A dark strain in the *Fusarium solani* species complex isolated from primary subcutaneous sporotrichoid lesions associated with traumatic inoculation via a rose bush thorn

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*Fusarium* species are hyaline hyphomycetes widely distributed in nature and documented agents of both superficial and systemic infections in humans. In this paper, we report a darkly-pigmented and initially non-sporulating isolate in the *Fusarium solani* species complex (FSSC) causing a post-traumatic sporotrichoid infection in an otherwise healthy, male patient. Sequencing of multiple loci showed that the isolate represented an otherwise unknown lineage, possibly corresponding to a separate species, within the multi-species *F. solani* complex. In prolonged culture, the non-sporulating isolate produced revertant wild-type subcultures with typical *Fusarium* conidiation. This suggests that the original dense, dark, non-sporulating isolate was a host-adapted form selected *in vivo* for characters compatible with human pathogenicity. The production of such forms by *Fusarium* species is increasingly recognized now that sequencing has allowed the identification of highly atypical isolates. *In vitro* antifungal susceptibility of the isolate was investigated against seven conventional and two newly approved antifungal agents. The isolate showed *in vitro* resistance to amphotericin B, but appeared susceptible to itraconazole and terbinafine. A cure was ultimately achieved with combined terbinafine/itraconazole therapy with prolonged itraconazole follow-up therapy.

**Keywords** *Fusarium solani* species complex, sporotrichoid infection, primary subcutaneous fusariosis, chlamydospores, acid fast fungal cells

**Introduction**

Patients who have had fungal inoculum introduced by penetrating injury of the skin may, in some cases, develop a pattern where the infectious agent spreads via proximal lymph nodes, causing a series of lymphocutaneous, ulcerative, nodular lesions. This pattern is described as ‘sporotrichoid’ since it is most familiar from infections caused by the dimorphic fungus *Sporothrix schenckii*. The list of etiological agents responsible for skin lesions mimicking sporotrichosis includes bacteria, fungi and protozoa [1,2]. *Fusarium* species are hyaline molds that are widely distributed plant pathogens and saprotrophs in nature. *‘Fusarium solani’* is the traditional name for a visually recognizable *Fusarium* ‘species’ that is now known through molecular study to
comprehend a large complex of morphologically similar, related species [3]. The *Fusarium solani* species complex (FSSC) is one of the most common and widespread fusarial clades, and is among the most frequently isolated fungi from soil and plant debris. It is also associated with severe opportunistic infections in healthy or immunocompromised human hosts [4–6]. Here we report a darkly pigmented member of the FSSC isolated from a case of a post traumatic infection in an otherwise healthy, male patient.

**Case report**

The patient was a 44-year-old, otherwise healthy man with no significant past medical history. He worked as an administrator and did gardening for recreation. The injury occurred when he was pricked by a rose bush in the right hand between the little and ring fingers about one week prior to development of the first nodule at the wound site (Fig. 1). The first lesion began as a painless swelling with redness around the puncture, but it worsened in time and became an erythematosus nodule and began to drain. He received multiple unsuccessful courses of antibacterial therapy. Over 8 weeks, a gradually extending series of new papules and nodules erupted on his hand and arm. He was admitted to Cerrahpasa Medical Faculty Dermatology Department on 27 April 2004 with multiple erythematous papules, fistulas and ulcerated, crusted, red nodular lesions 2–5 cm diameter on his right hand and arm extending up the antecubital fossa along lymph nodes and vessels (Fig. 2). Routine clinical laboratory data were unremarkable. There was no history of diabetes mellitus or steroid use. An excisional biopsy was performed on one of the lesions on his arm for pathological and microbiological examinations. Histological examination revealed active chronic supplicative granulomas in connective tissue and a granulomatous infiltrate in the deep dermis with edema and increased myofibroblastic activity. Histopathological findings were consistent primarily with deep mycotic infection; however, Ehrlich-Ziehl-Neelsen (EZN) and Periodic acid-Schiff stained sections did not reveal any detectable specific agent. Microbiological evaluation of Gram-stained smears from biopsy tissue specimens revealed no bacteria. No bacterial organisms were cultured from the specimen. Special stains for acid fast bacteria and mycobacterial cultures also were negative. Giemsa-stained tissue was negative for *Leishmania* amastigotes. Mycological examination of Gram, EZN, Giemsa and methylene blue (MM) stained imprinted tissue and smeared pus preparations revealed globose fungal cells, a finding lacking specificity for any particular etiologic agent. Because the expected lymphocutaneous pathogen, *Sporothrix schenckii*, has a relatively long incubation period, two additional punch biopsy samples were taken from arm nodules along with two more samples of purulent materials expressed from the hand. These samples were referred to the Deep Mycoses laboratory on different days, each approximately one week apart. The later specimens again showed fungal cells consistent with those seen in the first biopsy. Cultures yielded pure growth of a dark pigmented mycelial fungus. Treatment with itraconazole (ITZ) (200 mg 1×1 daily *per os*) was started as suggested by the *in vitro* antifungal susceptibility test results. Two months later, on follow up, the patient showed some regression of lesions but infection was
still evident, and therapy was continued several months further. Six months after the beginning of antifungal therapy, regression of the lesions halted and the arm remained unchanged. Therefore, therapy was changed to 200 mg ITZ 2×1 daily. During follow up, the lesions remained stable in size and number, but the scars of some healed lesions were also evident (Fig. 2). On 29 December 2004, after further examination of in vitro antifungal susceptibility test results, terbinafine (TRB), 250 mg 1×1 daily, was added. The patient responded to this therapy. Combination therapy was continued for two more months and then, since regression of lesions had restarted, therapy was continued as ITZ 200 mg 2×1 daily. On follow up in early spring of 2006, there was a marked flattening of the lesions (Fig. 3). Individual lesions completely resolved at different time intervals. Therapy was continued until all lesions were healed. The initial lesion healed last. The healing process required approximately 3 years. There was no sign of recurrence 6 months later.

Materials and methods

Isolation and phenotypic identification

Three biopsy specimens from arm nodules and two samples of pus expressed from the hand were examined by the Deep Mycosis Laboratory. Direct microscopic examination was performed using Gram, EZN, Giemsa, and MM stained slide preparations of imprinted tissue sections and pus. Purulent materials were further examined by India ink preparations. Aseptically divided pieces of the tissue specimens were inoculated into Sabouraud dextrose agar (SDA) (Oxoid Ltd, Basingstoke, Hampshire, England), Brain heart infusion agar (BHIA) (Oxoid Ltd, Basingstoke, Hampshire, England), Potato dextrose agar (PDA) (Oxoid Ltd, Basingstoke, Hampshire, England), Czapek dextrose agar (CDA) (Oxoid Ltd, Basingstoke, Hampshire, England) and Malt extract agar (MEA) (Oxoid Ltd, Basingstoke, Hampshire, England) plates and incubated at 35, 30 and 25°C. Purulent material was streaked onto the same set of agars and incubated at the same temperatures. All plates were sealed with parafilm (American National Can, Chicago, IL, USA) to maintain adequate humidity over a prolonged incubation time. Microscopic morphology of the isolate was examined by staining with lactophenol cotton blue and using unstained wet mounts with physiological saline. A glass smear prepared from a suspension of dark buff mycelial colonies was stained with EZN to search for the acid fast fungal cells that had been observed in tissue preparations. The strain was subcultured on SDA and PDA and incubated at 37±1°C for two weeks to assay growth at 37°C. Phenotypic identification employed classical mycologic techniques and was based on macroscopic and microscopic morphologic features.

Molecular analyses

For sequence-based identification, the FastDNA Kit (Bio 101, Carlsbad, CA) was used according to the manufacturer’s instructions to extract DNA. Amplification was performed using primers V9G and LR5 [7,8] designed to span the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (rDNA), and primers EF-1 and EF-2 for translation elongation factor alpha (EF) amplification as recommended by O’Donnell et al [3]. In a PCR System 9700 thermocycler (PE Applied Biosystems, Foster, CA), initial denaturation was set at 94°C for 2 min, followed by 35 cycles of 94°C for 35 sec, 55°C for 50 sec, and 72°C for 2 min, followed by a final extension at 72°C for 6 min and chilling to 4°C. The reaction mixtures of 50 μl contained 1 μl genomic DNA extract, 25 pmol of each primer, 200 μmol of each dNTP (Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, Buckinghamshire, UK), 1 U of Taq polymerase (Super Taq®, HT Biotechnology, Ltd., Cambridge, UK), and 1X standard PCR buffer supplied together with the Taq polymerase. After purification of the PCR products with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and verification of final concentration, PCR products were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems) with primers ITS1 and ITS4 [9] for the ITS region and primers EF-3 and

Fig. 3 Marked flattening of the lesions.
EF22T for the EF region [3]. For ITS, sequencing was carried out on an ABI Prism 3700 instrument (Applied Biosystems). Sequence editing was done in Seqman II software (DNASTar, Madison, WI). New and published sequences were aligned in BioNumerics 3.5 (Applied Maths, Sint Martens-Latem, Belgium). Sequencing and phylogenetic analysis for EF are described in detail by O’Donnell et al. [9].

Antifungal susceptibility tests

In vitro susceptibility of the isolate against conventional and newly approved antifungals were examined according to the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) M38-A reference broth macrodilution method [10]. Nine antifungal agents were studied. These included amphotericin B (AMB) (Bristol-Meyers Squibb, Wallingford, Conn.), fluconazole (FLZ; Pfizer, Istanbul, Turkey), flucytosine (5-FC, Sigma Chemical Co., St. Louis, Mo.), itraconazole (ITZ; Janssen Pharmaceuticals, Beerse, Belgium), ketoconazole (KTZ; Milen, Istanbul, Turkey), miconazole (MCZ; Selectchemie AG, Zürich, Switzerland), voriconazole (VRZ, Pfizer, Istanbul, Turkey), posaconazole (PSZ, Schering-Plough, Istanbul, Turkey) and terbinafine (TRB; Novartis, Basel, Switzerland). The newer antifungal agents, VRZ and PSZ, were obtained from their respective manufacturers. Antifungal concentrations ranged from 64–0.125 μg/ml for FLZ, and 16–0.03 μg/ml for all other antifungals. Morpholine propanesulfonic acid (MOPS, Sigma) buffered (to a pH of 7.0) standard RPMI 1640 medium, with glutamine and without bicarbonate (for the remaining antifungal agents) supplemented with 2% glucose were used as test media. Growth and sterility controls were included. The isolate was subcultured onto PDA slopes and incubated for 48 h at 35°C and then until day 7 at 25°C. Inoculum suspension was prepared using a mixture of conidia and hyphae as described in the M38-A protocol. The final inoculum concentration was approximately \(0.4 \times 10^4\) to \(5 \times 10^4\) CFU/ml. *Paecilomyces variotii* ATCC 22319 (American Type Culture Collection, Manassas, VA, USA), *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258 with known minimum inhibitory concentrations (MICs) were used for quality control (QC). MICs were determined visually after 48-h incubation at 35°C without agitation. The MIC for AMB, ITZ, VRZ, and PSZ was defined as the lowest concentration of the drug that inhibited all visible growth. For the remaining antifungals, the MIC was the lowest concentration of the drug that inhibited 50% of the growth (MIC 2) with respect to growth control (drug-free) tube [10, 11].

**Results**

**Mycology**

Direct microscopic examination of stained imprinted tissue slides and smears of purulent materials revealed the presence of thick walled round fungal cells which were stained acid fast by the EZN procedure.

*Fig. 4* Direct microscopic examination of stained imprinted tissue slides and smears of purulent materials revealed the presence of thick walled round fungal cells which were stained acid fast by the EZN procedure.
pigment. Microscopic examination of phaeoid colonies detected only intercalary and terminal chlamydospores but no micro- or macroconidia (Fig. 4). The hyaline colony phenotype produced microscopic characteristics typical of saprobic growth in the FSSC. These hyaline colonies also grew at 37°C.

Molecular analysis
The identity of the isolate was confirmed as a member of the FSSC by sequencing the ITS and EF regions. Recently, the isolate was included in an extensive phylogenetic study by O’Donnell et al. [3] and recognized as a unique multilocus sequence type (MLST), labelled 6-f, that is not represented by any other isolate in the extensive holdings of the NRRL collection. The ITS sequence for this isolate has been deposited into GenBank under the accession number EU329685. The sequence of the RNA polymerase II gene sequence used in MLST analysis by O’Donnell et al. [3] is also available as EU329638.

The isolate is preserved as UTHSC R-3564 at the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, TX, USA, as CBS 117608 in the collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, and as NRRL 37640 in the collection of the Microbial Genomics and Bioprocessing Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, USA.

Antifungal susceptibility test results
MICs (μg/ml) were >16 for AMB, 32 for FLZ and 5-FC, 1 for ITZ, 4 for KTZ, MCZ and VRZ, 8 for PSZ and 2 for TRB. The isolate showed in vitro resistance to amphotericin B, but appeared to be susceptible to itraconazole and terbinafine. MICs against the QC strains were within the expected limits (ranges specified in CLSI M38-A document) [10, 11].

Discussion
Skin infections with a classic ‘sporotrichoid’ pattern in an otherwise healthy patient can be ascribed to a limited number of etiologies [1, 2, 12]. The dimorphic fungus Sporothrix schenckii, agent of sporotrichosis, is one of the causes of primary cutaneous fungal infection and the only regularly seen cause of lymphocutaneous mycosis. Since roses can spread sporotrichosis, it is one of a few diseases that has been referred to as rose-thorn or rose-gardener’s disease. However, several other fungal species have the ability to cause ‘sporotrichoid’ skin lesions when they enter the body through the skin [1, 2, 12–14]. In the present case a unique member of the FSSC was isolated from sporotrichoid lesions in a patient who had a history of rose-thorn injury. This case is the first we are aware of that shows that Fusarium spp. may cause primary cutaneous ulcerated and crusted lesions that progress along dermal and subcutaneous lymphatics.

Fusarium is a hyaline fungus; however, darkly pigmented hyphae have also been reported from infected human and rat tissue [15, 16]. In the present case, the initially isolated colonies on SDA and PDA plates and on microscope preparations were phaeoid in appearance, which raised the possibility of phaeohyphomycosis. The rounded fungal cells seen in direct tissue microscopy did not eliminate the possibility of phaeohyphomycosis due to Exophiala spp., or even sporotrichosis. In some subcultures, the strain produced
characteristic ‘wild type’, normal FSSC colonies with hyaline hyphae and micro- and macroconidia. However, during maintenance on laboratory stocks, it produced variable colony phenotypes, wild-type and phaeoid (Fig. 6); the latter colonies resembled the primary isolates from infected tissue. Remarkably, microscopic preparations of phaeoid colonies revealed no conidial production. Colonies consisted of round chlamydospores produced on thin hyphae without conidia (Fig. 7). Segal et al. [15] reported the presence of chlamydospores along with phaeoid hyphae in tissue infected with Fusarium. In the present case, the round EZN positive cells seen in direct microscopic examination of imprinted tissue preparations were retrospectively recognized as possible chlamydospores which are a characteristic microscopic feature of the FSSC growing in culture.

The production of dense, relatively slow-growing colonies with reduced or no conidiation, compared to wild-type, is increasingly recognized as characteristic of Fusarium isolates that have become adapted to human host conditions during protracted infections such as mycetoma [17]. Some such isolates are completely nonsporulating and can only be identified by sequencing [18]. Many others are initially mistaken for Acremonium isolates [19]. In fact, a relatively prevalent mycetoma agent in South America, Acremonium falciforme, was revealed by sequencing to consist entirely of slow-growing, microconidial isolates in the FSSC [17]. This species was therefore recombined as Fusarium falciforme. Recently, O’Donnell et al. [3], in MLST studies of F. solani complex isolates from clinical sources, showed that the host-adapted Acremonium-like

F. falciforme isolates grouped with morphologically wild-type isolates in the combined MLST group 3+4 and that, in fact, the wild-type and host-adapted morphs together in this MLST assemblage formed the numerically largest group of medically important FSSC isolates. The name F. falciforme was extended to all isolates in the MLST group. The isolate from the present study is in a remotely related MLST type, type 6-f, but its primary isolates and later phaeoid sectors show reduced conidiation, slow, dense growth, and intense production of diffusing soluble pigments as are seen in host-adapted F. falciforme. No existing species name at the modern level of resolution applies to this MLST type, though it can still be correctly referred to as ‘F. solani ss. lat.’ (‘sensu lato’ = ‘in the broad sense’) or as a member of the FSSC.

The in vivo features seen in this case, pigmentation and chlamyospore production, may be selected for by host conditions, and may therefore be related to virulence factors aiding the maintenance and dissemination of the fungus within the host. Members of the FSSC are considered to be among the most aggressive opportunistic pathogenic groups within the genus, i.e., the groups causing most of the disseminated fusarial infections in humans and experimental models [5,20].

In general, most Fusarium spp. strains are resistant to antifungal agents in vitro. Resistance to 5-FC and imidazoles is practically universal, as shown elsewhere [21–23]. Amphotericin B has been the most investigated drug in vitro against Fusarium spp. and gives relatively high MIC values [24]. The case isolate showed in vitro resistance to AMB, but appeared to be susceptible to itraconazole and terbinafine. The patient initially respond to ITZ therapy, but clinical improvement stopped and complete cure was only obtained with combined terbinafine/ITZ therapy and follow-up ITZ as detailed above. This unusual regimen may relate to the genetic distinctiveness of the case isolate and may show the value of ongoing susceptibility testing in the management of infections caused by members of the FSSC.

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