Sequence analysis of isolates of Aspergillus in chronic and allergic aspergillosis reveal a spectrum of cryptic species

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Abstract

Aims

To establish the prevalence and antifungal susceptibilities of *Aspergillus* cryptic species from respiratory samples.

Methods

Retrospective susceptibility data on *Aspergillus* species cultured between 2015 and 2017 by "high volume culture" [HVC] vs. "conventional" culture techniques.

Results

Fifty-six (2.5%) isolates were identified as *Aspergillus* cryptic species by sequencing of ITS, BenA and CalM gene loci. Recovery was higher in HVCs compared to conventional cultures.

Common cryptic species were *A. montevidensis* (n=15), *A. creber* (n=11), *A. sydowii* (n=5), and *A. calidoustus* (n=4). Eighteen (32.1%) isolates had MIC values ≥4mg/L to amphotericin B, and 19.1% to 60.1% had MIC values ≥8mg/L to the triazoles.

Conclusions

HVC increases the likelihood of recovery of cryptic species. MIC values to antifungals were high.

Keywords: Aspergillus, cryptic species, triazole antifungals, amphotericin B, high volume culture, molecular identification

Executive Summary

- Cryptic *Aspergillus* species are morphologically indistinguishable from the main *Aspergillus* sections. They constitute up to 10-15% of the clinical isolates from patients with aspergillosis, and are known to exhibit high MIC values to systemic antifungals.
- The present study aimed at establishing the prevalence and antifungal susceptibilities of *Aspergillus* cryptic species from respiratory samples processed at the Mycology Reference Centre Manchester (MRCM).
- We retrospectively collected data on susceptibility of *Aspergillus* species cultured between 2015 and 2017 by "high volume culture" [HVC], where up to 1 mL of undiluted specimen is cultured and in those that were conventionally cultured. *In vitro* testing of susceptibility was performed and MIC values were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. Molecular sequencing of the ITS, β-tubulin (BenA) and calmodulin gene loci was performed for species-level identification of isolates.
- Fifty-six (2.5%) isolates were identified as *Aspergillus* cryptic species by sequencing of ITS, BenA and CalM gene loci. In the sections *Aspergillus* (*A. montevidensis*, n=15; *A. pseudoglaucus*, n=1; *A. chevalieri*, n=1), *Versicolores* (*A. creber*, n=11; A. sydowii, n=5; *A. jensenii*, n=1; *A. tabacinus*, n=1), *Usti* (A. calidoustus, n=4; *A. pseudodeflectus*, n=1; *A. insuetus*, n=1), *Fumigati* (*A. hiratsukae*, n=2; *A. thermomutatus*, n=2; *A. aureoles*, n=1), *Nidulantes* (*A. rugulosus*, n=1; *A. spinulosporus*, n=1; *A. unguis*, n=1), *Circumdati* (*A. pallidofulvus* , n=1; *A. westerdijkiae*, n=1), *Cremei* (*A. wentii* ,n=2), Flavi (Tamarii, n=1), Nigri (A. uvarum, n=1), and *Clavati* (*A. giganteus*, n=1).
- Forty-six (82.1%) of the cryptic species of *Aspergillus* were recovered from HVCs and the recovery rate was significantly higher from HVCs than from conventional cultures (4.4% (46/1,043) vs.0.8% (11/1,200), p<0.0001). All (n=56) isolates had MIC values to amphotericin B, Itraconazole, voriconazole, and posaconazole. However, only 47 (83.9%) isolates had MICs to isavuconazole. Eighteen (32.1%) isolates had MIC values ⍰4mg/L to amphotericin B. For

the triazoles, MIC values ≥8mg/L to itraconazole was observed in 34 (60.1%) of the isolates, to voriconazole in 15 (26.8%), to posaconazole in 11 (19.6%), and in 9 (/47, 19.1%) of the isolates to isavuconazole. All isolates from the section *Usti* had MIC values to the triazoles >4mg/L. Very high (>8mg/L) MICs to itraconazole was observed mainly in the section *Aspergillus* and *Versicolores*.

- In the present study, HVC was found to increase the likelihood of recovery of cryptic species. Consistent with previously published studies, our isolates had high MIC values to both the triazoles and amphotericin B. The clinical significance, in terms of severity, manifestation and prognosis of aspergillosis caused by these species remains unclear.

Introduction

Aspergillus species are ubiquitous and opportunistic moulds that survive and thrive in a wide array of environmental and metabolic conditions [1]. The genus *Aspergillus* encompasses over 300 species, some of which have just recently been described [2]. However, only about 50 of these *Aspergillus* species are recognised as human pathogens [3]. *Aspergillus fumigatus* is the most frequent species of *Aspergillus,* implicated in over 60% of the various clinical syndromes of aspergillosis [4].

Recently, cryptic species that are morphologically indistinguishable from the main *Aspergillus* sections have been described, mainly in Europe and the United States of America [5,6]. They exhibit high minimum inhibitory concentration (MIC) values to systemic triazoles *in vitro* and are associated with multi-azole and pan-azole resistant infections [7,8]. Previous studies have shown that these cryptic species constitute up to 10-15% of the clinical isolates from patients with aspergillosis [5,7–10]. Two recent studies from Korea have shown prevalence of cryptic species as high as 23.1% [11] and 25.7% [12].

Very limited data exist on the frequency of cryptic *Aspergillus* species and their antifungal susceptibility patterns in the UK. The aim of this study at the Mycology Reference Centre Manchester (MRCM) was to establish the rate of recovery of cryptic species of *Aspergillus* from respiratory samples and their antifungal susceptibilities.

Materials and Methods

This was a retrospective study evaluating laboratory records of respiratory samples processed using a high volume culture (HVC) technique [13] and reported from the MRCM, Wythenshawe Hospital, Manchester University NHS Foundation Trust, Manchester UK between 2015 and 2017. Environmental isolates were excluded.

Data collected included 1) specimen type, 2) culture method i.e. conventional vs. HVC techniques, 3) morphological identification, 4) molecular identification, 5) MIC values to the triazoles (itraconazole, voriconazole, posaconazole, and isavuconazole) and amphotericin B, and 6) clinical diagnoses of the patients.

In brief, for conventional culture, sputum was homogenized by mixing with an equal volume of 0.1% dithiothreitol solution and diluted 500-fold in sterile water. Ten µL of the diluted specimen was cultured on Sabouraud dextrose agar (2 plates) and incubated at 37°C and 45°C for up to 5 days. For HVC, the entire undiluted specimen (up to 1 mL) was cultured on Sabouraud dextrose agar (up to 2 plates) and incubated at 30°C for up to 14 days [13].

In vitro testing of susceptibility was performed and MIC values were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method [14]. *Candida krusei* ATCC 6258 was used as reference strain for quality control for each antifungal susceptibility test. Molecular methods were performed for species-level identification of isolates with atypical morphological features, using Internal Transcribe Sequences (ITS) 5 and 4, β-tubulin (BenA) Bt_2a and Bt_2b, and calmodulin 5 and 6 primers as previously described [2].

This was a service audit and because of its retrospective nature it was exempt from ethical review. Patient information was anonymized and deidentified prior to analysis, and no information that could lead to patient identification was used. Microsoft Excel ® was used for data management, data cleaning and summary statistics. Social Science Statistics chi-square calculator [15] was used to

compare the rate of recovery of cryptic *Aspergillus* species between HVCs and conventional cultures. P<0.05 was considered statistically significant.

Results

A total of 2,243 clinical isolates of *Aspergillus* spp. were reported from 1,151 unique patients with chronic and allergic bronchopulmonary aspergillosis. Of the 2,243 isolates, 2,004 (89.3%) were recovered from sputum samples and the remainder 239 (10.7%) from bronchoalveolar (BAL) lavage samples. By morphological data, 1,909 (85.1%) *isolates were from section Fumigati, 163 (7.3%) isolates from section Nigri, 35 (1.6%) isolates from section Flavi, 25 (1.1%) from section Nidulantes, 21 (0.9%) from section Versicolores, 19 isolates (0.8%) from section Terrei and 71 (3.2%) isolates were reported as Aspergillus spp*. One thousand and two hundred (53.5%) isolates were recovered from conventional cultures and 1,043 (46.5%) from HVCs.

Overall, 7.6% (171/2,243) of the recovered isolates were sequenced. Ninety (52.6%) of which were identified as *A. fumigatus*, 9 (5.3%) *A. versicolor*, 8 (4.7%) *A. nidulans*, 7 (4.1%) *A. flavus*, and 1 (0.6%) *A. terreus*. The remainder 56 (32.7% of 171 and 2.5% of 2,243), all of which were identified as *Aspergillus* spp. morphologically were subsequently identified as cryptic species of *Aspergillus* as summarised in **Table 1**. The 56 isolates were from 49 patients, 28 of whom were patients being managed for chronic pulmonary aspergillosis (CPA), 8 with *Aspergillus* bronchitis and the remainder 13 were patients with either allergic bronchopulmonary aspergillosis (ABPA) or severe asthma with fungal sensitisation (SAFS). Forty-six (82.1%) of the cryptic species of *Aspergillus* were recovered from HVCs and the recovery rate was significantly higher from HVCs than from conventional cultures (4.4% (46/1,043) vs.0.8% (11/1,200), p<0.0001).

All (n=56) isolates had MIC values to amphotericin B, Itraconazole, voriconazole, and posaconazole. However, only 47 (83.9%) isolates had MICs to isavuconazole. Eighteen (32.1%) isolates had MIC values ≥4mg/L to amphotericin B. For the triazoles, MIC values ≥8mg/L to itraconazole was observed in 34 (60.1%) of the isolates, to voriconazole in 15 (26.8%), to posaconazole in 11 (19.6%), and in 9 (/47, 19.1%) of the isolates to isavuconazole (**Table 1**). All isolates from the section *Usti* had MIC values to the triazoles >4mg/L. Very high (>8mg/L) MICs to itraconazole was observed mainly in the section *Aspergillus* and *Versicolores* (**Figure 1**).

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Discussion

The present study suggests that cryptic species of *Aspergillus* constitute about 2.5% of our respiratory isolates, often in samples of patients with CPA. This is slightly lower than those from previous studies reporting prevalence between 5% and 26% [6,12,16–18]. This is not surprising, as, because of costs, we do not routinely perform identification of *Aspergillus* isolates by sequencing the β-tubulin or calmodulin genes. Isolates with either atypical morphological or irregular susceptibility patterns compared to those observed for the main, typical Aspergillus species are sequence. The prevalence of previously unknown (cryptic) species of *Aspergillus* in clinical samples is on the rise [5]. The identification of cryptic *Aspergillus* species is crucial; these species show high frequency of antifungal drug resistance [6]. Another very interesting finding from our study is that the rate of recovery of these cryptic species is significantly higher when HVCs are performed instead of conventional cultures. This has not been previously reported.

Identification to *Aspergillus* species–complex level is reached at by examination of both micro and macroscopic characteristics [19]. For isolates with atypical macroscopic features, sequencing of the β-tubulin or calmodulin genes are required for species level identification [2,20]. Recent evidence suggests that thermotolerance testing is useful in the discrimination of closely related species within the sections, for example, *A. ustus* Vs. *A. calidoustus* in section *Usti* [21]. Multi-locus sequencing (MLST) and Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-ToF MS) provide the means to accurately identify both common and cryptic *Aspergillus* species [6].

The presence of cryptic species should always be suspected when a putative *Aspergillus* isolate displays surprising morphological characteristics, such as a defect in sporulation and/or unusual antifungal susceptibility profile [5]. However, definitive species identification requires advanced nucleic acid amplification and sequencing analyses of the ITS as well as beta-tubulin or calmodulin genes, not available in most laboratories [22]. In clinical practice, MALDI-TOF MS has shown promising results for rapid and accurate distinction between *A. fumigatus* and other *Aspergillus* spp.

of the section *Fumigati* [5]. However, this technique is expensive, not widely available and requires trained personnel to operate.

At the MRCM, susceptibility testing is performed on all *Aspergillus* isolates, mainly to triazoles and amphotericin B but also to echinocandins and terbinafine. We saw high-level of multi- and pan-azole resistance, and frequent triazole cross-resistance among species of *Aspergillus* spp. especially for patients on long azole therapy [23]. Multi-resistant aspergillosis due to cryptic species such as *A. calidoustus* or *A. lentulus* is also increasingly reported in hematopoietic stem-cell transplant or organ transplant recipients, in whom it is associated with a particularly high mortality rates [5]. Consistently, in the present study, all 4 isolates of *A. calidoustus* were pan-azole resistant; interestingly these isolates were susceptible to amphotericin B, though with a high MICs. Echinocandins and terbinafine have been shown to be highly active against clinical isolates, including cryptic *Aspergillus* species from section *Versicolores*, *Aspergillus*, and *Circumdati* [16–18].

The mainly limitation of this study is that there are currently no breakpoints for cryptic species of *Aspergillus*.

Conclusion

In conclusion, the present single-centre study with <8% molecular identification rate reports a 2.5% rate of recovery of cryptic species of *Aspergillus*, these isolates had high MIC values to the testes triazoles and amphotericin B. The true prevalence and clinical significance of these cryptic species in this setting remains to be determined. Novel to our study is that HVC increases the likelihood of recovery of cryptic species from respiratory samples and allows resistance profiling of these isolates.

Future Perspective

Cryptic *Aspergillus* species are emerging issues in the medical mycology laboratories, identification is not straightforward and molecular methods are always required to confirm identification. The most urgent need is in the establishment of clinical breakpoints and ecological cut off values for these

species. The key virulence factors, spectrum of diseases manifestation caused by these species, and novel resistant mechanisms unique to cryptic species needs to be examined.

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'*' – of interest,

"**" – of considerable interest

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Table 1: Frequency of molecular identification of cryptic *Aspergillus* species over a 3-year period

S.No	Section	Species	Isolation	Number of Isolates (%)	Minimum inhibitory concentration values to antifungals				
					Amphotericin B	Itraconazole	Voriconazole	Posaconazole	Isavuconazole
1	*Aspergillus*	*A. montevidensis*	HVC	15 (26.8)	0.5	>8	0.5	0.125	
2		*A. montevidensis*	HVC		>8	>8	>8	>8	>8
3		*A. montevidensis*	HVC		0.125	>8	4	0.5	2
4		*A. montevidensis*	HVC		0.06	0.5	1	0.125	1
5		*A. montevidensis*	HVC		0.06	>8	2	0.25	2
6		*A. montevidensis*	HVC		0.25	>8	4	0.5	4
7		*A. montevidensis*	HVC		0.25	>8	4	0.25	4
8		*A. montevidensis*	HVC		0.125	>8	2	0.25	4
9		*A. montevidensis*	HVC		0.5	0.5	1	0.06	1
10		*A. montevidensis*	HVC		1	>8	0.25	>8	0.5
11		*A. montevidensis*	HVC		0.5	>8	4	0.25	8
12		*A. montevidensis*	HVC		>8	>8	>8	>8	>8
13		*A. montevidensis*	HVC		0.06	>8	2	0.125	1
14		*A. montevidensis*	HVC		0.25	>8	4	0.25	4
15		*A. montevidensis*	HVC		0.5	>8	>8	0.5	>8
16		*A. pseudoglaucus*	HVC	1 (1.8)	>8	0.25	>8	0.03	
17		*A. chevalieri*	HVC	1 (1.8)	0.125	0.06	0.5	0.03	0.5
18	*Versicolores*	*A. creber*	HVC	11 (19.6)	2	>8	1	0.5	0.5
19		*A. creber*	HVC		1	1	1	0.125	0.5
20		*A. creber*	HVC		2	0.5	0.5	0.25	0.5
21		*A. creber*	HVC		2	2	1	0.25	0.5
22		*A. creber*	HVC		4	>8	1	0.5	0.5
23		*A. creber*	HVC		4	>8	1	0.5	1
24		*A. creber*	HVC		2	>8	0.5	0.25	0.25
25		*A. creber*	HVC		>8	>8	>8	0.25	>8
26		*A. creber*	HVC		8	>8	1	0.5	0.5

27		*A. creber*	HVC		2	2	0.5	0.5	1
28		*A. creber*	HVC		4	>8	1	0.5	1
29		*A. sydowii*	HVC	5 (8.9)	1	0.5	1	0.125	1
30		*A. sydowii*	HVC		1	0.5	1	0.25	0.5
31		*A. sydowii*	HVC		1	0.5	0.5	0.125	0.5
32		*A. sydowii*	HVC		4	>8	2	1	4
33		*A. sydowii*	HVC		4	>8	4	1	4
34		*A. jensenii*	HVC	1 (1.8)	>8	>8	>8	>8	>8
35		*A. tabacinus*	HVC	1 (1.8)	4	>8	2	1	4
36	*Usti*	*A. calidoustus*	Conventional	4	1	>8	8	>8	
37		*A. calidoustus*	HVC		1	>8	8	>8	4
38		*A. calidoustus*	Conventional		2	>8	>8	>8	
39		*A. calidoustus*	Conventional		1	>8	8	>8	8
40		*A. pseudodeflectus*	HVC	1 (1.8)	>8	>8	>8	>8	>8
41		*A. insuetus*	HVC	1 (1.8)	0.5	>8	>8	>8	4
42	*Fumigati*	*A. hiratsukae*	Conventional	2 (3.6)	>8	>8	>8	>8	>8
43		*A. hiratsukae*	HVC		1	0.125	0.5	0.06	0.5
44		*A. thermomutatus*	Conventional	2 (3.6)	2	>8	8	0.5	
45		*A. thermomutatus*	Conventional		0.25	>8	8	0.5	2
46		*A. aureolus*	HVC	1 (1.8)	2	>8	0.25	0.5	0.125
47	*Nidulantes*	*A. rugulosus*	Conventional	1 (1.8)	1	0.5	0.5	0.5	1
48		*A. spinulosporus*	Conventional	1 (1.8)	0.25	0.25	0.5	0.125	
49		*A. unguis*	HVC	1 (1.8)	1	>8	0.25	1	0.5
50	*Circumdati*	*A. pallidofulvus*	HVC	1 (1.8)	>8	1	1	0.5	1
51		*A. westerdijkiae*	HVC	1 (1.8)	>8	0.25	0.5	0.25	
52	*Cremei*	*A. wentii*	Conventional	2 (3.6)	8	0.5	2	0.125	2
53		*A. wentii*	HVC		>8	0.125	0.5	0.03	1
54	*Flavi*	*A. tamarii*	Conventional	1 (1.8)	0.5	0.5	1	0.125	
55	*Nigri*	*A. uvarum*	HVC	1 (1.8)	0.125	0.5	0.5	0.125	1

56	*Clavati*	*A. giganteus*	HVC	1 (1.8)	0.25	0.25	1	0.125	

HVC: High volume culture

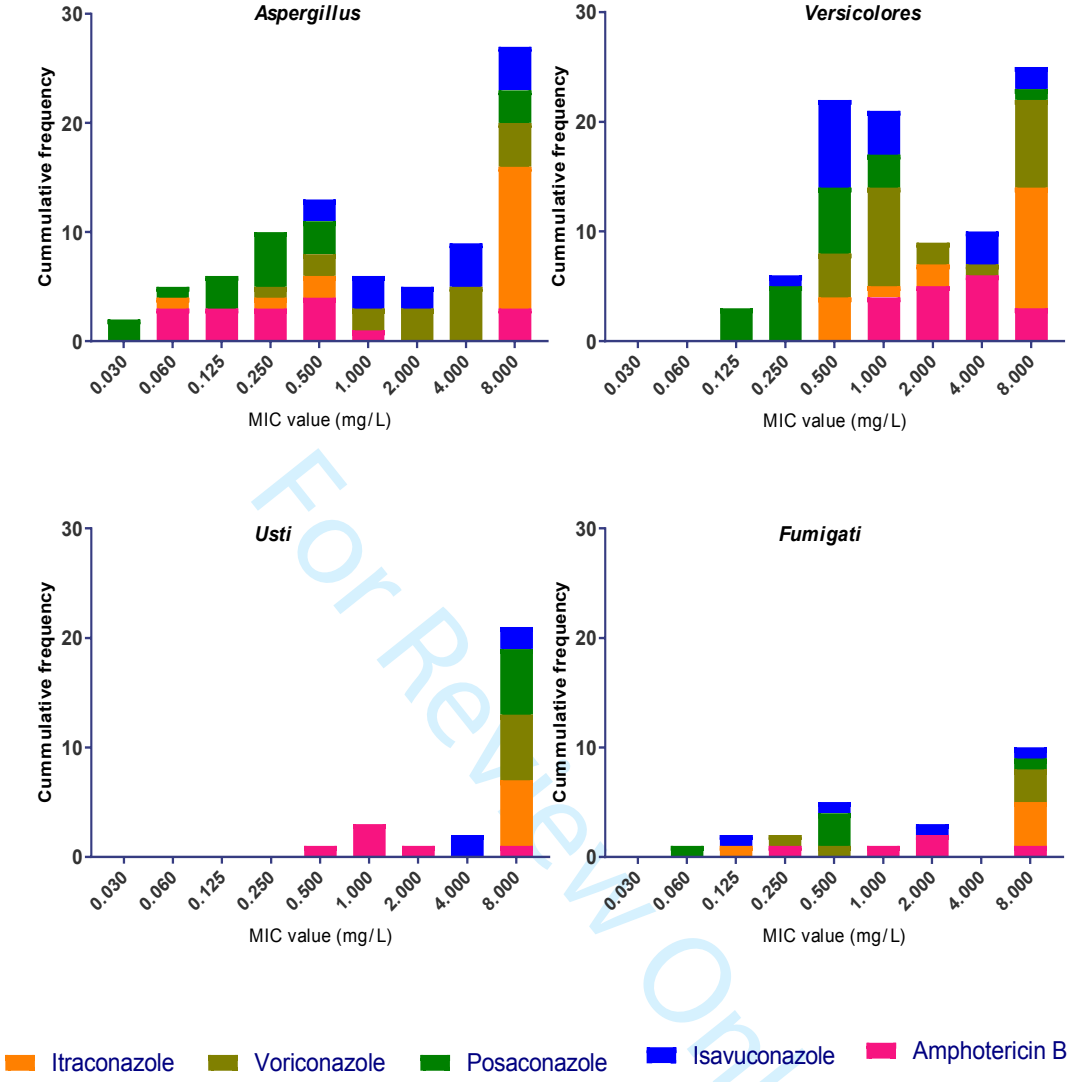

Figure 1: Distribution of minimum inhibitory concentration (MIC) values in cryptic species of the four main section of *Aspergillus*. All isolates from the *Usti* section had MIC values >4mg/L to the triazoles. Very high (>8mg/L) MICs to itraconazole was observed mainly in the section *Aspergillus* and *Versicolores*.