



Screening of cryptic species among clinical *Aspergillus* isolates collected during one year period in a Portuguese reference laboratory



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ABSTRACT

Correct identification of *Aspergillus* species is important given that sibling species may show variable susceptibilities to multiple antifungal drugs and also because sharper definition of species may facilitate epidemiological studies. Thus, we screened *Aspergillus* clinical isolates from Portuguese hospitals to determine which, if any, of the cryptic species of *Aspergillus* were involved in patient infections.

Over a one year period, *Aspergillus* isolates from Portuguese health institutions were collected. These isolates were identified on the basis of microscopic morphology and through the use of molecular tools. Genomic DNA was prepared from each isolate and the sequencing of the Internal Transcribed Spacers (ITS) regions, specifically the ITS1 and ITS2 non-coding regions flanking the 5.8S rDNA was used to determine the species complex, whereas β -tubulin and calmodulin sequencing was done to achieve the correct species identification.

Over the study period, 57 *Aspergillus* isolates from clinical samples were collected from 10 Portuguese health institutions. According to the morphological observations, 29 isolates were identified as *Aspergillus fumigatus*, 11 *A. flavus*, 8 *A. niger*, 3 *A. nidulans*, 2 *A. terreus*, 2 *A. candidus* and 2 *Aspergillus* sp. Among those isolates, six species-complexes were detected by ITS sequencing, and were distributed as follows: *Fumigati* (50.1%), *Flavi* (21.0%), *Nigri* (15.8%), *Terrei* (5.3%), *Nidulantes* (3.6%) and *Versicolores* (3.6%). β -tubulin and calmodulin sequencing resulted in ten (17.5%) cryptic species being identified among the 57 isolates. Seven of those isolates belonged to the *Nigri* complex (*A. brasiliensis*, *A. awamarii*, *A. tubigensis*), two to the *Versicolores* complex (*A. sidowii* and *A. fructus*), one to the *Fumigati* complex (*A. lentulus*) and one to the *Nidulantes* complex (*Emmericella echinulata*).

With rigorous application of molecular tools, cryptic species of *Aspergillus* are not uncommon in the clinic. The identification of cryptic species among the collected clinical isolates of *Aspergillus* alerts the clinician to isolates with reduced susceptibilities to antifungal drugs and emphasizes a correct identification to species level.

➤ Isolates no. 12-47 and INSA7 were identified morphologically only to the genus level (*Aspergillus* sp.), but both were classified as belonging to the *Versicolores* complex by molecular methodologies.

➤ Eleven isolates (19.3%) belonging to seven *Aspergillus* cryptic species were identified (Table 1, in red). Seven of those isolates belonged to the *Nigri* complex (cryptic species *A. brasiliensis*, *A. awamarii* and *A. tubigensis*), two belonged to the *Versicolores* complex (cryptic species *A. sidowii* and *A. fructus*), one to the *Fumigati* complex (cryptic species *A. lentulus*), and one to the *Nidulantes* complex (cryptic species *Emmericella echinulata*)

Table 1. Morphological and molecular identification of the collected clinical isolates

Isolate code	Origin	Sample	Morphological identification	Species complex (ITS sequencing)	Species (β -Tubulin and calmodulin sequencing)
12-26	Hospital # 1	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-27	Hospital # 1	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-28	Hospital # 1	Bronchial secretions	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-29	Hospital # 1	Bronchial secretions	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-30	Hospital # 1	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-31	Hospital # 1	Bronchoalveolar lavage	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-32	Hospital # 1	Bronchoalveolar lavage	<i>A. flavus</i>	<i>Flavi</i>	Not identified to species level
12-33	Hospital # 1	Bronchoalveolar lavage	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-34	Hospital # 2	Sputum	<i>A. flavus</i>	<i>Flavi</i>	Not identified to species level
12-35	Hospital # 2	Sputum	<i>A. niger</i>	<i>Nigri</i>	<i>A. niger</i>
12-36	Outpatient NIH	Unknown	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-37	Hospital # 3	Bronchial secretions	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-38	Outpatient NIH	Stool	<i>A. niger</i>	<i>Nigri</i>	<i>A. tubigensis</i>
12-39	Hospital # 1	Bronchoalveolar lavage	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-40	Hospital # 1	Auricular exudate	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-41	Hospital # 1	Sputum	<i>A. niger</i>	<i>Nigri</i>	<i>A. awamarii</i>
12-42	Hospital # 4	Auricular pus	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-43	Hospital # 3	Bronchial secretions	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-44	Outpatient NIH	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-45	Hospital # 5	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-46	Hospital # 6	Palate biopsy	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-47	Outpatient NIH	Bronchoalveolar lavage	<i>Aspergillus</i> sp.	<i>Versicolores</i>	<i>A. sidowii</i>
12-48	Hospital # 7	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-49	Hospital # 7	Bronchial secretions	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-50	Hospital # 7	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-51	Hospital # 7	Bronchoalveolar lavage	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-52	Hospital # 7	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-53	Hospital # 7	Ascitic fluid	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-54	Hospital # 7	Auricular exudate	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-55	Hospital # 7	Skull biopsy	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-56	Hospital # 7	Bronchial secretions	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-57	Hospital # 7	Unknown	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-59	Hospital # 7	Bronchoalveolar lavage	<i>A. candidus</i>	<i>Terrei</i>	<i>A. terreus</i>
12-60	Hospital # 7	Auricular exudate	<i>A. candidus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-62	Hospital # 7	Bronchial secretions	<i>A. niger</i>	<i>Nigri</i>	<i>A. tubigensis</i>
12-63	Hospital # 7	Nasal sinus aspirate	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
12-64	Portuguese NIH	Bronchoalveolar lavage	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-65	Portuguese NIH	Auricular exudate	<i>A. niger</i>	<i>Nigri</i>	<i>A. awamarii</i>
12-66	Portuguese NIH	Auricular exudate	<i>A. niger</i>	<i>Nigri</i>	<i>A. niger</i>
12-67	Portuguese NIH	Auricular exudate	<i>A. niger</i>	<i>Nigri</i>	<i>A. brasiliensis</i>
12-68	Portuguese NIH	Bronchial secretions	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
12-69	Portuguese NIH	Sputum	<i>A. fumigatus</i>	<i>Fumigati</i>	Not identified to species level
12-70	Portuguese NIH	Sputum	<i>A. terreus</i>	<i>Terrei</i>	<i>A. terreus</i>
INSA1	Outpatient NIH	Bronchoalveolar lavage	<i>A. flavus</i>	<i>Flavi</i>	<i>A. flavus</i>
INSA2	Outpatient NIH	Bronchoalveolar lavage	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
INSA3	Hospital # 3	Unknown	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
INSA4	Outpatient NIH	Bronchoalveolar lavage	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
INSA5	Hospital # 3	Unknown	<i>A. nidulans</i>	<i>Nidulantes</i>	<i>Emmericella echinulata</i>
INSA6	Hospital # 8	Unknown	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
INSA7	Outpatient NIH	Bronchoalveolar lavage	<i>Aspergillus</i> sp.	<i>Versicolores</i>	<i>A. fructus</i>
INSA8	Hospital # 3	Unknown	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
INSA9	Hospital # 3	Unknown	<i>A. nidulans</i>	<i>Fumigati</i>	<i>A. lentulus</i>
INSA10	Outpatient NIH	Sputum	<i>A. niger</i>	<i>Nigri</i>	<i>A. awamarii</i>
INSA11	Hospital # 3	Unknown	<i>A. nidulans</i>	<i>Nidulantes</i>	<i>Emmericella nidulans</i>
INSA12	Outpatient NIH	Sputum	<i>A. fumigatus</i>	<i>Nigri</i>	<i>A. tubigensis</i>
INSA13	Hospital # 9	Mammary gland Biopsy	<i>A. fumigatus</i>	<i>Fumigati</i>	<i>A. fumigatus</i>
INSA14	Outpatient NIH	bronchial secretions	<i>A. terreus</i>	<i>Terrei</i>	<i>A. terreus</i>

NIH – National Institute of Health

➤ Isolates identified as belonging to the *Fumigati* complex were found to be more frequently recovered in clinical samples than were the other complexes ($P < 0.001$), being more frequently recovered from respiratory specimens ($P < 0.001$).

INTRODUCTION

Correct identification of *Aspergillus* species is important given that sibling/ cryptic species may show different susceptibilities to antifungal and also because sharper definition of species may facilitate epidemiological studies.

OBJECTIVE

Clinical isolates of *Aspergillus* from Portuguese hospitals were screened to determine which, if any, of the cryptic species of *Aspergillus* were involved in patient infections.

METHODS

Over a one year, isolates of *Aspergillus* were collected from 10 health institutions in Portugal.

All isolates were plated for growth as single colonies on malt extract agar (2%) with chloramphenicol (0.05 g/L). These isolates were identified on the basis of macro and microscopic morphology and through the use of molecular tools.

Genomic DNA was prepared from each isolate and the sequencing of the Internal Transcribed Spacers (ITS) regions was performed to achieve the species-complex, whereas β -tubulin and calmodulin sequencing was done to achieve the correct species identification.

RESULTS & DISCUSSION

➤ A total of 57 *Aspergillus* isolates were collected.

➤ Among the collected isolates, six species-complexes were detected by ITS sequencing, and were distributed as follows: *Fumigati* (29 isolates; 50.9%), *Flavi* (12 isolates; 21.0%), *Nigri* (9 isolates; 15.8%), *Terrei* (3 isolates; 5.3%), *Nidulantes* (2 isolates; 3.6%) and *Versicolores* (2 isolates; 3.6%) (Table 1).

➤ Three misidentifications at the species-complex level (based on morphology) were resolved by ITS sequencing (isolates 12-59, 12-60 and INSA9) (Table 1).

CONCLUSION

Cryptic species of *Aspergillus* have significant prevalence in the clinic. The identification of *Aspergillus* cryptic species among the collected clinical isolates emphasizes the importance of a correct identification to species level, since sibling species may have different antifungal susceptibilities.