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A review of German *Scedosporium prolificans* cases from 1993 to 2007

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Scedosporium prolificans is one of the most life-threatening fungal opportunistic pathogens due to its high resistance to common systemic antifungal agents. While a close relative of *Pseudallescheria boydii*, *S. prolificans* has a more limited geographic range being primarily found in Australia, USA and Spain. Infections have also been reported from several other European countries and from Chile. Twenty patients with *Scedosporium prolificans* infection or colonization from August 1993 to May 2007 were retrospectively reviewed in Germany. They had all been identified at or reported to the Reference Laboratory for *Pseudallescherial Scedosporium* spp. in Berlin. Twelve of 13 patients with haematological disorders and/or on immunosuppressive therapy developed a fatal invasive scedosporiosis. Colonization of the respiratory tract was reported for one patient after heart-lung-transplantation, all six patients with cystic fibrosis and one with chronic sinusitis. Molecular studies of the *S. prolificans* isolates confirmed that parts of the 18S, the Internal Transcribed Spacer (ITS) regions and the D1/D2 domain of the 28S region of rDNA are monomorphic. However, sequencing of parts of the translation elongation factor EF1-alpha (EF-1α) and the chitin synthase (CHS-1) genes revealed the presence of three and two distinct genotypes, respectively. Two informative mutations were found in EF-1α and a single nucleotide exchange in the CHS-1 gene.

Keywords *Scedosporium prolificans*, ITS, D1-D2 sequencing, genotype, translation elongation factor EF-1a, chitin synthase CHS-1, *in vitro*-susceptibility

Introduction

Scedosporium prolificans Guého & de Hoog (formerly *Lomentospora prolificans* Hennebert & Desai and *Scedosporium inflatum* Malloch & Salkin), an emerging pathogen, is highly resistant to antimycotic agents [1,2]. Due to its dark conidia, it is considered to be the most common agent of disseminated phaeohyphomycosis [3]. The fungus is a member of the order Microascales and more specifically of the *Pseudallescheria/Scedosporium* species complex [4]. In contrast to the more common species *Pseudallescheria boydii* and *Scedosporium apiospermum*, *S. prolificans* has a somewhat more limited geographic distribution. According to current knowledge, the organism is distributed mainly in Australia, USA and Southern Europe, especially in Spain [5–10]. This geographic presence suggests an association with semi-arid climates. It is more rarely reported from other European countries, e.g., France [11], UK [12], Germany [13], The Netherlands [14], as well as from Chile [15].

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Given the emergence of this fungus in temperate climates, we conducted a review of the recovery of *S. prolificans* in Germany over a 14 year-period. An epidemiological typing system was developed by multilocus sequencing studies and the *in vitro* antifungal susceptibility of the strains was analyzed.

Material and methods

Case reports

Each case report associated with *Scedosporium prolificans*, documented at the Reference Laboratory for *Pseudallescheria boydii* / *Scedosporium prolificans* at the Robert Koch Institute in Berlin, Germany, was retrospectively evaluated. If possible, patients were analysed for: (1) their underlying disease, (2) the origin of the isolate, (3) the clinical relevance of *S. prolificans* (colonization *versus* infection), (4) whether the patient was already under antimycotic treatment, (5) therapeutical strategies after identification of *S. prolificans*, and (6) clinical outcome. Infection was defined by the recovery of *S. prolificans* from a sterile body site of a patient who developed clinical symptoms compatible with a deep seated fungal infection and/or histopathologically proven hyphomycosis. Isolation of *Scedosporium prolificans* from only the respiratory tract (sputum, sinus maxillaris), without any histologically proven hyphomycosis, was defined as colonization.

Strains

All clinical isolates were identified on the basis of macro- and micromorphological criteria, temperature maxima, cycloheximide susceptibility [16] and by PCR with species-specific primers [17]. Reference strains from Spain, Belgium, UK, Australia and USA were included in the genotyping studies. Isolates were deposited in the fungal collection at the Robert Koch-Institut, Berlin. Stocks were stored on ceramic beads at −70°C (Microbank, MAST-Diagnostica, Reinfeld, Germany). All available isolates have also been studied by amplification and sequencing of parts of the 18S, the ITS regions and the D1/D2 domain of the 28S rDNA, the chitin synthase (CHS-1) and the translation elongation factor 1-alpha (EF-1α) [18] in order to determine diversity within the species, as well as their potential value in genotyping.

DNA extraction

Mycelia (approximately 50 mg, wet weight) were homogenized in 300 µl of cetyltrimethylammonium-bromide (CTAB) buffer containing glass beads (0.45–0.50 mm in diameter; B. Braun, Melsungen, Germany) as previously described [17]. After 15 min of sonication in a water bath, followed by an incubation step at 65°C for 10 min, then 300 µl ethanol were added and samples were homogenized by vortexing. Total DNA was purified on silicagel columns (Qiamp Mini Kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at −20°C.

Amplification

Fragments of rDNA were amplified with the primer pairs NS7-ITS2, ITS4-ITS5 [19], ITS3-R635 and F63-R635 [20], EF 1-728 F [CAT CgA gAA gTT CgA gAA gg] and EF 1-986 R [TAC TTg AAg gAA CCC TTA CC] and CHS-79 [Tgg ggC AAg gAT gCT Tgg AAg AAg]and CHS-354 [Tgg AAg AAC CAT CTg TgA gAg TTg] [18] in a reaction mixture containing 25 µl distilled water, 5 µl of PCR buffer ($MgCl_2$, 10 ×), 8 µl 0.25 mM dNTP (Roche Diagnostics GmbH, Germany), 1 µl of each primer and 1.25 U of Taq BioTherm DNA polymerase (Rapidozym, Germany). The PCR reaction (in brackets for the amplification of the translation elongation factor EF1-α and CHS-1) started with an initial denaturation step at 96°C, 5 min (95°C, 10 min) followed by 30 cycles 96°C, 30 s (35 cycles 95°C, 15 s); 52°C, 30 s (55°C, 20 s); 72°C, 75 s (72°C, 60 s). The last elongation step at 72°C was prolonged with 3.5 min (5 min).

DNA sequencing

The amplified products were purified by QIAquick Gel Extraction Kit (Qiagen). Direct sequencing was performed with an ABI PRISM 3130 XL Sequencer (Applied Biosystems).

Sequences obtained were aligned with the computer program BioEdit sequence alignment editor version 7.0.5.

Antifungal susceptibility testing

Susceptibility testing was performed according to Clinical and Laboratory Standards Institute methods [21]. The combined effects of voriconazole/terbinafine and voriconazole/caspofungin were studied by a checkerboard microdilution method with 17 and 15 isolates, respectively. Briefly, the spore suspensions were adjusted spectrophotometrically to yield a final inoculum of 1×10^4 to 5×10^4 CFU/ml. Testing was done in 0.165 M MOPS-buffered RPMI medium (pH 7.0) in microtiter plates, incubated at 35°C for 48 h. Final concentrations ranged from 0.03–16 µg/ml for voriconazole and from 0.25–16 µg/ml for terbinafine and

caspofungin. MIC studies were conducted in duplicate, with at least one rerun. Results were read visually with the help of a reading mirror by two laboratory persons acting independently of each other.

MICs of voriconazole and terbinafine were defined as the lowest concentrations that completely inhibited growth (score of 0, growth inhibition of 100%). A partial inhibition (score of ≤ 2, growth inhibition greater than or equal to 50%) was considered for the MIC of caspofungin. For comparison with published data and for antifungal combinations, a score of ≤ 2 was also used. The combination effects were calculated by the fractional inhibitory concentration index (FIC-Index) [22] and interpreted according to Odds: ≤ 0.5 synergy, >0.5–4.0 no interaction and >4.0 antagonism [23].

Results

Patients

Twenty *S. prolificans* positive patients were identified and/or registered at the Reference Laboratory (Table 1) from 1993 to 2007. Thirteen out of the 20 patients had a hematologic malignancy and/or underwent bone marrow or solid organ transplantation or received long term corticosteroids. Twelve of these 13 patients developed proven invasive fungal infections, 8 of which involved fungemia. Ten of the patients died of scedosporiosis and two others died as a result of their underlying diseases associated with their disseminated fungal infections. The lungs of one patient who underwent heart-lung transplantation seven years prior to the present studies were colonized with *S. prolificans* for nearly three years, but without the development of proven infection. He died after the development of a motile thrombus in the heart from a non-sclerotic cardiac infarction. Colonization of the respiratory tract was noted with all 6 CF patients and one with chronic sinusitis. In some patients with CF, *S. prolificans* was isolated once, while in others it was repeatedly recovered without signs of invasive infections. The longest period of colonization as revealed by repeated isolation of the fungus from the respiratory tract was 3 years. Instances of locally invasive infections due to *S. prolificans* were not found in our CF-patients. Diagnosis of scedosporiosis was made by positive blood culture in 8/12 (66%) of the patients with proven invasive fungal infection and in 50% of them this occurred less than a week before death.

Molecular results

A total of 35 isolates (23 clinical isolates from 18 patients in Germany and 12 reference strains, Table 2) were included in these investigations. Analysis of sequences of nearly 1,500 basepairs (bp) from the terminal region of the SSU, the ITS 1, 5.5S and ITS 2 regions to the D1/D2 domain of LSU rDNA were monomorphic for all isolates. The alignment of the 326 to 328 bp long sequences of an intron of the translation elongation factor EF-1α revealed three clusters (genotypes). The first type, EF-1α A, differed from type EF-1α B by 2 nucleotide exchanges (C/T) and (T/G) at position 104 and 233 and from type EF-1α C by 5 nucleotide exchanges at five different positions (28, 48, 82, 247-8 and 266). The nucleotides C at position 104 and T at position 233 were specific for genotype EF-1 α B. Type EF-1α A was associated with 9 German patients, 4/6 reference strains from Spain, as well as in WCH 07-201141 from Australia and CBS 114.90 from the USA. Type EF1-α B was found in 5 German patients, as well as in the *S. prolificans* type strain CBS 467.74 from Belgium, FMR 6643 from Spain, NCPF 7685 from the UK and WCH 07-201137 from Australia. Type EF-1α C was found in isolates from another 4 German patients, as well as in FMR 6641 from Spain and WCH 07-327433 from Australia. Genotypes were identical in sequential isolates from the same patient, i.e., three from one patient and four isolates from a second patient. The alignment of 254 bp long sequences of the chitin synthase gene (CHS-1) revealed a single nucleotide exchange (C instead of T) at position 27, which was detectable in three isolates from Germany, one from Australia, and the one from USA, but not in isolates from Spain (Table 2). The nucleotide sequences of each EF-1α genotype and of the two CHS-1 genotypes (CHS-1 A and CHS-1 B) were deposited in GenBank (Accession numbers EU596512-EU596513-EU596514 and FJ179541-FJ179542).

In-vitro susceptibility testing

Ranges of MICs (μg/ml) as score 0 (score of ≤ 2 in brackets) were 16 to >16 (2 to 16) for voriconazole, 8 to >16 (2 to >16) for terbinafine and (4 to >16) for caspofungin. Synergy of voriconazole and terbinafine was observed in 11/17 (65%) of the isolates but no interaction was found in the remaining 6/17 (35%) isolates. Using the score of ≤ 2, synergy increased to 82% (14/17) and indifference (no interaction) was seen in 18% of the isolates. The voriconazole MIC (score of ≤ 2) of three isolates was 2 μg/ml, whereas the geometric mean MIC of voriconazole against the other

Table 1 Patients in Germany with isolation of *Scedopsorium prolificans* and clinical data

RKI-No.	Other collection numbers	Origin/Patient no.	Geographical origin	Genotype EF-1α
Reference strains				
	CBS 467.74	Greenhouse soil	Belgium	EF-1α B
	CBS 114.90	Man	USA	EF-1α A[#]
	NCPF 7685	Man	UK	EF-1α B
	FMR 6641	Man	Spain	EF-1α C
	FMR 6643	Man	Spain	EF-1α B
	FMR 6696	Man	Spain	EF-1α A
	FMR 6702	Man	Spain	EF-1α A
	FMR 6722	Soil	Spain	EF-1α A
	FMR 6724	Soil	Spain	EF-1α A
	WCH 07-327433	Man	Australia	EF-1α C
	WCH 07-201141	Man	Australia	EF-1α A
	WCH 07-201137	Man	Australia	EF-1α B[#]
Clinical strains		Patient no.	Germany:	
RKI 93-2809		1	Frankfurt, HE	EF-1α C[#]
RKI 93-3775	CBS 494.92	2	Rostock, MV	EF-1α A
RKI 94-2399	CBS 100390	3	Boppard, RP	EF-1α B[#]
RKI 95-1482	CBS 100391, ATCC 201214	4	Leipzig, SN	EF-1α A
RKI 00-0363		5	Leverkusen, NW	EF-1α A
RKI 01-0720		7	München, BY	EF-1α A
RKI 02-0664		9	Hamburg, HH	EF-1α A
RKI 07-0265	92-03-1399 (796)	10	Homburg, RP	EF-1α B
RKI 03-0507		11	Essen, NW	EF-1α A
RKI 04-0283		12	Erlangen, BY	EF-1α B
RKI 04-0286	CBS 102176	13	Mainz, RP	EF-1α C
RKI 04-0407		14	Hamburg, HH	EF-1α A
RKI 05-0040		15	Homburg, SL	EF-1α A
RKI 07-0264	311-6-05 (783)	15	"	EF-1α A
RKI 05-0706		15	"	EF-1α A
RKI 05-0707		15	"	EF-1α A
RKI 07-0266	05-04-278 (764)	16	Winnenden, BW	EF-1α B
RKI 07-0263	63-05-05 (190)	17	Bonn, NW	EF-1α C
RKI 06-0470		18	Hamburg, HH	EF-1α C
RKI 07-0093		19	Berlin, BE	EF-1α A
RKI 07-0187		19	"	EF-1α A
RKI 07-0188		19	"	EF-1α A
RKI 07-0130		20	Augsburg, BY	EF-1α B[#]

Abbreviations: AIDS, acquired immunodeficiency syndrome; AMB, amphotericin B; AML, acute myelogenous leukemia; BAL, bronchoalveolar lavage; BMT, bone marrow transplantation; CAS, Caspofungin; CF, cystic fibrosis; CML, chronic myelogenous leukemia; FLZ, fluconazole; HLTX, heart-lung transplantation; ITZ, itraconazole; LTX, lung transplantation; MCZ, miconazole; PCZ, posaconazole; RTX, renal transplantation; TRB, terbinafine; TS, tracheal secretion; VCZ, voriconazole.
[#]This case report has been published [13].

14 isolates was 5.1 μg/ml (data not shown). The highest synergistic effect of voriconazole plus terbinafine was noticed in the three isolates with the lowest MIC. For the combination of voriconazole and caspofungin, synergy was found in only 2/15 isolates (13%), independently from the endpoint used (score of 0 or ≤ 2). Antagonism was not observed in any of the combinations.

Discussion

Since 1993, reports have documented the importance of *Scedosporium prolificans* as the cause of life threatening hyphomycosis in the temperate climate of Germany. Development of an infection after exposure to *S. prolificans*, as with other opportunistic fungi, clearly depends on the immunological status of the host. As published previously, *S. prolificans* may colonize the respiratory tract for months or even years, especially in patients with cystic fibrosis (CF) [10,24,25], without becoming invasive. This colonization can be transient, as noted in two of our patients, not requiring antifungal therapy. Neither the CF patients nor the patient with chronic sinusitis developed a scedosporiosis. Nevertheless the risk of developing an infection after long term colonization by *S. prolificans* cannot be excluded

Table 2 Analyzed strains, their genotypes and geographical origin

First isolation of *S. prolificans*	Patient No.	Age (years) sex	Underlying disease	Site of isolates	Clinical manifestation	Antifungal treatment before isolation of *S. prolificans*	Antifungal treatment after isolation of *S.prolificans*	Outcome following diagnosis[#]
1993	1	54, F	RTX	Blood	Fever, pneumonia, sepsis, skin	None	AMB liposomal + 5FC; MCZ	Died d 9
1993	2	52, M	AML/sepsis	Skin biopsy	Sinusitis maxillaris, diss. Skin lesions	FLZ, AMB+5-FC	none	Died d 1
1994	3	53, F	AML	Blood (5x)	Pneumonia, sepsis, endophthalmitis, skin	AMB+5FC	AMB + 5FC	Died d 7
1995	4*	60, M	AIDS, Burkitt lymphoma	Brain	Sepsis (lung, spleen, kidney, heart, cerebrum	FLZ 100 mg	diagnosis post mortem	Died
2000	5	20, F	HLTX	Sputum	Colonized 34 month	ITZ, AMB per inhalation	AMB per inhalation, VCZ	Died of cardiac infarction
2000	6	61, F	Long term corticosteroids	TS, blood	Fever, pneumonia, multi organ failure	AMB	diagnosis post mortem	Died
2001	7	15, M	CF	Sputum	Colonized	n.a.	none	*S.p.* not invasive
2001	8	15, M	CF	Sputum	Colonized	n.a.	none	*S.p.* not invasive
2002	9	44, F	CML, BMT	BAL, urine, catheter, blood	Pneumonia, sepsis	FLZ, CAS	CAS	Died d 5
2002	10	12, F	CF	Sputum	Routine CF screening		none	*S.p.* transient
2003	11	n.a., M	BMT	Blood	Sepsis, endophthalmitis	n.a.	PCZ	Died d n.a.
2004	12	55, M	AML	BAL	Sepsis, lung, heart, spleen, kidney	AMB	n.a.	Died d 2
2004	13	40, M	AML	Blood	Sepsis, multi organ failure	AMB	AMB	Died d 0
2004	14	64, M	AML	Blood	Diss. Into brain	ITZ	Unknown	Died d 5
2004	15	9, M	CF	Sputum	Alike ABPA	AMB per inhalation; ITZ	CAS; VCZ+TRB	Fungus ball by *S. p.* suspected
2005	16	n.a., M	CF	Sputum	Colonized	n.a.	n.a.	*S.p.* not invasive
2005	17	10, F	CF	Sputum	Colonized, routine CF screening	n.a.	None	*S.p.* transient
2006	18	60, M	Chronic idiopathic myelofibrosis, BMT	BAL, blood	Sepsis	AMB, VCZ	VCZ, CAS	Died d 16
2007	19	47, F	COPD, LTX	BAL, vitreous body	Sepsis, endophthalmitis	VCZ	PCZ + CAS; AMB Liposomal; VCZ	Died d 19
2007	20	18, F	Allergic rhinopathia, sinusitis maxillaris	Sinus maxillaris	Sinusitis maxillaris	None	Surgery	Cured

Acronyms of strain collections: CBS, Centraalbureau voor Schimmelcultures; RKI, Robert Koch-Institute. Abbreviations of the Federal States in Germany: BW, Baden-Württemberg; BY, Freistaat Bayern; BE, Berlin; HH, Freie und Hansestadt Hamburg; HE, Hessen; MV, Mecklenburg-Vorpommern; NW, Nordrhein-Westfalen; RP, Rheinland-Pfalz; SL, Saarland; SN. Freistaat Sachsen.

[#]Isolates with single nucleotide exchange in the CHS 1 gene (genotype CHS-1 B).

in this patient population. This was indicated recently in an *S. prolificans*-infected patient with double lung transplantation (LTX) and GVHD [26], although it was not documented with certainty whether or not the patient had been colonized by *S. prolificans* prior to LTX. Serological tests to detect *S. prolificans* specific antibodies are not routinely performed, although they could assist in the diagnosis of allergic bronchopulmonary mycosis and the early detection of an invasive infection due to *S. prolificans*. The question of whether colonization of the respiratory tract by *S. prolificans* is a contraindication for lung transplantation in CF patients remains under discussion. One of the reported CF patients (patient 15) developed a dramatic respiratory failure at the same time as the first isolation of *S. prolificans* after treatment which included voriconazole and terbinafine. This young boy finally developed a fungus ball in the lung, but investigation is still ongoing to identify the infectious agent.

All but one of the patients receiving immunosuppressive therapy developed fatal, disseminated scedosporiosis. Nearly all of them were receiving antifungal treatment, but none received a combination therapy of voriconazole (or posaconazole) plus terbinafine. The short survival period of most of the patients with deep infection is alarming. In eight patients with haematological disorder and documented onset of invasive scedosporiosis, the survival period, on average, was 5.6 days. This observation underlines the need for earlier detection, e.g., with molecular methods.

Fever was one of the main clinical symptoms and was used as an indicator for the collection of blood samples for culture. The high percentage of successful isolation of *S. prolificans* from blood cultures, as reported previously [6,8], is remarkable when compared to other hyphomycoses. The detection of *S. prolificans* obviously is nearly unaffected by ongoing antimycotic treatment. Breakthrough *Scedosporium* infections are frequently found in patients with neutropenia under antifungal prophylaxis [7]. This reflects the extreme resistance of this species to these drugs as opposes other opportunistic hyphomycetes, e.g., *P. boydii* and *S. apiospermum*. *Scedosporium prolificans* is known to be multi-drug resistant, especially to amphotericin B, 5-flucytosine, and most azoles. While there is limited data on the successful treatment of patients with deep seated *S. prolificans* infection, the combination of voriconazole plus terbinafine appears to be the most effective [27–29]. The *in vitro* studies of *S. prolificans* isolates from Germany may underline the recommendation for the use of this therapeutic regimen. Interestingly, three isolates recovered from patients who had not receive prior voriconazole therapy showed their MICs to be significantly lower than those from other patients. The susceptibility of *S. prolificans* isolates susceptible to voriconazole have been reported rarely [30,31], which suggests that there is a need for further studies on susceptibility and resistance mechanisms of *S. prolificans*.

Due to inadequate surveillance of systemic fungal infections, an assessment of the incidence of this particular mycosis in Germany is not possible. A recent telephone survey at three University institutes for microbiology with mycological expertise revealed that isolation of *S. prolificans* is still rare or has never been noticed. However, we have to take into account the fact that about 50% of the clinical isolates arriving at the Reference Laboratory had been sent as 'unidentified hyphomycetes'. Thus one can speculate that *S. prolificans* is an underdiagnosed in many laboratories or even neglected as a contaminant. Analysis of all clinical *Scedosporium* isolates at the Reference Laboratory during the period 1993–2007 revealed that 18% of patients were colonized or infected by *S. prolificans*. In CF patients the rate was even higher, 6/18 (33%) of CF patients at an ambulatory clinic in Muenchen, Bavaria, 2001–2005, being colonized by *S. prolificans*.

The sequencing results presented here confirm the high homology within *S. prolificans*, which is known to be highly conserved in the ITS1/2 region [17,32] and the D1/D2 domain of the large rDNA subunit. In contrast, molecular typing by random amplification of polymorphic DNA (RAPD) and PCR-fingerprinting [33] and especially by inter-simple-sequence-repeat polymerase chain reaction [34] revealed so many diverse molecular patterns that the appropriate method for epidemiological studies in *S. prolificans* is still under discussion. Our data document the existence of different genotypes in *Scedosporium prolificans*. Three EF-1α genotypes and two CHS-1-genotypes were found in isolates from patients in Germany, Spain and Australia. Until now only one CHS-1 genotype was found in the limited number of Spanish isolates. So far no correlation of these genotypes to the clinical outcome and antifungal susceptibility has been found. Further studies will be necessary to determine if there is a correlation between the CHS-1 genotypes and the geographical origin of the isolates.

The EF-1 α genotypes in *S. prolificans* are distributed on a worldwide basis, similar to the airborne species *Aspergillus fumigatus* [35]. However, marked differences in regional prevalence of *Scedosporium* species are observed [10,36] which is probably explained by prevailing environmental conditions. The mechanism of dispersal of *S. prolificans* and the route of transmission are still poorly understood, since the slimy conidia

are only rarely detected in studies of airborne fungi. In species with low effective dispersal, such as *Penicillium marneffei* [37] and *Fonsecaea* species [38], whose conidia are supposed to travel only small distances, populations tend to show regional structuring.

The observation of differing EF-1α and CHS-1 genotypes may be helpful in further epidemiological studies on *S. prolificans* and especially in the development of a MLST scheme, to explore the question of *S. prolificans* population structures, as well as species epidemiology.

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