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Current section and species complex concepts in *Aspergillus:* recommendations for routine daily practice

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The identification of fungi by molecular techniques has generated important changes in fungal taxonomy. The use of molecular tools in taxonomic studies has led to the description of some cryptic species that were placed into a complex of morphologically similar organisms and were subsequently misidentified. There are still limited data available on the prevalence of cryptic species of *Aspergillus* in the clinical setting, although some studies report 10–14% of the total number of *Aspergillus* species. In addition, the main concern about the emergence of *Aspergillus* cryptic species is that they can be more resistant to antifungal agents. The rise in the incidence of fungal infections and the changing landscape of epidemiology, together with the description of new pathogens and their different susceptibility profiles, make the identification by molecular methods and/or antifungal susceptibility testing the best options available for the correct management of these infections.

Keywords: *Aspergillus*; resistance; molecular identification

Introduction

The population at risk for invasive fungal infections is expected to increase in the coming years as new and evolving predisposing factors continue to rise. Despite advances in diagnosis and treatment, mortality and morbidity rates remain high. Although *Candida albicans* and *Aspergillus fumigatus* are the main etiologic agents of disease, the epidemiology of fungal infections is changing and the number of species able to infect humans is constantly growing.[1–4] This change in the epidemiology accounts for both yeast and molds. However, while the incidence of yeast has remained just about constant, in recent years an increase in the incidence of mold infections has been documented.[5,6] Furthermore, in some groups of patients molds are more frequently encountered than yeast.[6] The reasons for the changing epidemiology have been attributed to several reasons, such as the growing number of people at risk with impaired immunity, extensive use of antifungal agents (producing a shift toward more resistant organisms), and advances in diagnosis and detection methods that have generated tools to achieve more accurate identification of fungal pathogens.

While yeasts are quite easily identified by their biochemical properties, mold identification in clinical laboratories relies mainly on the observation of morphological characteristics. For years the identification and the description of fungal species have been based on the observation of phenotypic characters. Morphological, and in some cases biochemical, properties were used to separate species. With the arrival of the genomic era the identification of fungi by molecular techniques generated (and still does) important changes in their taxonomy. Although there is no consensus for species delimitation in fungi, the phylogenetic species recognition (PSR) concept[7] has been demonstrated to be useful in several taxonomic studies.[2,3,8,9] This method is based on the sequencing of several targets to perform phylogenetic analysis for species recognition. In fact, based on PSR, new cryptic and sibling (from hereon "cryptic") species within the most frequent pathogens have been described.[2,3,10] Furthermore,

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other species have been reclassified under a different genus.[8,11]

The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) have been the gold standard for identifying pathogenic fungi.[12] However, in some genera, such as *Fusarium, Scedosporium,* and *Aspergillus,* ITS regions identify only to the species "complex level" (that is, species that are morphologically or biochemically similar and otherwise indistinguishable by classical methods and/or ITS sequencing). Therefore, other targets have been selected as the preferred marker for classification purposes. Some of these new species are more resistant to current antifungal drugs, making the identification to species level of the etiologic agent of an infection decisive for the correct management of the patients. In this paper we will focus on the identification and susceptibility profile of the *Aspergillus* spp. isolates found in the clinical setting.

Aspergillus species in the clinical setting

Aspergillus spp. are the most frequent molds isolated from clinical samples. The genus *Aspergillus* contains about 250 species divided into eight subgenera (*Aspergillus, Fumigati, Circumdati, Candidi, Terrei, Nidulantes, Warcupi,* and *Ornati*),[13] which in turn are subdivided into several sections or species complexes. The most frequent cause of aspergillosis is *A. fumigatus,* followed by *A. flavus, A. terreus,* and *A. niger,* but many other species have been described in human infections. *A. terreus* has been particularly associated with lethal infections,[14,15] and *A. flavus* has been described as the most common species of *Aspergillus* isolated in some centers.[16]

The use of molecular tools in taxonomic studies has led to the description of some cryptic species that were misidentified and underestimated, as they were classified as complexes of morphologically similar species.[17] Until now, the consensus is that ITS sequencing allows classifying an isolate of *Aspergillus* within its species complex.[12] However, to reach a correct identification to a species level, the sequence of other targets, such as β-tubulin, calmodulin, or rodlet A genes,[17,18] has become necessary.

Taxonomic studies using molecular identification have produced important changes in the classification of *Aspergillus* species, cryptic species have been described and studies to reclassify strains have been conducted.[1,19–21] Nevertheless, the real distribution of these cryptic species in clinical samples remains unclear. In a prospective study of fungal infections in transplant patients in the U. S.,[22] 218 *Aspergillus* isolates were identified. Using molecular identification, 147 (67.4%) were classified as *A. fumigatus* complex, 29 (13.2%) as *A. flavus* complex, 19 (8.7%) as *A. niger* complex, 11 (7.4%) as *A. terreus* complex, 6 (2.7%) as *A. ustus* complex, 5 (2.3%) as *A. versicolor* complex, and 1 as *A. nidulans* complex. Comparable results have been found in a prospective study performed in Spain (unpublished data) with samples from deep body sites. From a total of 278 *Aspergillus* isolates, 162 (50.3%) belonged to the *A. fumigatus* complex, 43 isolates (13.4%) to the *A. nigri* complex, 30 (9.3%) to the *A. flavus* complex, 27 (8.3%) to the *A. terreus* complex, 8 (2.9%) to the *A. nidulans* complex, 6 (2.2%) to the *A. usti* complex, and 1 to *A. versicolor* complex. Table 1 shows the distribution of species in both studies. Although the frequency and distribution of species are comparable between works, some differences can be noted. Thus, *Neosartoria udagawae* and *A. versicolor* were only identified in the U.S. study, while *A. nidulans* sensu stricto was only found in the Spanish study. In addition, the frequency of *A. tubingensis* and *A. terreus* was significantly lower in the U.S. study. These differences can be attributed to the study design, since the U.S. study included transplant patients only[22] and in the Spanish study samples were from patients with diverse underlying conditions; however, more studies are needed in order to know if these differences are stable and could be due to other factors such as differences in geographical distribution of *Aspergillus* species. In the U.S. study,[22] 10.6% (23 out of 218) isolates belonged to cryptic species of *Aspergillus,* and in the Spanish survey (unpublished data) up to 14.4% (40 out of 278) of the *Aspergillus* strains belonged to species that were not possible to identify by classical methods. Within the cryptic species, the most frequent ones were *A. tubingensis,* with 6 strains (2.8%) in the United States and 22 (7.9%) in Spain, *A. calidoustus,* with 6 isolates (2.8%) in the U. S. and 4 isolates (1.4%) in Spain, and *A. lentulus,* with 4 (1.8%) in the U. S. and 3 (1.1%) isolates in Spain. Other cryptic species such as *N. udagawae* and *A. alliaceus* were found only in one study (see Table 1). Many of these cryptic species have been previously described in human infections.[17,19,22–25] Moreover, some of them have been found to be in equal or greater frequency in clinical samples than their

Table 1. *Aspergillus* species distribution according to epidemiological surveys from Spain and the U. S.[22]

		Transnet		Spanish study	
Species	Section	N isolates	%	N isolates	%
A. fumigatus	Fumigati	139	63.8	156	56.1
A. lentulus	Fumigati	4	1.8	3	1.1
A. udagawae	Fumigati	3	1.4	0	0.0
N. peudofischeri	Fumigati	1	0.5	1	0.4
A. viridinutans	Fumigati	0	0.0	1	0.4
A. fumigatiafinis	Fumigati	0	0.0	1	0.4
A. flavus	Flavi	29	13.3	27	9.7
A. alliaceus	Flavi	0	0.0	3	1.1
A. terreus	Terrei	11	5.0	26	9.4
A. carneus	Terrei	0	0.0	1	0.4
A. tubingensis	Nigri	6	2.8	22	7.9
A. niger	Nigri	13	6.0	21	7.6
A. calidoustus	Usti	6	2.8	4	1.4
A. insuetus	Usti	0	0.0	1	0.4
A. keveii	Usti	0	0.0	1	0.4
A. sydowii	Versicolores	2	0.9	1	0.4
A. versicolor	Versicolores	3	1.4	0	0.0
E. quadrilineata	Nidulantes	1	0.5	0	0.0
A. nidulans	Nidulantes	0	0.0	8	2.9
A. westerdijkiae	Circumdati	0	0.0	1	0.4
Total		218	100	278	100

well-known relatives,[1,4,20,21] as it was found in the Spanish study with more strains of *A. tubingensis* than of *A. niger* (Table 1). Thus, the use of molecular identification seems to become an important tool in the clinical setting.

Antifungal susceptibility

One of the main concerns about the emergence of the cryptic species is that they can be more resistant to the antifungal drugs. The EUCAST Subcommittee on AFST, based on epidemiological studies,[26] has proposed breakpoints for voriconazole and posaconazole with regard to *A. fumigatus* and *A. terreus,* amphotericin B (AMB) with regard to *A. fumigatus* and *A. niger,* and itraconazole with regard to *A. fumigatus, A. flavus, A. terreus,* and *A. nidulans*.[27] Thus, strains with MIC (minimal inhibitory concentration) values > 2 mg/L for AMB, itraconazole, and voriconazole have been defined as resistant, while for posaconazole MIC values > 0.25 mg/L are considered resistant, according to the EUCAST methodology. Taking into account these breakpoints, resistance has been analyzed in several studies. Table 2 shows geometric mean, MIC_{50} (MIC causing inhibition of 50% of isolates), MIC_{90} (MIC causing inhibition of 90% of isolates), and the range for the *Aspergillus* sections to which the most common clinical isolates belong. As showed in Table 2, sections Flavi and Terrei show higher MICs values to AMB. The reduced susceptibility of *A. terreus* to amphotericin B is well known.[28] Infections caused by these fungi have been associated with a lower response rate and poorer outcomes.[29] The reduced susceptibility of Flavi can be attributed to *A. alliaceus* (Table 2), which has been described as resistant to this drug.[30,31] Reduced susceptibility to AMB has also been found in cryptic species such as *A. lentulus,*[19,32] *A. fumigatiaffinis,*[19,22] *A. niveus,*[33] *A. carneus*,[34] or *A. calidoustus.*[1] It should be noted that the prevalence of these cryptic species is very low (<5% of isolates). Table 3 shows geometric mean, MIC_{50},

Table 2. Geometric mean, range, MIC_{50} and MIC_{90} from the species isolated from clinical samples grouped in *Aspergillus* sections. Data from the collection of the Spanish National Centre for Microbiology. MIC values in mg/L

Section (N)		Amphotericin B	Itraconazole	Voriconazole	Posaconazole
Fumigati (161)	GM	0.28	0.18	0.51	0.05
	Range	0.06–16	0.06–1	0.12–2	0.015–0.5
	MIC_{50}	0.25	0.25	0.5	0.06
	MIC_{90}	0.5	0.25	1	0.12
Flavi (30)	GM	2.15	0.19	0.63	0.09
	Range	0.5–32	0.03–1	0.12–4	0.015–0.25
	MIC_{50}	1	0.25	0.5	0.12
	MIC_{90}	32	1	1	0.12
Terrei (27)	GM	1.59	0.13	0.95	0.05
	Range	0.5–8	0.06–0.25	0.5–2	0.03–0.12
	MIC_{50}	1	0.12	1	0.06
	MIC_{90}	4	0.25	2	0.12
Nigri (43)	GM	0.12	0.43	0.74	0.10
	Range	0.06–1	0.06–16	0.25–2	0.015–0.25
	MIC_{50}	0.12	0.5	1	0.12
	MIC_{90}	0.25	1	1	0.12
Nidulantes (8)	GM	1.53	0.12	0.21	0.08
	Range	0.12–32	0.06–0.5	0.12–1	0.03–0.12
	MIC_{50}	1.5	0.12	0.25	0.12
	MIC_{90}	16	0.25	0.5	0.12
Usti (6)	GM	0.56	4	5.66	3.17
	Range	0.25–1	1–16	4–16	2–16
	MIC_{50}	0.5	8	4	2
	MIC_{90}	1	16	16	16
Versicolores (1)	MIC	0.50	1.00	1.00	0.50
Circumdatii (1)	MIC	32.00	0.50	0.50	0.12
All	GM	0.39	0.21	0.60	0.07
	Range	0.06–32	0.015–16	0.12–16	0.015–16
	MIC_{50}	0.25	0.25	0.5	0.06
	MIC_{90}	2	0.5	1	0.12

N, number of isolates tested; GM, geometric mean of MICs; MIC_{50}, MIC causing inhibition of 50% of isolates; MIC_{90}, MIC causing inhibition of 90% of isolates.

MIC_{90}, and range for the most frequent cryptic species.

Reduced susceptibility to azole drugs have been extensively investigated in *A. fumigatus*.[35–38] Several reports describe an increasing incidence of strains with acquired resistance associated with an elevated mortality, but such emergence of resistance strains has only been found in some European countries.[39,40] On the other hand, some cryptic species within *A. fumigatus* species complex, such as *A. lentulus, A. viridinutans, N. pseudofischeri, A. fumigatiafinnis,* have been described to be resistant to all azoles.[19,22,39,40] This pattern of resistance has been also described in species from other complexes, such as *A. tubingensis* (*A. niger* complex)[19,21] and *A. calidoustus* (*A. ustus* complex) that have an azole multiresistant susceptibility profile.[1] *In vitro* susceptibility data for these species are shown in Table 3 (data from the Spanish National Centre for Microbiology).

Table 3. Geometric mean, range, MIC_{50} and MIC_{90} from most common cryptic species of *Aspergillus* with decreased susceptibility to antifungal agents. Data from the Spanish National Centre for Microbiology. MIC values are expressed as mg/L

Species (N)		Amphotericin B	Itraconazole	Voriconazole	Posaconazole
A. lentulus (17)	GM	6.40	3.57	3.22	0.20
	Range	1–16	0.25–16	2–8	0.06–2
	MIC_{50}	8	8	4	0.12
	MIC_{90}	16	16	8	2
A. alliaceus (25)	GM	21.71	0.21	0.54	0.15
	Range	0.5–32	0.125–16	0.25–2	0.06–16
	MIC_{50}	32	0.125	0.5	0.12
	MIC_{90}	32	2	2	0.5
A. tubingensis (22)	GM	0.09	0.51	0.78	0.10
	Range	0.06–1	0.06–16	0.25–2	0.015–0.25
	MIC_{50}	0.12	0.5	1	0.12
	MIC_{90}	0.12	1	2	0.12
A. calidoustus (12)	GM	0.80	8.31	5.88	7.41
	Range	0.5–2	1–16	4–8	2–16
	MIC_{50}	1	16	8	8
	MIC_{90}	2	16	8	16

N, number of isolates tested; GM, geometric mean of MICs; MIC_{50}, MIC causing inhibition of 50% of isolates; MIC_{90}, MIC causing inhibition of 90% of isolates.

Conclusions

Fungal infections are becoming more frequent in the clinical setting. Despite technical advances and the availability of more antifungal compounds, the survival rate among these patients remains low. Proper identification of the isolate and early initiation of an effective antifungal therapy are essential for patient recovery. Recent advances in molecular tools have allowed for the description of new cryptic species among different groups of fungi. This has been particularly significant in *Aspergillus* since new species are almost impossible to differentiate by classical morphological tools and some are more resistant to antifungal drugs. ITS sequencing allows classifying an isolate within its species complex but to reach a proper identification to species level it is necessary to sequence other targets such as beta tubulin. The frequency of these cryptic species is still not clear, some data report between 10 and 14% of the total *Aspergillus* strains. But more studies are warranted in order to elucidate the real prevalence of these species in the clinical setting.

The rise in the incidence of fungal infections, the changing landscape of epidemiology, together with the description of new pathogens and their different susceptibility profile, make the identification by molecular methods and/or antifungal susceptibility testing the best options available for the correct management of these infections.

The classification of isolates by molecular methods could be useful for the clinical management of patients, since over 10% of *Aspergillus* strains could belong to cryptic species, some of which are more resistant *in vitro* to antifungal agents. More studies should be done to understand the local epidemiology in each area.

Some recommendations can be made for routine daily practice. When dealing with strains on cultures from deep sites we recommend performing identification by β-tubulin sequencing. Where such sequencing is not available, other strategies for *A. fumigati* complex, such as matrix-assisted laser deroption/ionication/time of flight mass spectrometer[41] or luminex assay,[42] appear to be useful for identifying cryptic species. When other strategies are not available, we advise performing antifungal susceptibility testing of the strains to assess their susceptibility profile *in vitro* and then sending the

strains to the reference centre for correct classification and epidemiological analysis. It has to be taken into account that although proper identification can speed up directed antifungal therapy, these techniques cannot detect strains with secondary resistance. Therefore, susceptibility testing is an essential tool and should be performed routinely for better management of antifungal therapy.

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Conflicts of interest

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