Re-identification of clinical isolates of the *Pseudallescheria boydii*-complex involved in near-drowning

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Summary

Fungal infections caused by the members of the genera *Pseudallescheria* and/or *Scedosporium* are important complications in patients after near-drowning. As the taxonomy of *Pseudallescheria* and *Scedosporium* has been revised, clinical isolates from 11 patients, after near-drowning, previously identified as *P. boydii* or *S. apiospermum* had to be re-identified. *S. apiospermum*, now separated from *P. boydii* as a distinct species, was found most frequently ($n = 8$), while *S. aurantiacum*, recently described as new species and *P. boydii* were less common ($n = 2$ and $n = 1$, respectively). Three patients near-drowned during the Tsunami 2004 were infected by different species of the *P. boydii* complex. *In vitro* testing resulted in lowest minimal inhibitory concentration (MICs) for voriconazole (range 0.25–2.0 μg ml^{-1}).

Key words: *Pseudallescheria boydii, Scedosporium apiospermum, Scedosporium aurantiacum,* identification, ITS sequencing, near-drowning, *in vitro*-susceptibility.

Introduction

In 2005, the WHO adopted the following definition of fatal and non-fatal drowning: 'Drowning is the process of experiencing respiratory impairment from submersion/immersion in liquid'. 'Near-drowning' is the survival of such an accident for more than 24 h.[1] Near-drowned people are at high risk for the development of infections, mainly in the lungs, as a result of aspiration of microorganisms.

Depending on the source of water, salt tolerant bacteria such as *Shewanella putrefaciens* or *Vibrio* spp., or other bacteria such as *Aeromonas hydrophilia* can be isolated from the site of infection.[2] Fungal infections caused by *Aspergillus* spp. and species of the family *Mucoraceae* [3–5] have been reported after near-drowning, but most frequently, infections were caused by the members of the *P. boydii*-complex.[6–8] These organisms have been isolated not only from the respiratory tract but also particularly from the brain and the lesions becoming apparent weeks or months after near-drowning. Sources of infection were reported to be brackish water, fresh and rain water, water in butts, ditches, ponds, mountain lakes and oceans. Mortality rates of scedosporiosis/pseudallescheriosis after near-drowning are high. Isolates are frequently resistant to amphotericin B.

Molecular studies of a representative set of isolates classified previously as *P. boydii* (teleomorph) or *S. apiospermum* (anamorph) revealed that several species of a complex were concerned. Recently, *P. boydii* and *S. apiospermum* have been recognised as distinct species, while *P. minutispora*, *S. aurantiacum* and *S. dehoogii* were proposed as new species.[9,10]

Differences in susceptibility to antifungal agents could be noticed. *S. aurantiacum* has been discussed to show the highest resistance of all species in the *P. boydii*-complex.[11] Hence, correct identification of the members of the *P. boydii*-complex is indispensable for the epidemiological analysis and the development of treatment regimen. Therefore, available isolates from near-drowned individuals in Europe were re-identified and susceptibility testing was performed. In addition, avail-

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able data of the patients' history have been collected and analysed.

Material and methods

Strains

Clinical isolates of 11 near-drowned patients have been studied. These isolates routinely had been identified on the basis of macro- and micro-morphological criteria, temperature maxima, cycloheximide susceptibility [12] and by PCR with species-specific primers [13] and were deposited as '*P. boydii*' (teleomorph) or '*S. apiospermum*' (anamorph) according to former taxonomic concepts in the collection for fungi at the Robert Koch-Institute, Berlin, Germany. The study included the following reference strains: CBS 101.22^{T} *P. boydii,* RKI 93-2956 *S. apiospermum*, CBS 116910 *S. aurantiacum,* CBS 101721 *S. dehoogii*, RKI 94-866 *P. minutispora*, CBS 100391 *S. prolificans*. Stock cultures were stored on ceramic beads at −70 °C (Inverness Medical, Köln, Germany). The isolates from near-drowned patients are listed in Table 1. All available isolates have been re-identified by sequencing of the internal transcribed spacer (ITS) regions of rDNA and by common phenotypical methods.

DNA extraction

Mycelia and conidia were homogenised in 300 µl cetyltrimethylammoniumbromide buffer containing glass beads as previously described.[13] After 15 min of sonication in a water bath, followed by an incubation step at 65 °C for 10 min, 300 µl of ethanol was added and samples were homogenised by vortexing. Total DNA was purified by 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 ITS4-ITS5 in a reaction mixture containing 25 µl distilled water, 5 µl of PCR buffer ($MgCl_2$, 10×), 8 µl 0.25 $mmol^{-1}$ dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 1 µl of each primer and 1.25 U of Taq BioTherm^{TM} DNA polymerase (Rapidozym, Berlin, Germany). The PCR reaction was performed with an initial denaturation step at 96 °C for 5 min, followed by 30 cycles of 96 °C for 30 s, 52 °C for 30 s and 72 °C for 75 s. A final elongation step at 72 °C was conducted for 3.5 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, Darmstadt, Germany). Sequences obtained were aligned with the computer program BIOEDIT sequence alignment editor version 7.0.5.

Case reports

Case reports of *pseudallescheriosis/scedosporiosis* after near-drowning with available clinical isolates, some of them already published [6,14–17] or documented at the Consultant Laboratory for *P. boydii/S. prolificans* in Germany, have been evaluated retrospectively. If data were available, they were analysed for the habitat, where the accident happened, the patients' history, the origin of the isolate, therapeutical strategies after identification of pseudallescheriosis/scedosporiosis and clinical outcome.

Susceptibility testing

All isolates of patients after near-drowning were tested for their *in vitro* susceptibility to amphotericin B, voriconazole, posaconazole, caspofungin and terbinafine. After subculturing twice on potato dextrose agar, the inocula were prepared according to the Clinical and Laboratory Standards Institute guidelines and adjusted photometrically. Testing was performed in microtitre plates with 0.165 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffered RPMI 1640 medium, pH 7.0. After an incubation period of 48 h at 35 °C, the MICs were read visually by two independently operating persons using a reading mirror. The MIC was defined as the lowest concentration of antifungal substance, which inhibited the growth greater or equal to 95% (for amphotericin B 100%). The MIC of caspofungin was defined as the minimal concentration reducing the growth distinctly, as seen with the help of a reading mirror. *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains.

Results

Re-identification of the clinical isolates by sequencing of the ITS regions revealed that eight patients were infected by *S. apiospermum* (*P. boydii*-complex clade 4), two patients by *S. aurantiacum* and one patient by *P. boydii* (*P. boydii*-complex clade 5) (Table 1). Multiple isolates of the same patients were identical except that of

Table 1 Patients' data and related strains.

Patient no. [Reference]	Age (year/ month	Sex	Collection number	Re-identified species	Origin of isolates	Suspected source of *Pseudallescheria/ Scedosporium*	Clinical manifestation	First identification of *Scedosporium*	Therapy	Outcome
1 [14]	2/6	male	RKI 97-249	*S.apiospermum*	Cerebral biopsy	Pond	Brain abscesses	Postmortem	AMB, 5-FC, FCZ	died
2	1/10	female	RKI 98-222	*S.apiospermum*	TS	Water butt	Brain abscess	Day 12	MCZ	died day 21
3 [16]	2	male	CBS 111588	*S.apiospermum*	Cerebral biopsy	Water butt	Brain abscesses	Day 38	VCZ, TBF, CAS	survived
4	37	female	RKI 05-0107	*S.apiospermum*[1]	Pleura	Car-accident, roadside ditch	Brain abscess, pleuritis, autoptic proven	Postmortem, aspergillosis susp.	most probably AMB	died
5	1/5	male	RKI 05-180	*S.apiospermum*	BAL	Water butt	n.a.	n.a.	VCZ	n.a.
6	71	male	RKI 05-459	*S.apiospermum*	Skin-abscess, multiple	Tsunami 2004	Skin abscesses	Month 8	CAS	died of cancer, deep seated skin abscesses
7	1/3	male	RKI 06-0282	*S.aurantiacum*	BAL	Water butt	Transient flora, invasion unproven	Day 4	AMB (3 wk)	not applicable
8 [6]	39	male	CBS 118233	*S.apiospermum*	Brain	Car-accident, mountain lake	Brain abscesses	Week 16	CAS; VCZ	died
9 [17]	59	male	RKI 07-0432	*P.boydii*	Paravertebral biopsy	Tsunami 2004	Spondylodiscitis	Week 12	surgical; VCZ i.v.	survived
10 [17]	51	female	RKI 07-0433	*S.aurantiacum*	Brain	Tsunami 2004	Brain abscess	Week 8	surgical; VCZ i.v. (6 mo)	survived
11	n.a.	n.a.	RKI 07-0517	*S.apiospermum*	BAL	Near drowning in The Netherlands	n.a.	n.a.	n.a.	n.a.

i.v., intravenous; n.a., data not available; AMB, amphotericin B; BAL, bronchial alveolar lavege; 5-FC, flucytosine; FCZ, fluconazole; MCZ, miconazole; TS, tracheal secretion; VCZ, voriconazole; TBF, terbinafine; CAS, caspofungin.

[1]two different genotypes.

patient no. 4 who harboured two different genotypes of *S. apiospermum*. Correct species identification by micromorphology alone was not possible, as for example the *P. boydii* isolate did not develop cleistothecia (teleomorph), even after 6 weeks of incubation on oatmeal agar with lupine stem. The *S. aurantiacum* isolates had very long synnemata (400–800 μm) of the *Graphium* anamorph and did not assimilate saccharose, but production of diffusible yellow pigments as reported in the protologue remained unclear. All isolates were able to grow between 40 °C and 42 °C and were resistant to 0.04% cycloheximide.

Five of eleven patients were children under 3 years of age, four of them had nearly drowned in a water butt. Adults were subjects of traffic accident or of the Tsunami in South-east Asia. Six patients developed brain abscesses, one spondylodiscitis and one deep seated skin infections. The latter was the only one, who had underlying diseases before the near-drowning event. The 71-year-old male patient suffered from diabetes mellitus and a melanoma had been diagnosed 3 years earlier. *S. apiospermum* was most probably a part of the transient flora in one patient without clinical manifestation. Only in three cases, survival after scedosporiosis is definitely known;[16,17] these patients received voriconazole in combination with surgical intervention or combined with caspofungin and terbinafine.

Results of *in vitro* susceptibility testing for amphotericin B, voriconazole, posaconazole, caspofungin and terbinafine are shown in Table 2. The highest variation was noticed for voriconazole (ranges 0.25–2.0 μg ml^{-1}). Despite a range from 2 to >16 μg ml^{-1} for amphotericin B 10/11 isolates had a MIC ≥16 μg ml^{-1}. All isolates were resistant *in vitro* to terbinafine.

Discussion

The first report of an infection caused by *Pseudallescheria/Scedosporium* species after near-drowning was published indirectly in 1980 by Van der Vliet *et al.* [18] who analysed two patients who had died of scedosporiosis after kidney transplantation. It was revealed that the donor had died after near-drowning and his kidneys were infected with this mould. Since that time, several cases of pseudallescheriosis/scedosporiosis after near-drowning have been published in Europe.[6,8,14–17,19,20]

In drowning and near-drowning, three main age groups have been reported: (i) children younger than 5 years of age, mainly near-drowned in their own or in the neighbours' garden; (ii) adolescents between 15 and 21 years of age, often after alcohol abuse and (iii) adults over 35 years of age, mainly because of cardiac problems. Half of the cases presented here belong to the first category.

In contrast to other patients, who are at risk to develop life threatening, deep seated hyphomycosis, near-drowned individuals are primarily immunocompetent. Only patient 6, a 71-year-old male who became infected by *S. apiospermum* during the Tsunami in December 2004, had several underlying diseases. In February 2005, metastases of the melanoma were diagnosed in the skeleton as well as metastases of a low grade cholangiocellular carcinoma in the liver. Radiation was applied for skeleton metastases and chemotherapy for liver metastases. Two months later, the first skin abscess occurred without sufficient diagnostics. Scedosporiosis was definitely diagnosed in August 2005 when a deep abscess had developed within a few days below the left eye, localised in a former soft tissue injury during the Tsunami. A cerebral tumour which was discussed as potential septic focus of the *Scedosporium* infection or as a metastasis, postmortem turned out to be a metastasis of the melanoma. The patient died of gram-negative septic multi-organ failure. A therapy with itraconazole and caspofungin was ineffective as a result of delayed diagnosis, onset of treatment and his continuously high immunosuppression. Autopsy revealed a progressive abscess in his right arm and around the bulbus infiltrating the eye.

Scedosporioses resulting from the Tsunami in December 2004 obviously are quite different from infections in children: hyphomycoses had been acquired by aspira-

Table 2 *In vitro* test results.

	Antifungal substance MIC (μg ml^{-1})				
Species	AMB	VCZ	PCZ	TBF	CAS
S. apiospermum (*n* = 8)	2 >16	0.52 (GM 1.19)	12 (GM 1.83)	>16	216 (GM 4.36)
S. aurantiacum (*n* = 2)	>16	0.51	12	>16	16 >16
P. boydii (*n* = 1)	>16	0.25	1	>16	0.5

AMB, amphotericin B; VCZ, voriconazole; PCZ, posaconazole; TBF, terbinafine; CAS, caspofungin.

tion and/or by multiple trauma of the skin,[17] such as in the 71-year-old male patient. Interestingly enough, all the three patients, after the Tsunami, in this study had been infected by a distinct species. Reliable re-identification so far seems to be possible by molecular methods only. Sequencing results presented here confirm that the isolates from patients after near-drowning are taxonomically less homogeneous than supposed to be until recently. *S. apiospermum* was the most common species found to cause infections after near-drowning, but *S. aurantiacum* and *P. boydii* (anamorph: *S. boydii*) were also identified. Re-identification of isolates after near-drowning in the Tsunami[17] revealed for the first time that *S. aurantiacum* is also able to cause cerebral infections. None of the isolates belonged to the species *P. minutispora*, *S. dehoogii* or *S. prolificans*. *S. prolificans* is distantly related to the *P. boydii* complex and is a causative agent of lethal infections in severely immunocompromised patients from Europe.[21,22] The species thus far has never been described from patients after near-drowning; this matches with the observation that it is not found in brackish or polluted water like *P. boydii* and most other *Scedosporium* species.

The *in vitro* results of the limited number of *S. aurantiacum* isolates in this study cannot confirm that *S. aurantiacum* should be less susceptible to voriconazole than other species of the *P. boydii* complex.[11] Also the successful outcome of the *S. aurantiacum* infection in patient 10[17] by a combination of voriconazole and draining of the brain abscess indicates a susceptibility of this isolate. But for caspofungin, *S. apiospermum* isolates had a wider range of MICs, whilst the two *S. auranticacum* isolates had high MICs, which is comparable to the *in vitro* resistance of *S. auranticaum* to micafungin.[11] Results of susceptibility testing demonstrate the need for individual testing of the clinical isolates. Although more than 90% of the isolates are highly resistant to amphotericin B *in vitro* and also *in vivo*, there are certain strains, which are susceptible *in vitro* to amphotericin B.

As the highly effective drug miconazole is no longer available, *in vitro* studies indicate that voriconazole, itraconazole, posaconazole or ravuconazole plus caspofungin might be the most effective drug combinations to be applied in pseudallescheriosis or scedosporiosis after near-drowning.[23–25] A combination of voriconazole or posaconazole with terbinafine, as an *ultima ratio* in *S. prolificans* infections, seems not to be a promising treatment option in an infection caused by *P. boydii*, *S. apiospermum* or *S. aurantiacum*. It is therefore doubtful whether the successful outcome of a child (patient 3) after a long-term triple therapy was influenced by the combination with terbinafine at all.

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Conflicts of interest

The authors have declared no conflicts of interest.

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