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

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1 1 **Abstract**

2 2 **Aims**

3 3 To establish the prevalence and antifungal susceptibilities of *Aspergillus* cryptic species from
4 4 respiratory samples.

5 5 **Methods**

6 6 Retrospective susceptibility data on *Aspergillus* species cultured between 2015 and 2017 by “high
7 7 volume culture” [HVC] vs. “conventional” culture techniques.

8 8 **Results**

9 9 Fifty-six (2.5%) isolates were identified as *Aspergillus* cryptic species by sequencing of ITS, BenA and
10 10 CalM gene loci. Recovery was higher in HVCs compared to conventional cultures.

11 11 Common cryptic species were *A. montevicensis* (n=15), *A. creber* (n=11), *A. sydowii* (n=5), and *A.*
12 12 *calidoustus* (n=4). Eighteen (32.1%) isolates had MIC values $\leq$ 4mg/L to amphotericin B, and 19.1% to
13 13 60.1% had MIC values $\leq$ 8mg/L to the triazoles.

14 14 **Conclusions**

15 15 HVC increases the likelihood of recovery of cryptic species. MIC values to antifungals were high.

16 16 **Keywords:** *Aspergillus*, cryptic species, triazole antifungals, amphotericin B, high volume culture,
17 17 molecular identification

19 Executive Summary

- 20 • Cryptic *Aspergillus* species are morphologically indistinguishable from the main *Aspergillus*
- 21 sections. They constitute up to 10-15% of the clinical isolates from patients with aspergillosis,
- 22 and are known to exhibit high MIC values to systemic antifungals.
- 23 • The present study aimed at establishing the prevalence and antifungal susceptibilities of
- 24 *Aspergillus* cryptic species from respiratory samples processed at the Mycology Reference
- 25 Centre Manchester (MRCM).
- 26 • We retrospectively collected data on susceptibility of *Aspergillus* species cultured between
- 27 2015 and 2017 by “high volume culture” [HVC], where up to 1 mL of undiluted specimen is
- 28 cultured and in those that were conventionally cultured. *In vitro* testing of susceptibility was
- 29 performed and MIC values were determined using the European Committee on
- 30 Antimicrobial Susceptibility Testing (EUCAST) method. Molecular sequencing of the ITS, β -
- 31 tubulin (BenA) and calmodulin gene loci was performed for species-level identification of
- 32 isolates.
- 33 • Fifty-six (2.5%) isolates were identified as *Aspergillus* cryptic species by sequencing of ITS,
- 34 BenA and CalM gene loci. In the sections *Aspergillus* (*A. montevicensis*, n=15; *A.*
- 35 *pseudoglaucus*, n=1; *A. chevalieri*, n=1), *Versicolores* (*A. creber*, n=11; *A. sydowii*, n=5; *A.*
- 36 *jensenii*, n=1; *A. tabacinus*, n=1), *Usti* (*A. calidoustus*, n=4; *A. pseudodeflectus*, n=1; *A. insuetus*,
- 37 n=1), *Fumigati* (*A. hiratsukae*, n=2; *A. thermomutatus*, n=2; *A. aureoles*, n=1), *Nidulantes* (*A.*
- 38 *rugulosus*, n=1; *A. spinulosporus*, n=1; *A. unguis*, n=1), *Circumdati* (*A. pallidofulvus*, n=1; *A.*
- 39 *westerdijkiae*, n=1), *Cremeri* (*A. wentii*, n=2), *Flavi* (*Tamaris*, n=1), *Nigri* (*A. uvarum*, n=1), and
- 40 *Clavati* (*A. giganteus*, n=1).
- 41 • Forty-six (82.1%) of the cryptic species of *Aspergillus* were recovered from HVCs and the
- 42 recovery rate was significantly higher from HVCs than from conventional cultures (4.4%
- 43 (46/1,043) vs. 0.8% (11/1,200), $p < 0.0001$). All (n=56) isolates had MIC values to amphotericin
- 44 B, Itraconazole, voriconazole, and posaconazole. However, only 47 (83.9%) isolates had MICs
- 45 to isavuconazole. Eighteen (32.1%) isolates had MIC values ≥ 4 mg/L to amphotericin B. For

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the triazoles, MIC values ≥ 8 mg/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 >4 mg/L. Very high (>8 mg/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.

57 Introduction

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

63 Recently, cryptic species that are morphologically indistinguishable from the main *Aspergillus*
64 sections have been described, mainly in Europe and the United States of America [5,6]. They exhibit
65 high minimum inhibitory concentration (MIC) values to systemic triazoles *in vitro* and are associated
66 with multi-azole and pan-azole resistant infections [7,8]. Previous studies have shown that these
67 cryptic species constitute up to 10-15% of the clinical isolates from patients with aspergillosis [5,7-
68 10]. Two recent studies from Korea have shown prevalence of cryptic species as high as 23.1% [11]
69 and 25.7% [12].

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

74

75 **Materials and Methods**

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

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

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

89 *In vitro* testing of susceptibility was performed and MIC values were determined using the European
90 Committee on Antimicrobial Susceptibility Testing (EUCAST) method [14]. *Candida krusei* ATCC
91 6258 was used as reference strain for quality control for each antifungal susceptibility test.
92 Molecular methods were performed for species-level identification of isolates with atypical
93 morphological features, using Internal Transcribe Sequences (ITS) 5 and 4, β-tubulin (BenA) Bt₂a
94 and Bt₂b, and calmodulin 5 and 6 primers as previously described [2].

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

- 99 compare the rate of recovery of cryptic *Aspergillus* species between HVCs and conventional cultures.
- 100 $P < 0.05$ was considered statistically significant.

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101 **Results**

102 A total of 2,243 clinical isolates of *Aspergillus* spp. were reported from 1,151 unique patients with
103 chronic and allergic bronchopulmonary aspergillosis. Of the 2,243 isolates, 2,004 (89.3%) were
104 recovered from sputum samples and the remainder 239 (10.7%) from bronchoalveolar (BAL) lavage
105 samples. By morphological data, 1,909 (85.1%) isolates were from section *Fumigati*, 163 (7.3%)
106 isolates from section *Nigri*, 35 (1.6%) isolates from section *Flavi*, 25 (1.1%) from section *Nidulantes*, 21
107 (0.9%) from section *Versicolores*, 19 isolates (0.8%) from section *Terrei* and 71 (3.2%) isolates were
108 reported as *Aspergillus* spp. One thousand and two hundred (53.5%) isolates were recovered from
109 conventional cultures and 1,043 (46.5%) from HVCs.

110 Overall, 7.6% (171/2,243) of the recovered isolates were sequenced. Ninety (52.6%) of which were
111 identified as *A. fumigatus*, 9 (5.3%) *A. versicolor*, 8 (4.7%) *A. nidulans*, 7 (4.1%) *A. flavus*, and 1
112 (0.6%) *A. terreus*. The remainder 56 (32.7% of 171 and 2.5% of 2,243), all of which were identified
113 as *Aspergillus* spp. morphologically were subsequently identified as cryptic species of *Aspergillus* as
114 summarised in **Table 1**. The 56 isolates were from 49 patients, 28 of whom were patients being
115 managed for chronic pulmonary aspergillosis (CPA), 8 with *Aspergillus* bronchitis and the remainder
116 13 were patients with either allergic bronchopulmonary aspergillosis (ABPA) or severe asthma with
117 fungal sensitisation (SAFS). Forty-six (82.1%) of the cryptic species of *Aspergillus* were recovered
118 from HVCs and the recovery rate was significantly higher from HVCs than from conventional
119 cultures (4.4% (46/1,043) vs.0.8% (11/1,200), $p<0.0001$).

120 All (n=56) isolates had MIC values to amphotericin B, itraconazole, voriconazole, and posaconazole.
121 However, only 47 (83.9%) isolates had MICs to isavuconazole. Eighteen (32.1%) isolates had MIC
122 values ≥ 4 mg/L to amphotericin B. For the triazoles, MIC values ≥ 8 mg/L to itraconazole was observed
123 in 34 (60.1%) of the isolates, to voriconazole in 15 (26.8%), to posaconazole in 11 (19.6%), and in 9
124 (/47, 19.1%) of the isolates to isavuconazole (**Table 1**). All isolates from the section *Usti* had MIC
125 values to the triazoles >4 mg/L. Very high (>8 mg/L) MICs to itraconazole was observed mainly in the
126 section *Aspergillus* and *Versicolores* (**Figure 1**).

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128 **Discussion**

129 The present study suggests that cryptic species of *Aspergillus* constitute about 2.5% of our
130 respiratory isolates, often in samples of patients with CPA. This is slightly lower than those from
131 previous studies reporting prevalence between 5% and 26% [6,12,16–18]. This is not surprising, as,
132 because of costs, we do not routinely perform identification of *Aspergillus* isolates by sequencing the
133 β -tubulin or calmodulin genes. Isolates with either atypical morphological or irregular susceptibility
134 patterns compared to those observed for the main, typical *Aspergillus* species are sequence. The
135 prevalence of previously unknown (cryptic) species of *Aspergillus* in clinical samples is on the rise
136 [5]. The identification of cryptic *Aspergillus* species is crucial; these species show high frequency of
137 antifungal drug resistance [6]. Another very interesting finding from our study is that the rate of
138 recovery of these cryptic species is significantly higher when HVCs are performed instead of
139 conventional cultures. This has not been previously reported.

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

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

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

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

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

167 Conclusion

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

173 Future Perspective

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

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177 species. The key virulence factors, spectrum of diseases manifestation caused by these species, and
178 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	<i>Aspergillus</i>	<i>A. montevidensis</i>	HVC	15 (26.8)	0.5	>8	0.5	0.125	
2		<i>A. montevidensis</i>	HVC		>8	>8	>8	>8	>8
3		<i>A. montevidensis</i>	HVC		0.125	>8	4	0.5	2
4		<i>A. montevidensis</i>	HVC		0.06	0.5	1	0.125	1
5		<i>A. montevidensis</i>	HVC		0.06	>8	2	0.25	2
6		<i>A. montevidensis</i>	HVC		0.25	>8	4	0.5	4
7		<i>A. montevidensis</i>	HVC		0.25	>8	4	0.25	4
8		<i>A. montevidensis</i>	HVC		0.125	>8	2	0.25	4
9		<i>A. montevidensis</i>	HVC		0.5	0.5	1	0.06	1
10		<i>A. montevidensis</i>	HVC		1	>8	0.25	>8	0.5
11		<i>A. montevidensis</i>	HVC		0.5	>8	4	0.25	8
12		<i>A. montevidensis</i>	HVC		>8	>8	>8	>8	>8
13		<i>A. montevidensis</i>	HVC		0.06	>8	2	0.125	1
14		<i>A. montevidensis</i>	HVC		0.25	>8	4	0.25	4
15		<i>A. montevidensis</i>	HVC		0.5	>8	>8	0.5	>8
16		<i>A. pseudoglaucus</i>	HVC	1 (1.8)	>8	0.25	>8	0.03	
17		<i>A. chevalieri</i>	HVC	1 (1.8)	0.125	0.06	0.5	0.03	0.5
18	<i>Versicolores</i>	<i>A. creber</i>	HVC	11 (19.6)	2	>8	1	0.5	0.5
19		<i>A. creber</i>	HVC		1	1	1	0.125	0.5
20		<i>A. creber</i>	HVC		2	0.5	0.5	0.25	0.5
21		<i>A. creber</i>	HVC		2	2	1	0.25	0.5
22		<i>A. creber</i>	HVC		4	>8	1	0.5	0.5
23		<i>A. creber</i>	HVC		4	>8	1	0.5	1
24		<i>A. creber</i>	HVC		2	>8	0.5	0.25	0.25
25		<i>A. creber</i>	HVC		>8	>8	>8	0.25	>8
26		<i>A. creber</i>	HVC		8	>8	1	0.5	0.5

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

56	<i>Clavati</i>	<i>A. giganteus</i>	HVC	1 (1.8)	0.25	0.25	1	0.125	
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HVC: High volume culture

For Review Only

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*.