

## A 1-year *Aspergillus terreus* surveillance study at the University Hospital of Innsbruck: molecular typing of environmental and clinical isolates

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### ABSTRACT

*Aspergillus terreus* appears to have become an increasingly frequent cause of opportunistic infections in the University Hospital of Innsbruck (UHI) and is of serious concern because of *in vivo* and *in vitro* resistance to amphotericin B. In order to determine the possible relationship between environmental contamination by *A. terreus* and the occurrence of invasive aspergillosis, a 1-year prospective study (2004–2005) was carried out in the UHI. Isolates obtained from air samples of various high-risk settings and those from surveillance cultures of proven and probable aspergillosis (EORTC/MSG criteria) were examined by genotyping. Within 1 year, 34 and 15 *A. terreus* isolates were collected from the environment and from patients, respectively. Genotypic analysis with rapid amplification of polymorphic DNA (RAPD) PCR and the combination of three different primers (R108, CII, P4) revealed 46 distinct genotypic profiles (types 1–46). No strain similarity was detected among and within the patients and environmental areas, indicating a great genomic diversity in *A. terreus*, which is common in the environment of Innsbruck and a source of invasive infections in immunosuppressed patients. Genotypical diversity was found in clinical and environmental *A. terreus* isolates.

**Keywords** *Aspergillus terreus*, molecular genotyping, surveillance

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### INTRODUCTION

Invasive fungal infections due to *Aspergillus* species have become a major cause of morbidity and mortality among immunocompromised patients [1,2]. *Aspergillus fumigatus* is most frequently isolated from clinical specimens, but other species of clinical importance include *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus* [3–5]. *A. terreus* appears to have become an increasingly frequent cause of opportunistic infections and is of serious concern because of *in vivo* and *in vitro* resistance to amphotericin B [6]. At the University Hospital of Innsbruck (UHI), a tertiary-care hos-

pital with c. 2000 beds, infections due to *A. terreus* have been noted since 1994 [7]. A laminar airflow unit was instituted in 2000 for patients at risk; this resulted in a decrease in the incidence of invasive aspergillosis (IA), but *A. terreus* infections still occurred, albeit less frequently. The reason for the frequency of IA caused by *A. terreus* at the UHI is not clear, nor is the reason for the density of *A. terreus* in the hospital environment. The molecular typing of *Aspergillus* has proven useful in many epidemiological situations. One of the most widely used genotyping methods for *A. terreus* is the random amplification of polymorphic DNA PCR (RAPD PCR), a technically relatively simple and rapid procedure [8,9].

In this study, the source of *A. terreus* strains collected prospectively over a 12-month period from indoor air of the UHI, outdoor air of Innsbruck and clinical samples (from colonized

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or infected patients) was investigated. Another objective was to determine the frequency of isolation of *A. terreus* in the air of Innsbruck.

## MATERIALS AND METHODS

### Environmental monitoring

Between October 2004 and October 2005, environmental samples were collected prospectively twice weekly inside and outside the UHI. The haematology unit, the bone marrow transplant unit, the solid organ transplantation unit and the paediatric cancer unit were investigated in detail.

All these settings are controlled via highly efficient particulate air (HEPA) filter systems. Samples were taken during the course of normal hospital activity. In parallel, nine places outside the hospital buildings were screened for *A. terreus*.

Air sampling was performed with a Surface Air System Sampler (Admeco-Impactor, Type FH2, Switzerland); samples of 150 and 100 L were collected inside and outside, respectively. The Surface Air System Sampler was loaded with autoclaved Sabouraud dextrose agar (SAB) plates, and measurements were made c. 100 cm above the floor; three specimens per sample collection were taken and incubated at 37°C for 3–5 days. CFUs were counted and extrapolated to CFU/m<sup>3</sup> according to the manufacturer's instructions.

The morphological identification of *A. terreus* and *Aspergillus* spp. was based on a standard method [10]. Isolates were preserved at room temperature in 5% glycerol for further molecular typing.

### Patients

Patients hospitalized in the respective settings were screened for nasal *A. terreus* colonization twice weekly. Swabs were taken for inoculation of SAB plates and broth, and processed further as described above.

Any other clinical *A. terreus* isolates obtained from patients suffering from aspergillosis according to EORTC/MSG criteria were collected and subjected to molecular typing [11].

### Molecular genotyping

*A. terreus* isolates were taken from stock cultures and subcultured on SAB. If there was more than one colony, a mixture of several colonies from the same specimen was genotyped. Chromosomal DNA of all isolates was extracted using the QIAamp Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RAPD PCR was performed with three different primers previously shown to have good discriminatory power for *A. terreus*: R108 (5'-GTATTGCCCT-3'), CII (5'-GCGCACGG-3') and P4 (5'-GATAGATAGATA-3') [9,12]. A 5-μL template containing 50 ng of *A. terreus* chromosomal DNA, 2.5 μL of one primer (20 pmol) and 17.5 μL of water were mixed with ReadyTo-Go RAPD analysis beads (Amersham Pharmacia Biotech, Freiburg, Germany) containing PCR buffer (30 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 2.5 μg of bovine serum albumin, 3 mmol/L MgCl<sub>2</sub>, 0.4 mmol/L dNTPs) and 1 unit of Amplitaq DNA polymerase. Thermocycling was done in a PCR cycler (UNO II; Bometra, Göttingen, Germany). The PCR products were analysed by electrophoresis in a 1.8% agarose gel (6 h at 80 V at room temperature) in 0.5 mmol/L Tris-borate-EDTA running

buffer. The gels were photographed under UV light and analysed. Highly related strains (groups) were defined as those having at least 90% homology on the basis of banding patterns. All experiments were carried out in duplicate to assess reproducibility.

## RESULTS

### Environmental and clinical samples

In total, 3498 air samples were collected, 1908 from inside and 1590 from outside the UHI. The results of species distribution are given in Fig. 1. The outdoor density of *Aspergillus* spp. ranged from 14 to 24.3 CFU/m<sup>3</sup> (mean 20.3 CFU/m<sup>3</sup>), with an increase from January to April; the highest density of *A. terreus* was detected in February. On average, 4.08 *A. terreus* isolates (range 0–8) were collected per month. Inside the UHI, the annual survey revealed very low fungal densities (mean 0.037 CFU/m<sup>3</sup>).

In total, 1873 nasal swabs from 368 patients were analysed, 442 from patients in the haematology unit, 340 from patients in the bone marrow transplantation unit, 376 from patients in the paediatric cancer unit, and 715 from patients in the surgical transplantation unit. Among all nasal swabs, 3.7% (taken from 66 patients) were positive for *Aspergillus* spp. Only one *A. terreus* carrier was identified; the other carriers had *A. fumigatus*. Among those *A. fumigatus* carriers, only one patient developed IA; the other patients suffering from IA were not identified as nasal *Aspergillus* carriers or were not included in the weekly surveillance project.

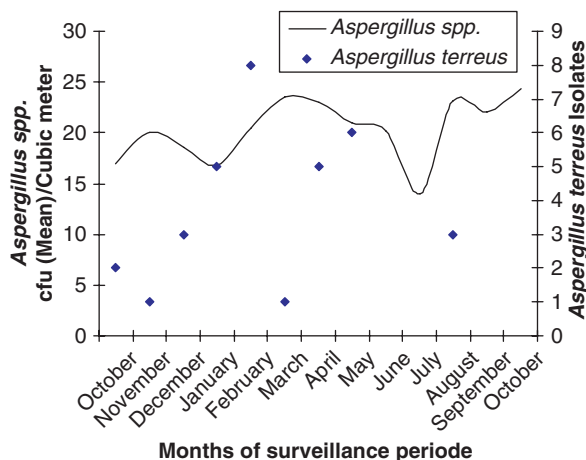


Fig. 1. Concentration of airborne *Aspergillus* spp. and *Aspergillus terreus* conidia (inside and outside) over the 12-month surveillance period (2004–2005).

**Table 1.** *Aspergillus terreus* and *Aspergillus* spp. isolates recovered from air samples during 2004 and 2005

Origin		No. of isolates	
		<i>A. terreus</i>	<i>Aspergillus</i> spp.
Inside	Haematology unit	0	8
	Organ transplantation unit	2	73
	Bone marrow transplantation unit	2	1
	Paediatric cancer unit	0	12
Outside	Environment	30	>2500

Overall, 49 *A. terreus* isolates were obtained during this 1-year study period. Thirty and four *A. terreus* isolates from outdoors and indoors were collected, respectively (Table 1).

14 clinical isolates were from patients suffering from aspergillosis (Table 2) within the study period.

### RAPD typing

Forty-six isolates were genotyped using RAPD PCR. Recultivation of three outdoor isolates failed.

Forty-one, 29 and 41 distinct strains were identified with the R108, CII and P4 primers, respectively. After combination of the results obtained with three different primers, 46 distinct profiles (types 1–46) were identified (Tables 2 and 3). No similarity was detected among and within the strains from patients and environmental strains, indicating great genomic diversity of *A. terreus*. RAPD was also performed using the clinical isolates obtained from 14 sources (Table 2).

### DISCUSSION

The present surveillance study shows *A. terreus* to be common in the environment of the UHI and to be common as a cause of aspergillosis in immunocompromised patients in Tirol, Austria. The genotypic, RAPD-derived profiles indicate that patients were infected with unique strains, making a collective source of transmission highly unlikely.

The composition and concentration of airborne fungi in external and internal environments have been the main focus of research [5,8,13,14], as *Aspergillus* infections increased significantly during the last decade. Fungal burden, and genus or species spread, depend on various factors such as season, wind speed, temperature and humidity [15]. In this study, *A. fumigatus* (97.17%) was predominant inside the hospital, followed by *A. terreus* and *A. niger* (3.92% each) and *A. flavus* (0.98%).

By contrast, Curtis *et al.* reported *A. niger*, *Aspergillus candidus*, *A. flavus* and *A. fumigatus* to be present inside the hospital [13]. Outside, *A. terreus* was present to a lesser extent (1.26%) but, overall, this species was common in the area of Innsbruck, as *A. terreus* was collected at a mean rate of 4.08 isolates per month. Others did not detect *A. terreus*, but showed [14,16,17].

Guinea *et al.* collected only two *A. terreus* strains out of 369 *Aspergillus* spp. when monitoring air and water [15]. The mean *Aspergillus* spp. load of 20.3 CFU/m<sup>3</sup> found in this study was higher than the 6.8 CFU/m<sup>3</sup> reported elsewhere

**Table 2.** Clinical characteristics of patients with *Aspergillus terreus* infections and rapid amplification of polymorphic DNA (RAPD) types of the corresponding isolates

Patient	Sex <sup>a</sup>	Age (years)	Disease	Source (date)	Primer-derived type			Combined type <sup>b</sup>
					CII	P4	R108	
1	M	53	Chronic aspergillosis <sup>c</sup> (post-transplantation)	Sputum (12 January)	E	O	F	8
				Sputum (13 January)	J	Z	U	19
				Sputum (18 January)	I	L	O	15
2	M	47	Solid organ transplantation <sup>c</sup>	Sputum (15 March)	K	Y	W	22
				Sputum (12 May)	K	A	P	23
				Sputum (15 May)	L	Y	P	26
				BAL (27 February)	G	J	N	12
3	M	72	Aspergilloma	Sputum (1 December)	V	A	H	38
4	M	8	Mucoviscidosis	BAL (14 October)	N	I	E	30
5	F	44	Invasive aspergillosis/CML <sup>c</sup>	Sputum (7 September)	Q	L	G	33
6	M	11	Mucoviscidosis	Sputum (3 March)	L	D	T	25
7	M	75	AML <sup>c</sup>	Larynx swab (14 September)	C	P	Z	46
8	M	53	Larynx cancer	Sputum (16 July)	A	I	B	2
9	F	18	Aspergilloma	Ear swab (3 August)	A	I	A	1
10	F	49	Otitis externa					

BAL, bronchial lavage; AML, acute myelogenous leukaemia; CML, chronic myelogenous leukaemia.

<sup>a</sup>M, Male; F, Female.

<sup>b</sup>Results of combined analysis of RAPD types produced by primers R108, CII and P4.

<sup>c</sup>Patients from the surveillance study (negative during screening).

**Table 3.** Rapid amplification of polymorphic DNA PCR typing results for environmental *Aspergillus terreus* isolates

Date of isolation (month/year)	Isolate no.	Primer-derived type			Combined type
		CII	P4	R108	
May/2005	36	B	I	V	3
December/2004	6	C	J	L	4
December/2004	7	C	K	K	5
May/2005	39	D	K	S	6
August/2005	41	D	X	Q	7
January/2005	13	E	H	G	9
April/2005	31	F	M	C	10
April/2005	29	F	Q	B	11
January/2005	12	H	R	J	13
February/2005	21	J	U	N	17
April/2005	28	J	C	K	21
April/2005	32	J	B	J	20
February/2005	19	J	V	M	18
May/2005	33	J	H	A	16
April/2005	30	K	D	K	24
May/2005	37	L	F	O	27
February/2005	20	M	W	L	28
February/2005	24	M	E	M	29
January/2005	11	O	I	H	31
February/2005	23	P	K	D	32
March/2005	26	R	L	G	34
November/2004	3	S	N	Y	35
December/2004	8	T	C	I	36
August/2005	43	U	G	F	37
January/2005	14	W	N	D	39
August/2005	44	X	T	R	40
May/2005	34	Y	E	I	41
October/2004	1	Z	G	E	43
October/2004	2	Z	B	E	42
May/2005	35	A	M	C	44
February/2005	16	B	F	X	45
March/2005	N*	H	S	M	14.
Total no.	31	23	28	26	31

N\*, nasal carrier.

[13]. This may be explained by ongoing renovation and construction within the UHI during sample collection. Several studies have confirmed that renovation leads to a higher density of viable conidia in the outdoor settings and in non-HEPA protected areas inside [16,18]. As shown in this study, HEPA filter systems reduced the level of contamination due to *Aspergillus* spp. inside the wards.

The lack of aspergilli in the respective risk areas indicates fungal uptake, most likely prior to hospitalization [19]. However, there is no consensus concerning the conidial density and the onset of IA.

Rhame reported a higher risk when the average fungal density inside the hospital was 0.9 CFU/m<sup>3</sup> [20].

Arnou *et al.* noted a marked decrease of infections when the density fell from 2 to less than 0.2 CFU/m<sup>3</sup> [21]. By contrast, two studies found no correlation between density of airborne aspergilli and incidence of IA [14,22].

Patients who are colonized with opportunistic fungi are at higher risk of developing invasive disease, and the growth of *Aspergillus* spp. in

cultures from the nose has been accepted as a surrogate marker of IA [2,23]. However, the value of isolating *Aspergillus* spp. from respiratory tract specimens is debatable, and it was shown that 37% of humans are colonized with *Aspergillus* spp. in deep lung segments without having signs of infection [19,24,25]. In this study, 3.7% of patients tested positive for *Aspergillus* spp. in nasal specimens, but only one patient developed aspergillosis due to *A. fumigatus*. Perfect *et al.* [26] made a similar observation, i.e. that most culture isolates from non-sterile body sites are not indicative of disease.

Surveillance isolates consisted, more or less, of *A. fumigatus*. Only one *A. terreus* carrier (who did not develop disease) was identified. Perfect *et al.* found *A. terreus* carriage to be always associated with invasive disease [26].

The molecular typing of *Aspergillus* has proven useful in many epidemiological situations [8]. One of the most widely used genotyping methods is RAPD PCR, a technically relatively simple and rapid procedure. Although RAPD PCR has been criticized for poor reproducibility, this method has been used successfully for *A. terreus* isolates, and primers R108, CII and P4 were found to be highly discriminatory [5,8,9]. No identical RAPD profiles were found among the strains from the patients or the environment, indicating great genetic diversity.

The great genetic diversity might be partly explained by the limitations of the RAPD approach. Similar findings have been observed for *A. fumigatus*, but the results depend on methodology and study design [27,28]. One study described two patients with IA that was caused by three *A. terreus* strains of different genotypes, a phenomenon also described, infrequently, in patients infected with *A. fumigatus* [29].

Efforts to identify a common source within the hospital have been unsuccessful. *A. terreus* is endemic in the environment of the UHI, although to a lesser extent than *A. fumigatus*. Worldwide, *A. terreus* accounts for a minority of cases of IA, ranging from 1% to 6–12% [8,26,30,31], and it is a rather common cause of IA in geographically distant centres, e.g. M.D. Anderson Cancer Center, Houston, Texas, TX, USA, University Hospital, Birmingham, Alabama, AL, USA, and UHI, Tirol, Austria [5,32]. The reason for the frequency of IA caused by *A. terreus* at the UHI is not clear. Many factors,

such as specific host-related characteristics, the state of immunosuppression of the affected patients, the extensive use of systemic antifungal agents for prophylaxis and empirical therapy, and geographical factors, may contribute to this phenomenon [33].

In summary, *A. terreus* has become a common cause of IA and is common in the environment of the UHI. The genotypic profiles indicate that patients were infected with unique strains, making a collective source of transmission highly unlikely. The onset of *A. terreus* infection depends, seemingly, not only on the degree of immunosuppression, but also on environmental exposure to *Aspergillus* spp. Finally, nasal surveillance cultures are not helpful in identifying patients at risk for *A. terreus* infections.

#### TRANSPARENCY DECLARATION

The authors declare no potential conflicts of interest.

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