MYCOLOGY

Value of different methods for the characterisation of *Aspergillus terreus* strains

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To evaluate different methods for strain differentiation, 10 isolates of *Aspergillus terreus* from Germany and two epidemiologically unrelated strains were investigated. The sources of the isolates were patients with cystic fibrosis (4), immunosuppression (2), otitis externa (2), sinusitis (1) and endocarditis (1). Environmental isolates were obtained from a contaminated cell culture and from soil. The isolates did not differ in their macroscopic and microscopic morphology, in their protein patterns analysed by SDS-PAGE and in their susceptibility to amphotericin B and itraconazole. The RFLP analysis of total genomic DNA digested by *EcoRI* resulted in patterns that were too faint for interpretation. However, after hybridisation of the digested DNA with a short DNA probe of repetitive sequence, six different patterns were found. Based on the patterns of the randomly amplified polymorphic DNA (RAPD) with three primers, nine different genotypes were discriminate. RAPD patterns discriminated the epidemiologically unrelated reference strains (endocarditis isolate from Thailand, soil isolate from the USA) and the isolates from Germany. It is concluded that, in contrast to the phenotypic methods, the analysis of RAPD patterns is useful for strain differentiation of *A. terreus*.

Introduction

Members of the genus *Aspergillus* are opportunist pathogens [1]. Most infections in man occur in severely immunocompromised patients [2]. *A. fumigatus* represents the most common agent of systemic infections, followed by *A. flavus* [2]. *A. terreus* is widespread in the environment, particularly in soil and on decaying vegetation [1]. It has also been found in the air of hospital areas [3, 4]. Invasive infections in man and animals have been reported [5, 7–11], showing that *A. terreus* is not simply a colonising organism. A nosocomial outbreak of invasive aspergillosis caused by this species has been described [12].

Various phenotypic and genotypic methods have been used successfully in fingerprinting *A. fumigatus*. Analysis of protein patterns by SDS-PAGE and immunoblotting were used for phenotypic fingerprinting [13, 14]. The analysis of restriction fragment length polymorphism (RFLP) [15, 16] patterns after hybridisation with probes of moderately repetitive sequences [17, 18] and the patterns resulting from

random amplification of polymorphic DNA (RAPD) were used for genotypic fingerprinting [19–24]. However, no comparable data are available for *A. terreus*.

To gain information on the typability of *A. terreus*, 12 isolates were investigated by the following methods: (i) macroscopic and microscopic morphology; (ii) susceptibility to amphotericin B and itraconazole; (iii) protein patterns in SDS-PAGE; (iv) DNA patterns after hybridisation of *EcoRI*-digested genomic DNA; and (v) DNA patterns resulting from RAPD-PCR.

Materials and methods

A. terreus isolates

The sources of isolates are listed in Table 1. Nine isolates (nos. 1–9) were from seven patients at the University Hospital of Essen, Germany. Paired isolates were obtained from sputum samples of two patients (A, B) with cystic fibrosis. Isolate no. 11 was found in a contaminated cell culture of eukaryotic cells at the University Hospital of Essen. Two isolates were from the Centraalbureau voor Schimmelcultures (CBS, Baarn and Delft, the Netherlands). One isolate (no. 10) was found on a cardiac valve of a patient with endocarditis who lived in Thailand (CBS 116.46). The other isolate (no. 12) was a soil isolate from the USA

Table 1. *A. terreus* isolates: sources, susceptibility testing, RAPD and hybridisation types

Strain No.	Source	Patient	Underlying condition	Date of isolation	MIC of AmB*		RAPD type†			Hybridisation type
					48 h	72 h	P1	P2	P3	
1	Sputum	A	Cystic fibrosis	31/7/1995	2	32	I	I	I	A
2	Sputum	A	Cystic fibrosis	26/4/1996	2	32	I	I	I	A
3	Sputum	B	Cystic fibrosis	10/10/1996	0.5	32	II	II	II	B
4	Sputum	B	Cystic fibrosis	13/11/1996	1	16	II	II	II	B
5	Sputum	C	Immunosuppression	13/8/1993	8	>64	I	I	I	A
6	Tracheal secretion	D	Immunosuppression	3/11/1994	8	16	III	III	I	A
7	External ear	E	Otitis externa	1/3/1994	2	4	III	IV	III	B
8	External ear	F	Otitis externa	26/8/1995	2	>64	VI	V	VI	C
9	Sinus maxillaris	G	Sinusitis	28/9/1995	2	4	V	VI	V	C
10‡	Cardiac valve	H	Endocarditis	No data	2	4	VI	VII	VI	D
11	Cell culture	—	—	12/4/1996	4	64	VII	VIII	VII	E
12‡	Soil	—	—	No data	1	2	VIII	IX	VIII	F

*MIC of amphotericin B (AmB) in mg/l.

†P1, P2, P3; number of primer.

‡Culture collection strains (CBS).

(CBS 469.81). The isolates from Essen were subcultured no more than five times before analysis.

Macroscopic and microscopic morphology

For species identification and investigation of the macroscopic and microscopic morphology, single spot cultures were performed on Czapek Solution Agar (Difco) and Malt Extract Agar (Difco). Cultures were incubated for 10 days at room temperature. The microscopic examination was performed on colonies grown on malt extract agar. The production of aleuriospores (globose to cavate hyaline cells produced on the vegetative submerged mycelium [5]) was investigated by microscopic examination of the reverse side of the culture plates at a magnification of 600.

Susceptibility testing

For determination of susceptibility to amphotericin B (amphotericin B/deoxycholate; Squipp-von Heyden, Munich, Germany) and itraconazole (Janssen, Olen, Belgium), micro-dilution broth assays with RPMI-1640 (with L-glutamine, without sodium bicarbonate; Sigma) buffered with 0.165 M MOPS (Sigma) were performed as described previously [25]. The range of the final concentrations in the tests was 0.125–128 mg/L for amphotericin B and 0.015–8 mg/L for itraconazole. Each well contained *c.* 10⁴ cfu in a final volume of 200 µl. One well without an antimycotic agent served as growth control. The plates were incubated at 35°C and the MICs were determined after incubation for 48 h. The MICs of amphotericin B were also determined after incubation for 72 h. The MIC for itraconazole was defined as the lowest concentration that inhibited *c.* 75% of growth compared to the growth control [25, 26]. For amphotericin B, the MIC was fixed at the lowest concentration with no visible growth [25, 26]. All experiments were performed twice. *A. fumigatus* ATCC 90906 with published MIC values for amphotericin B and itraconazole [27] was tested in parallel in each test series.

SDS-PAGE

The isolates were cultured on Sabouraud Glucose Agar (Unipath) at 33°C for 3 days. Conidia were harvested by swabbing and suspended in distilled water; *c.* 10⁶ conidia were suspended in 250 ml of Sabouraud Glucose Broth (Unipath). The suspensions were cultured on an orbital shaker (150 rpm) at 33°C for 72 h. Mycelia were harvested by filtration with Miracloth (Calbiochem, La Jolla, CA, USA) and were washed three times with NaCl 0.9% solution followed by a washing step in Tris-HCl-EDTA buffer (0.1 M Tris-HCl, pH 7.5, 0.01 M EDTA) containing 5 µg/ml (Boehringer Mannheim, Germany) and 0.01 M PMSF (Sigma). A 5-ml X-press chamber (Bachhofer, Reutlingen, Germany) was loaded with *c.* 8 g (wet weight) of the mycelial mass and was frozen at –20°C overnight. The cells were disrupted by pressing the frozen mycelium four times (pressure 1–1.5 t). This procedure resulted in *c.* 90% cell disruption, as determined by microscopic control. After thawing and centrifugation (10 000 g, 4°C, 20 min), the protein content of the clear supernates was determined by a modified Lowry assay (Pierce, Rockford, IL, USA). After boiling samples of the suspensions with an equal volume of buffer (0.125 M Tris-HCl, pH 6.8, SDS 4%, glycerol 20%, 2-mercaptoethanol 10%), samples containing 20 µg of protein were loaded on to 12.5% and 7.5% continuous SDS-polyacrylamide gels. Marker proteins (Promega, Madison, USA; Gibco, Eggenstein, Germany) were run in parallel. After electrophoresis, the gels were stained with Coomassie Blue (Serva, Heidelberg, Germany).

Preparation of DNA

The method of preparation of DNA was identical to that described previously [24]. Briefly, strains were cultured on Sabouraud glucose agar for 72 h at 33°C. Conidia were harvested by swabbing and inoculated in to five petri dishes (diameter 9.5 cm), containing 5 ml of Sabouraud glucose broth (Unipath) each. Cultures were incubated for 24 h at 33°C. The mycelial cells were harvested, washed and disrupted by vortex mixing

with glass beads at maximal speed. The DNA was isolated by phenol-chloroform extraction and precipitation in ammonium acetate and ethanol. The concentrations of DNA were determined, after digestion of RNA, by the DNA DipStick (Invitrogen, Leek, the Netherlands).

RFLP and hybridisation

DNA (c. 200 ng) was digested with *Eco*RI (Boehringer Mannheim) and applied to an agarose 1% gel. After electrophoresis in TAE (40 mM Tris acetate, 2 mM EDTA) the gels were stained with ethidium bromide. The DNA fragments were transferred to a nylon membrane (Boehringer Mannheim) and hybridised with digoxigenin-labelled DNA-probe (5'-(GTG)₅-3') at 45°C overnight in hybridisation buffer and detected as recommended by the manufacturers of the DIG Luminescent Detection Kit (Boehringer Mannheim). The membranes were placed on X-ray films (X-OMAT AR, Eastman Kodak, Rochester, NY, USA) for 3 h at 37°C.

RAPD-PCR

The primers used for polymerase chain reaction (PCR) were synthesised by MWG-Biotech (Ebersberg, Germany). Primer 1 (R108, 5'-GTATTGCCCT-3') was described by Aufavre-Brown *et al.* [19]. The sequence of primer 2 (5'-GCTGGTGG-3') was published by Loudon *et al.* [22]. The sequence of primer 3 was 5'-TCACCCTGA-3' [24]. DNA was amplified with the single primers and pairwise combinations of primers as described previously [24]. Briefly, amplification was performed in a mastermix (50 mM KCl, 3 mM MgCl₂, 10 mM Tris-HCl, pH 8.8) containing 200 mM (each) of dATP, dCTP, dGTP and dTTP (Biozym Diagnostik, Oldenburg, Germany), 1 µg of primer, 2.5 U of Taq DNA Polymerase (Promega, Madison, USA) and 5 µl (1 µg/µl) of template DNA in a final volume of 100 µl. A Minicycler (MJ Research, Watertown, USA) was used. After an initial cycle (94°C 5 min, 35°C 2 min, 72°C 2 min) 45 cycles of amplification (94°C 1 min, 35°C 2 min, 72°C 2 min) were followed by a final extension at 72°C for 10 min. All primers were run under the same conditions. The amplified products were electrophoresed at 100 V in a horizontal agarose 1.6% gel. Gels were photographed after staining with ethidium bromide. A mol.-wt marker (Pharmacia, Uppsala, Sweden) was run in parallel. RAPD patterns were compared visually. An isolate was defined as different when a band with a density equal to or greater than that of the 0.8-kb band of the marker (reference density) occurred and no corresponding band (not even a trace) was detectable in the other isolates. To demonstrate reproducibility of the patterns, all isolates were investigated three times independently, with different subcultures.

Results

Morphology

The 12 *A. terreus* isolates did not differ in their macroscopic and microscopic morphology on Czapek solution agar and malt extract agar. Colonies of all isolates exhibited a cinnamon-brown colour on Czapek solution agar and a wood-brown colour on malt extract agar. All isolates produced aleuriospores on both media. A brown exudate was produced on Czapek solution agar, but not on malt extract agar.

Susceptibility to amphotericin B and itraconazole

In susceptibility testing with amphotericin B, the MIC values of the *A. fumigatus* control strain remained constant at 0.25–0.5 mg/L during the 72-h incubation period. However, the MIC values of the *A. terreus* isolates increased from 0.5 to 8 mg/L after 48 h to 2–> 64 mg/L after incubation for 72 h. The MIC range against itraconazole was 0.03–0.125 mg/L after 48 h.

Protein patterns in SDS-PAGE

All isolates showed identical protein patterns in SDS-PAGE with numerous bands in the range 14–120 kDa. Major bands were seen at 20, 22, 26, 39, 53, 60, 78, 95 and 100 kDa.

RFLP and hybridisation patterns

The *Eco*RI-RFLP of cellular DNA resulted in numerous bands. The patterns were too faint for interpretation. However, after hybridisation with the DNA-probe 5'-(GTG)₅-3', six distinct patterns could be separated (Fig. 1 and Table 1). The isolates from patients A, C and D showed identical patterns (hybridisation type A), as did the isolates from patients B and E (hybridisation type B) and the isolates from patients F and G (hybridisation type C). The remaining isolates showed different patterns.

Patterns in RAPD

Further discrimination was obtained by RAPD analysis (Table 1). With primers 1 and 3, respectively, the 12 isolates could be discriminated into eight RAPD types. Primer 2 showed further discrimination, resulting in nine RAPD types (Fig. 2). The isolates from patients B and E, C and D, and F and G, respectively, could be discriminated from each other. In agreement with the hybridisation results, the paired isolates of patients A and B, respectively, were identical (RAPD type I and II). All German isolates showed a band at 770 bp with primer 2 which did not occur in the epidemiologically unrelated reference strains (nos. 10 and 12). The RAPD with pairwise combined primers resulted in no further discrimination. No differences in band patterns were found when testing different subcultures.

Fig. 1. Hybridisation patterns of 12 *A. terreus* isolates. The lane numbers represent the isolate numbers in Table 1. The mol. wts of marker bands (M) are given on the left side.

Fig. 2. RAPD patterns of 12 *A. terreus* isolates produced by primer 2 (1.6% agarose gel). The lane numbers represent the isolate numbers in Table 1. The mol. wts of the marker bands (M) are given on the left side. Isolates 1 and 2 showed identical patterns in the original picture.

Discussion

To our knowledge, this is the first study of *A. terreus* with different methods for strain differentiation. No differences were found in the macroscopic and microscopic morphology of the 12 *A. terreus* isolates. All isolates produced aleuriospores, which are not only found in *A. terreus*, but also in *A. flavipens* (white colonies), *A. niveus* and *A. carneus* [28]. However, no hülle cells, characteristic for *A. flavipens* and *A. carneus* [28], were found in the isolates under investigation.

In susceptibility testing a time-dependent increase of MICs for amphotericin B up to >64 mg/L was found. As the MIC values of the *A. fumigatus* reference strain remained constant over the incubation period of 72 h, no systematic error occurred. Similar results have been found by others [29]. The high MIC values of amphotericin B after prolonged incubation may have implications for the treatment of *A. terreus* infections. All isolates showed MIC values of 0.03–0.125 mg/L for itraconazole after incubation for 48 h. Therefore, susceptibility testing does not seem to be useful for strain differentiation. In studies on *A. fumigatus* strain-specific differences were found in SDS-PAGE protein patterns [13]. In contrast, no differences in SDS-PAGE were found in another study; however, only a few strains of this species were investigated [24]. In the present study, all isolates of *A. terreus* showed identical protein patterns in SDS-PAGE. Therefore, the analysis of protein patterns is not useful for strain differentiation in *A. terreus*.

Based on the results of the oligoblot hybridisation, six different patterns could be discriminated. However, interpretation of the patterns was difficult because of the numerous bands obtained by this method. RAPD analysis showed greater discrimination, resulting in nine different genotypes. Previously, this technique was used successfully in fingerprinting other *Aspergillus* species [19–24], showing a high genetic diversity in *A. fumigatus* and *A. flavus*. Similar results were also found for *A. terreus* in the present study. Therefore, RAPD seems to provide the best discriminatory power, i.e., the ability to demonstrate diversity within a species.

Both the epidemiologically unrelated reference strains could be separated from the German isolates based on the RAPD patterns with one primer. However, only a limited number of isolates was investigated in the present study and conclusions on the correlation of a specific RAPD pattern to the epidemiological origin of a strain could not be drawn. It is necessary to investigate epidemiologically unrelated outbreak strains to answer this important question. Unfortunately, no such strains of *A. terreus* exist in culture collections, i.e., NCPF, ATCC or CBS.

In both patients with cystic fibrosis, the paired isolates were genetically identical. These results indicate that this method is potentially useful in the analysis of nosocomial outbreaks of aspergillosis caused by *A. terreus*. However, the RAPD method is subject to some limitations. The reproducibility of patterns in one laboratory under the same experimental conditions is excellent, as described here. However, it has been reported that patterns depend on the Taq polymerases and the cyclor used [30], indicating that the reproducibility of patterns between laboratories might be problematic. Therefore, there is an urgent need for inter-laboratory standardisation of this technique.

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