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Epidemiological aspects and characterization of the resistance profile of *Fusarium* spp. in patients with invasive fusariosis

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ABSTRACT

Introduction. The remarkable intrinsic resistance of *Fusarium* species to most antifungal agents results in high mortality rates in the immunocompromised population.

Aims. This study aimed to investigate the epidemiology, clinical features and antifungal susceptibility of *Fusarium* isolates in patients with invasive fusariosis.

Methodology. A total of 27 patients admitted to a referral hospital from January 2008 to June 2017 were evaluated. Antifungal susceptibility testing of isolates was performed by broth microdilution according to the Clinical and Laboratory Standards Institute guidelines.

Results. Haematological malignancy was the predominant underlying condition, with an incidence of invasive fusariosis of 14.8 cases per 1000 patients with acute lymphoid leukaemia and 13.1 cases per 1000 patients with acute myeloid leukaemia. The *Fusarium solani* species complex (FSSC) was the most frequent agent group, followed by the *Fusarium oxysporum* species complex (FOSC). Voriconazole showed the best activity against *Fusarium*, followed by amphotericin B. Itraconazole showed high minimum inhibitory concentration values, indicating *in vitro* resistance. Clinical FSSC isolates were significantly (P<0.05) more resistant to amphotericin B and voriconazole than FOSC isolates.

Conclusion. The present antifungal susceptibility profiles indicate a high incidence of fusariosis in patients with haematological malignancy. Species- and strain-specific differences in antifungal susceptibility exist within *Fusarium* in this setting.

INTRODUCTION

Fusariosis is acquired by the inhalation of conidia, with subsequent haematogenous dissemination [1]. Occasionally, the skin may be a portal of entry at sites of tissue breakdown or onychomycosis [2–6]. Immunocompromised patients are at increased risk of developing invasive fusariosis as a result of profound and prolonged neutropaenia and/or severe T-cell immunodeficiency [7].

While a considerable body of knowledge about *Fusarium* has recently been accumulated in high-resource countries, in Latin America only a few groups of scientists have reported their experiences, mainly discussing epidemiological surveillance. A number of relevant reviews have recently become available, especially regarding the biology, epidemiology and clinical aspects of *Fusarium* species, as well as risk factors for infection, host characteristics, treatment and preventive strategies.

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Keywords: antifungal susceptibility; *Fusarium solani* species complex; *Fusarium oxysporum* species complex; invasive fusariosis.

Abbreviations: A, African descent; ALL, acute lymphoid leukemia; AMB, amphotericin B; AML, acute myeloid leukemia; B, brown; BAL, bronchoalveolar lavage; C, Caucasian; CML, chronic myeloid leukemia; ECVs, Epidemiological cutoff values; EORTC/MSG, European Organisation for Research and Treatment of Cancer/Mycoses Study Group; F, female; FDSC, *Fusarium dimerum* species complex; FFSC, *Fusarium fujikuroi* species complex; FIESC, *Fusarium incarnatum–Fusarium equiseti* species complex; FLC, fluconazole; FOSC, *Fusarium oxysporum* species complex; FSSC, *Fusarium solanispecies* complex; HCPA, Hospital de Clínicas de Porto Alegre; HL, Hodgkin's lymphoma; M, male; MICs, minimum inhibitory concentrations; ML, maximum-likelihood; MN, malignant neoplasm; NHL, non-Hodgkin's lymphoma; POSA, posaconazole; SDA, Sabouraud dextrose agar plus chloramphenicol; SEL, spinal epidural lymphoma; TEF1α, translation elongation factor 1-alpha; VRC, voriconazole; xMICA, micafungin.

The incidence and severity of diseases caused by *Fusarium* species vary according to geographical location. A study involving seven European countries described the *Fusarium fujikuroi* species complex (FFSC) as the predominant cause of invasive *Fusarium* infections [8]. This result is similar to that reported in a recent study conducted in Turkey, where FFSC was the most frequent agent group, followed by the *Fusarium solani* species complex (FSSC) [9]. However, in a study conducted in France, FSSC was the most prevalent, followed by the *Fusarium oxysporum* species complex (FOSC), the *Fusarium dimerum* species complex (FDSC) and, more rarely, the *Fusarium incarnatum–Fusarium equiseti* species complex (FIESC) [10].

Fusarium species are usually resistant to most antifungals. *In vitro* studies have shown lower minimum inhibitory concentrations (MICs) for amphotericin B, nystatin, ketoconazole, voriconazole and posaconazole for the *Fusarium* strains [11]. Most *Fusarium* species exhibit high minimum inhibitory concentrations (MICs) to currently used antifungals, especially azoles [12]. *Fusarium* species are intrinsically resistant to azole antifungals [13]. *F. solani*, in particular, is intrinsically resistant to echinocandins [14]. Recent diagnostic guidelines recommend amphotericin B and voriconazole as the drugs of choice for the treatment of deep and disseminated infections [10, 15]. However, *Fusarium nygamai* and *Fusarium thapsinum* have shown high MICs for all drugs other than amphotericin B and natamycin, indicating that identification and susceptibility testing of the aetiological agent is essential for guiding treatment [16].

Given the paucity of detailed studies on invasive fusariosis, the present study was designed to investigate the epidemiology, clinical features and antifungal susceptibilities of *Fusarium* isolates in patients with invasive fusariosis.

METHODS

Patients and methods

This study was conducted at Hospital de Clínicas de Porto Alegre (HCPA), located in Rio Grande do Sul, the southernmost state of Brazil. HCPA is a 750-bed university hospital that mainly provides care to patients from Porto Alegre, the state capital, and nearby towns, but also to those from further-out areas of the state. The state of Rio Grande do Sul has an area of 280 674 km^2. The region is unique in Brazil as it is characterized by a temperate humid subtropical climate with temperatures ranging from 31 to 39 °C in January and from 28.5 to 1 °C in July. Paediatric patients at HCPA are admitted to the 35-bed paediatric unit, while adults are admitted to the haematology unit. All patients admitted to these units from January 2008 to June 2017 were evaluated. The study was approved by the Research Ethics Committee of the institution.

The patients' medical records were reviewed for data on baseline characteristics, underlying diseases, treatment modalities, outcomes and mycological cultures positive for *Fusarium*. The isolates were obtained from a variety of clinical specimens from patients who had participated in a previous study approved by the Ethics Committee of the institution (ethics approval certificate number CAAE 52251115.4.0000.5327). All human subjects gave consent to participate in the study, with legal guardians providing consent for those under 18 years of age.

Patients were categorized according to the revised definitions of the European Organisation for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [17]. Invasive fusariosis was defined as any case with at least one positive blood culture or with the concurrent involvement of two or more noncontiguous sites. Neutropaenia was defined as an absolute neutrophil count ≤500 cells μl^{-1}.

DNA isolation, PCR and sequencing

Fusarium isolates were cultured on Sabouraud dextrose agar plus chloramphenicol (SDA; Difco Laboratories, Detroit, MI, USA). Culture plates were incubated at 26 and 37 ˚C and observed daily for growth up to 7 days. Initial identification of 27 *Fusarium* isolates was based on macroscopic colony morphology and microscopic features in a lacto-phenol wet mount preparation according to standard laboratory procedures. Final identification of 21 isolates was achieved using molecular methods. DNA extraction was performed as previously described by Dallé da Rosa *et al.* [18]. Fragments of the translation elongation factor 1-alpha (TEF1α) gene were amplified and sequenced using PCR protocols following the methods published by Al-Hatmi *et al.* [12]. Amplicons were purified from residual primers and dNTPs enzymatically with ExoSAP-IT (USB, Cleveland, OH, USA) and incubated at 37 °C for 30 min and at 80 °C for 15 min. Cycle sequencing was performed using the BigDye Terminator kit version 3.1 (Applied Biosystems, Inc., Foster City, CA, USA) and the extension products were purified with the BigDye XTerminator Purification kit (Applied Biosystems, USA) according to the manufacturer's instructions. Sequencing products were analysed on a 3500 Genetic Analyzer (Applied Biosystems, USA). Sequencing data visualization and sequence alignment were performed with Chromas v2.0 and CLC Main Workbench (CLC Bio, Denmark) software, respectively. Then, using BLAST, the consensus sequence was compared with reference sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The obtained sequences were deposited in the GenBank database and are shown in Table 1.

Phylogenetic analysis

Retrieved sequences were manually corrected to avoid mispaired bases. Consensus sequences were exported as FASTA files. Sequences of TEF1α were aligned with ClustalW. The best-fit model of evolution was determined by MEGA X [20]. Two maximum-likelihood (ML) phylogenetic trees were constructed from TEF1α sequences for FSSC and FOSC, with a bootstrap of 1000 repetitions.

Antifungal susceptibility

The clinical isolates were evaluated for susceptibility to voriconazole (Sigma-Aldrich, Saint Louis, USA), itraconazole

Table 1. Minimum inhibitory concentrations (MICs, μg ml^{-1}) of antifungal agents against the *F. solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC)

Species complex								
FSSC	**GenBank accession no.**	**Code**	**Amphotericin B**	**ECV***	**Voriconazole**	**ECV***	**Itraconazole**	**ECV***
N. gamsii	MN095848	F5	4	Below	2	Below	128	Above
F. keratoplasticum	MN095832	F7	4	Below	8	Below	128	Above
F. petroliphilum	MN095835	F10	2	Below	8	Below	128	Above
F. keratoplasticum	MN095830	F13	16	Above	32	Equal	128	Above
Neocosmospora sp.	MN095846	F14	4	Below	8	Below	64	Above
F. riograndense	KX534002	F17	1	Below	8	Below	128	Above
F. keratoplasticum	MN095828	F18	2	Below	1	Below	128	Above
F. petroliphilum	MN095834	F19	1	Below	2	Below	128	Above
F. keratoplasticum	MN095831	F22	16	Above	8	Below	128	Above
F. striatum	MN095845	F26	4	Below	16	Below	8	Equal
F. petroliphilum	MN095833	F30	1	Below	16	Below	64	Above
F. solani	MN095839	F32	16	Above	32	Equal	128	Above
F. keratoplasticum	MN095826	F27	16	Above	16	Below	16	Above
F. keratoplasticum	MN095827	F31	16	Above	32	Equal	128	Above
F. falciforme	MN095823	F6	2	Below	8	Below	128	Above
F. falciforme	MN095824	F8	4	Below	8	Below	>128	Above
FOSC								
Fusarium sp.	MN095836	F1	4	Below	1	Below	128	Above
Fusarium sp.	MN095838	F4	8	Equal	0.25	Below	128	Above
F. nirenbergiae	MN095844	F11	8	Equal	8	Below	128	Above
Fusarium sp.	MN095837	F12	2	Below	2	Below	128	Above
F. triseptatum	MN095847	F23	1	Below	2	Below	32	Above

*Epidemiological cutoff values (ECVs) of *Fusarium* according to Espinel-Ingroff *et al.* [19].

(Sigma-Aldrich, Saint Louis, USA), and amphotericin B (Sigma-Aldrich, Saint Louis, USA). MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method for filamentous fungi, M38-A2 [21]. The MIC was defined as the lowest concentration that showed 100% inhibition of visible growth as compared to the drug-free control well [21]. All tests were performed in duplicate. Two reference strains, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, were included as quality control isolates, with MIC ranges within pre-established limits [21]. The results were interpreted according to the CLSI epidemiological cutoff values (ECVs), as described by Espinel-Ingroff *et al.* [19], Therefore, considering >97.5 % of pooled statistically modelled MIC distributions, the ECVs were defined as follows: for amphotericin B, 8 μg ml^{-1} (FOSC and FSSC); for voriconazole, 16 μg ml^{-1} (FOSC) and 32 μg ml^{-1} (FSSC); and for itraconazole, 32 μg ml^{-1} (FOSC and FSSC).

Statistical analysis

Data were analysed with SPSS version 11.01 for Windows (SPSS, Chicago, IL, USA) software package. The groups (FSSC and FOSC) were compared with the chi-square test or adjusted residual analysis for categorical variables and the Mann–Whitney test for nonparametric continuous variables. Epidemiological data were compared with the chi-square test. The Mann–Whitney test was used to evaluate possible differences in MIC values for the different antifungal agents tested in the comparisons between FSSC and FOSC. Differences were considered statistically significant at a *P*-value of ≤ 0.05. Prevalence rates were calculated for invasive fusariosis

with *Fusarium* spp. fungi identified in haematopoietic stem cell transplant recipients (*n*=10), paediatric oncology patients (*n*=8), medical unit patients (*n*=8) and intensive care unit patients (*n*=1). The estimated incidence of invasive fusariosis during the study period (2008–2017) was calculated using the total number of admissions to the paediatric unit/haematology unit as the denominator, and rates were expressed as the number of cases per 1000 admissions.

RESULTS

A total of 27 cases of invasive fusariosis were identified from January 2008 to June 2017. All cases were classified as 'proven' based on the EORTC/MSG criteria. Table 2 shows the characteristics of the study population. Most patients were Caucasian descendants (88.9 %) living in the metropolitan area of Porto Alegre. The mean patient age was 22.7 years (range, 2–73 years). Infection was more prevalent in males (56 %) than in females (44 %). The most commonly affected sites were the nasopharynx (33 %) and skin (33 %), especially on the lower limbs (62.5 %). Blood culture was positive in 18.5 % of patients. The most common clinical presentations of invasive fusariosis were a persistently febrile patient (sustained temperature >38 °C) with profound and prolonged neutropaenia and disseminated metastatic skin lesions.

Nineteen strains were isolated from skin biopsies of the lower limbs and upper limbs, and from fingers, bone fragments, blood, the nasal cavity and ascites fluid; all of them from immunosuppressed patients. Twenty-one isolates were identified as FSSC [*Fusarium keratoplasticum* (*n*=7), *Fusarium falciforme* (*n*=3), *Fusarium petroliphilum* (*n*=3), *F. solani* (*n*=2), *Fusarium striatum* (*n*=1), *Fusarium riograndense* (*n*=1), *Neocosmospora gamsii* (*n*=1) and *Neocosmospora* sp. (*n*=1)] and FOSC [*Fusarium* sp. (*n*=3); *F. nirenbergiae* (*n*=1) and *F. triseptatum* (*n*=1)] (Table 1).

The most common underlying cause of immunodeficiency was haematological malignancy (63%), including acute myeloid leukaemia (AML, *n*=7) and acute lymphoid leukaemia (ALL, *n*=7). The incidence of invasive fusariosis was 14.8 cases per 1000 patients with ALL and 13.1 cases per 1000 patients with AML.

All patients received a daily 200 mg dose of fluconazole for prophylaxis against invasive fungal infections. Most patients were treated with voriconazole and amphotericin B, with an overall mortality rate of 40 %. The duration of treatment ranged from 5 to 120 days (mean, 22.6 days). Specific therapy was not recorded for three patients.

Phylogenetic analysis based on TEF1α sequences was conducted in order to position the isolates in the FSSC (Fig. 1) and FSOC (Fig. 2). FSSC was the most frequent agent group (16 cases, 76.2 %), followed by FOSC (5 cases, 23.8 %). Members of the FSSC were the most frequent aetiological agents in patients with haematological disease (14/16, 87.5 %). Our data suggest that these additional species might be of importance for human health.

The clinical strains showed resistance to amphotericin B (*n*=5/16, 31.25 %), with ECV >8 μg ml^{-1}. All strains showed high MICs for itraconazole. Voriconazole showed the best activity against *Fusarium*, followed by amphotericin B. The MICs ranged from 0.25 to 32 μg ml^{-1} for voriconazole and from 1 to 16 μg ml^{-1} for amphotericin B (Table 1). The clinical FSSC isolates were significantly (P<0.05) more resistant to amphotericin B and voriconazole than the FOSC isolates.

DISCUSSION

We analysed the relationship of *Fusarium* species with their *in vitro* resistance profile for three different antifungal agents in patients with invasive fusariosis. Determination of the pathogen at the species level and its susceptibility to the main antifungal agents is important for epidemiological studies and for the appropriate management of this difficult-to-treat infection. In our sample, FSSC was the most frequently encountered agent group, followed by FOSC. This result is similar to those reported in studies conducted in southern [22] and southeastern Brazil [23, 24], Italy [25, 26], Israel [27] and France [10], and may be related to the strong influence of European colonization in southern Brazil. Generally, the most frequent species causing fusariosis are *F. solani*, *F. oxysporum* and *Fusarium verticillioides* [28]. Based on the present results and on results from previous studies, it can be stated that, once *F. solani* is identified, clinical management should be started with a higher dose of antifungal agents because of its resistance, avoiding the use of fluconazole for prophylaxis.

The *Fusarium* species encountered and the prevalence of invasive fusariosis differ according to geographical location. The most frequent haplotypes reported in southeastern Brazil for invasive fusariosis are FSSC 2-d and FSSC 2 f, with these being described in immunosuppressed patients in the bone marrow transplant unit and in dermatology outpatients [21] However, in a previous study, FSSC2-h was the most commonly isolated species type, followed by FSSC1-a [26]. Our results were superior to those of previous studies, which showed a prevalence of invasive fusariosis of 0.1 % in patients with haematological malignancy, one of 0.24 % in haematopoietic stem cell transplant recipients and one of 0.4 % in patients with AML [25, 26]. In Europe, the incidence of *Fusarium* infections in patients with acute leukaemia is 0.06 % [29].

It is essential to evaluate the antifungal susceptibility profile of *Fusarium* species in patients with invasive fusariosis because most currently available antifungals show poor *in vitro* activity against the members of the FSSC, and fusariosis cases involving haematogenous dissemination result in high mortality [26]. With regard to azole susceptibility, FSSC isolates have shown higher MICs for voriconazole than FOSC isolates [19, 30]. Consistent with this, in the present study, FSSC isolates were more resistant to voriconazole than FOSC isolates. Also, as expected, itraconazole showed no activity against *Fusarium* isolates. In fact, only five isolates had MICs ≤64 μg ml^{-1} for itraconazole. Previous studies have shown that fluconazole and itraconazole have no activity against

Table 2. Characteristics of the 27 patients with *Fusarium* infection

Code	Sex	Race	Patient hometown	Age (years)	Pathogen source	Underlying condition	Treatment	Treatment duration (days)	Dead or alive
F5	M	C	Porto Alegre	34	Nasal	AML	AMB, VRC, liposomal AMB	74	Alive
F7	M	C	Campo Novo	45	Skin	CML	VRC (oral)	14	Dead
F10	M	C	Novo Hamburgo	12	Skin	NHL	VRC (oral)	45	alive
F13	F	C	Porto Alegre	54	Skin	AML	VRC, liposomal AMB	75	Dead
F14	F	C	Santa Cruz do Sul	15	Nasal	ALL	VRC (iv)	60	Alive
F17	M	C	Sapucaia do Sul	15	Nasal	ALL	VRC (iv and oral)	31	Alive
F18	M	C	Imbé	22	Skin	ALL	Deoxy AMB, VRC	5	Dead
F19	M	A	Porto Alegre	26	Skin	AML	Liposomal AMB, VRC	45	Alive
F22	F	C	Porto Alegre	65	Bone	AML	Liposomal AMB, VRC (iv)	10	Dead
F26	M	C	Uruguaiana	36	Nasal	AML	Liposomal AMB, VRC (iv)	35	Dead
F30	F	C	São Leopoldo	5	Nasal	ALL	AMB, VRC, POSA	52	Alive
F32	F	A	Porto Alegre	13	Nasal	ALL	Liposomal AMB	120	Alive
F27	F	C	Novo Hamburgo	31	Blood	HL	–	–	Dead
F31	M	C	Feliz	10	Skin	ALL	Liposomal AMB, VRC (iv)	14	Alive
F6	M	B	São Jerônimo	18	Nasal	Liver transplant	AMB, VRC (iv)	9	Dead
F8	F	C	Santo Antônio da Patrulha	73	Skin	Kidney transplant	AMB	30	Alive
F1	M	C	Porto Alegre	3	Skin	AML	VRC	28	Alive
F4	F	C	Putinga	2	Blood	Non-specific fever	AMB	14	Alive
F11	M	C	Viamão	71	Nasal	Peripheral vascular disease	–	–	Dead
F12	M	C	Jabotão dos Guarapés	15	Nasal	MN	MICA, VRC	60	Alive
F23	M	C	Porto Alegre	54	Peritoneum	Liver cancer and HIV	AMB	9	Dead
A1	F	C	São Lourenço do Sul	34	Blood	SEL	VRC	8	Alive
A2	F	C	Porto Alegre	10	Blood	ALL	VRC	7	Dead
A3	M	C	Novo Hamburgo	67	Blood	Pancreas cancer	–	–	Alive
A4	F	C	Nova Santa Rita	40	Skin	AML	AMB, VRC	15	Alive
A5	F	C	Guaíba	28	BAL	Non-specific fever	Deoxy AMB	12	Dead
A6	M	C	Glorinha	61	BAL	HIV	FLC	13	Alive

A, African descent; ALL, acute lymphoid leukemia; AMB, amphotericin B; AML, acute myeloid leukemia; B, brown; BAL, bronchoalveolar lavage; C, Caucasian; CML, chronic myeloid leukemia; F, female; FLC, fluconazole; HL, Hodgkin's lymphoma; M, male; MICA, micafungin; MN, malignant neoplasm; NHL, non-Hodgkin's lymphoma; POSA, posaconazole; SEL, spinal epidural lymphoma; VRC, voriconazole.

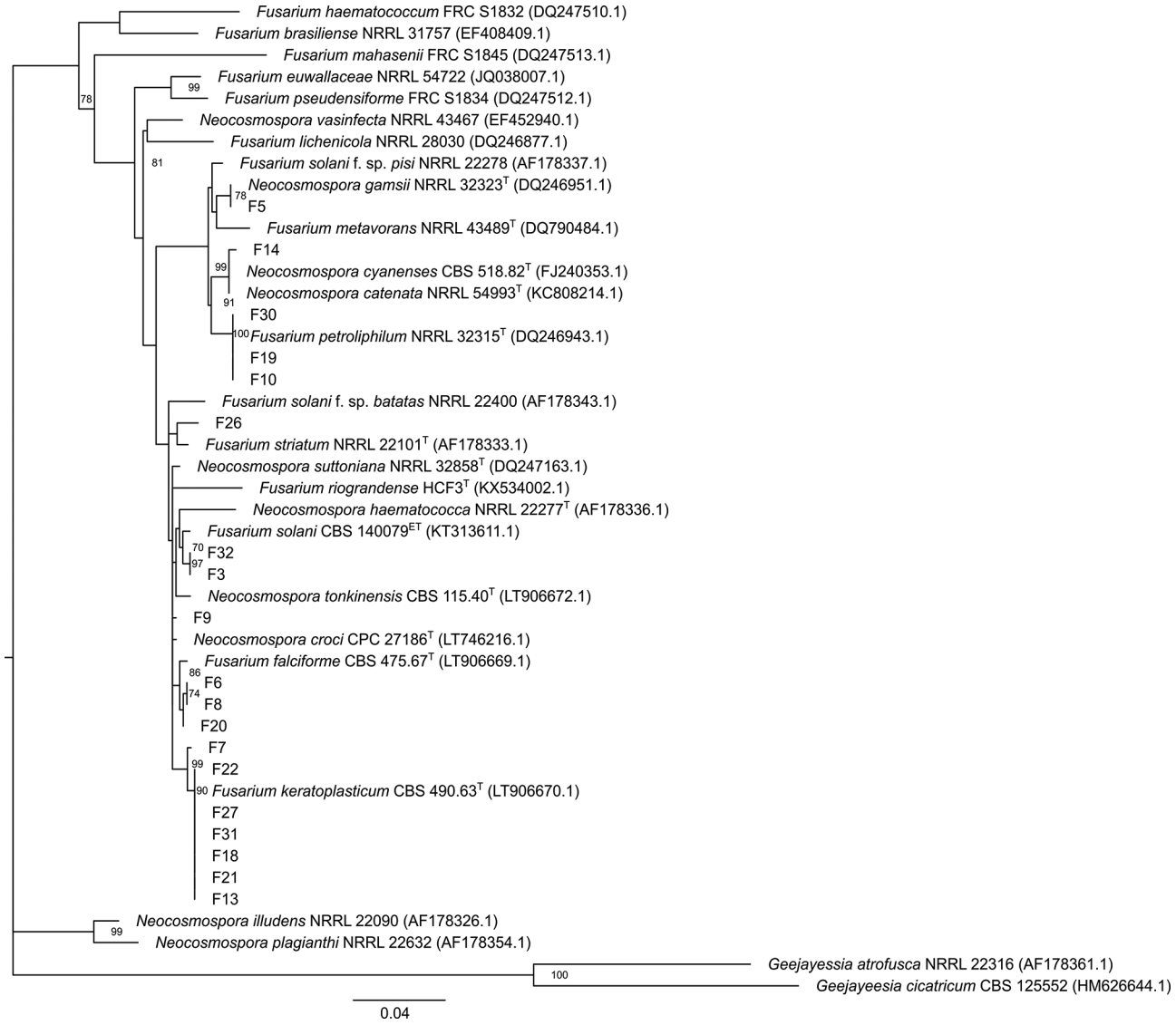

Fig. 1. Maximum-likelihood (ML) phylogenetic tree created from TEF1 sequences of 47 *Fusarium solani* complex (FSSC) sequences based on the Hasegawa–Kishino–Yano model. Total alignment length is 691 bp. The tree show values of bootstrap replications higher than 70% (*n*=1000). The tree was rooted with *Geejayeesia cicatricum* CBS125552. Evolutionary analyses were conducted in MEGA X [19].

Fusarium species [19, 26]. Consistent with the previously described observations, *F. solani* isolated from our patients showed higher amphotericin B MIC values than other *Fusarium* species.

Joint guidelines on the treatment of hyalohyphomycoses recommend amphotericin B and voriconazole for the treatment of systemic fusariosis [9]. In the present study, a similar proportion of patients received monotherapy vs combination therapy as initial treatment. In view of the highest rates of survival observed in our patients, voriconazole combined with intravenous amphotericin B and voriconazole alone were the most effective antifungal treatments for invasive fusariosis (77.8 and 66.7 %, respectively). With regard to the relationship between MIC, antifungal therapy and outcome, two of our patients infected with FSSC who died (F13 and F22) had been treated with amphotericin B and voriconazole. Isolates from these patients exhibited a broad resistance spectrum in susceptibility testing, including reduced susceptibility to voriconazole. In general, patients with a favourable outcome after antifungal treatment also showed high sensitivity to these drugs (83.3 %), with MIC values below the cutoff point suggested for their complexes.

Voriconazole was approved for the treatment of *Fusarium* infections in 2002 [10]. It has also been used prophylactically against *Fusarium* spp. [31]. Resistant profiles for amphotericin B and voriconazole, which are the currently recommended agents in the guidelines for the treatment of invasive fusariosis [32], and a late diagnosis may be associated with the high

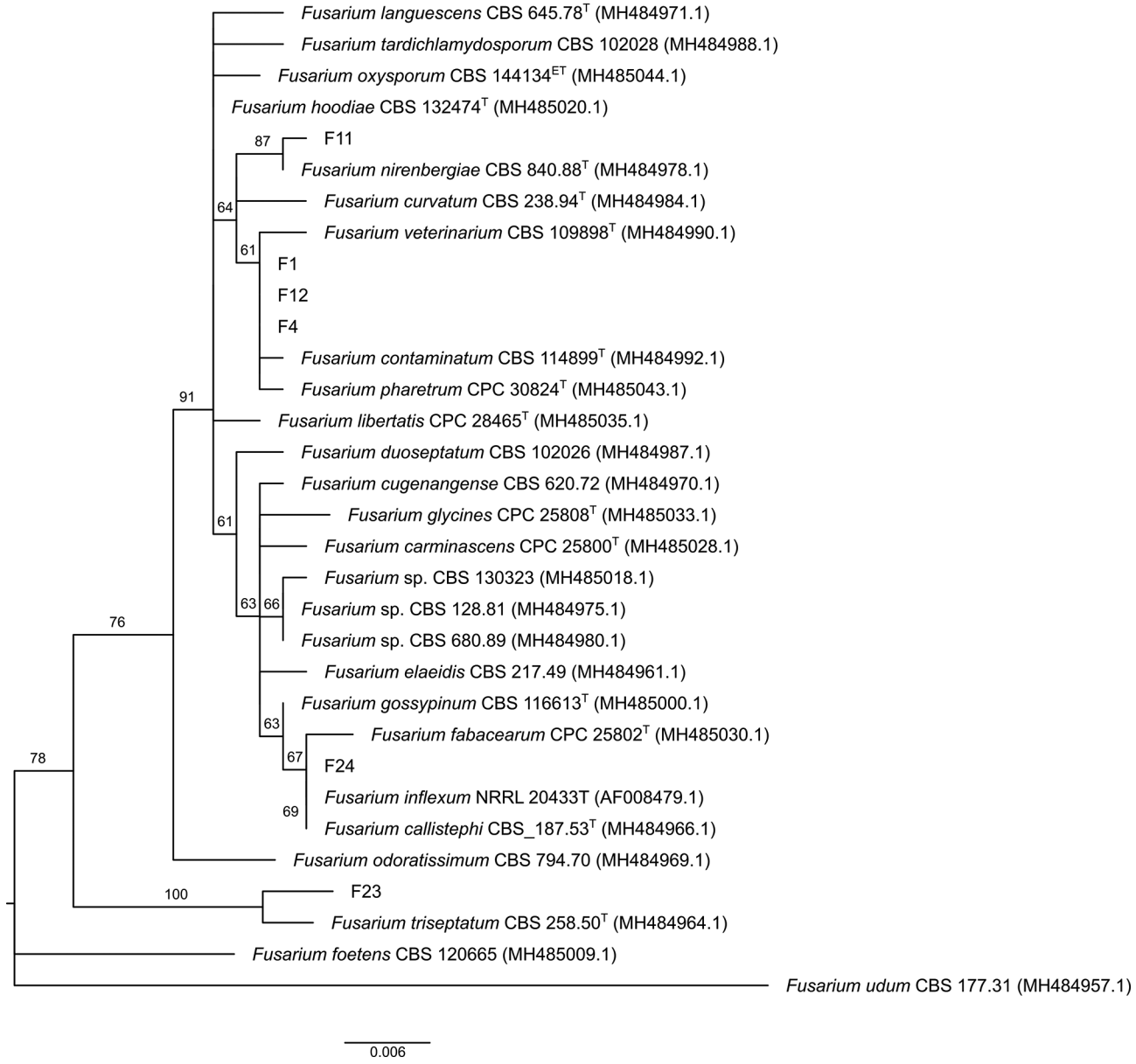

Fig. 2. Maximum-likelihood (ML) phylogenetic tree created from TEF1α sequences of 32 *Fusarium oxysporum* complex (FOSC) sequences based on the Kimura two-parameter model. Total alignment length is 616 bp. The tree show values of bootstrap replications higher than 60% (*n*=1000). The tree had *Fusarium udum* CBS 177.31 as an outgroup. Evolutionary analyses were conducted in MEGA X [20]

mortality rate observed in immunocompromised patients [33].

Consistent with reports in the literature, most patients with invasive fusariosis in the present study had haematological malignancy. An interesting feature of *Fusarium* infection in our study was the involvement of the skin and nasopharynx and a significant number of positive blood cultures, as previously described [34]. Only 7.4 % of our patients were HIV-positive, which is an uncommon event in this setting. Isolation of *Fusarium* from the respiratory tract was rare among our patients.

Given that *Fusarium* species are intrinsically resistant to most antifungal agents, new approaches are needed for this difficult-to-treat opportunistic mycosis. This study was relevant for establishing the epidemiological features of invasive fusariosis and, more importantly, identifying *Fusarium i*solates at the species level and testing them for antifungal susceptibility in the context of invasive fusariosis. The antifungal susceptibilities of isolates causing invasive fusariosis should be determined in all cases to ensure the accurate choice of drugs for appropriate patient care and successful treatment. It is important to increase our knowledge and promote the

importance of mycological examination in order to ensure appropriate treatment.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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