.

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Conflict of interest

The authors declare that they have no conflict of interest.

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