Original article

*Fusarium riograndense* sp. nov., a new species in the *Fusarium solani* species complex causing fungal rhinosinusitis

*Fusarium riograndense* sp. nov., une nouvelle espèce dans le complexe d’espèces de *Fusarium solani* causant une rhinosinuise fongique

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<sup>a</sup>Antifungal 
MLST 
Morphology 
Molecular phylogeny 
Original article

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**Abstract**

Invasive fusariosis has a high mortality and is predominantly observed in patients with leukemia. We report the first case of a novel species of *Fusarium*, *Fusarium riograndense* sp. nov, isolated from a lesion in the nasal cavity lesion of a patient with acute lymphoblastic leukemia. The etiological agent was identified by Multilocus Sequencing Typing (MLST), including RP2B, TEF-1α, and ITS-LSU sequences, the gold standard technique to identify new species of *Fusarium*. MLST and phenotypic data strongly supported its inclusion in the *F. solani* species complex (FSSC). The new species produced a red pigment in the Sabouraud Dextrose Agar similar to other members of the complex. The macroconidia developed from phialides on multibranched conidiophores which merge to form effuse sporodochia with a basal foot-cell instead of papilla in basal cell shape. The microconidia were ellipsoidal, 0–1-septated, produced from long monophialides. Chlamydospores were produced singly or in pairs. Amphotericin B (MIC 1 μg/ml) was the most active drug, followed by voriconazole (MIC 8 μg/ml). The patient was successfully treated with voriconazole. Our findings indicate another lineage within FSSC capable causing of invasive human infection.

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**Résumé**

La fusariose invasive a une mortalité élevée et est principalement observée chez les patients atteints de leucémie. Nous rapportons le premier cas d’une nouvelle espèce de *Fusarium*, *Fusarium riograndense* sp. nov, isolée de la lésion de la cavité nasale d’un patient atteint de leucémie lymphoblastique aiguë. L’agent étiologique a été identifié par le typage par séquençage multilocus (MLST), incluant les séquences RP2B, TEF-1α et ITS-LSU, la technique de référence pour identifier de nouvelles espèces de *Fusarium*. Le MLST et les données phénotypiques ont fortement soutenu son inclusion dans le complexe d’espèces *F. solani* (FSSC). La nouvelle espèce a produit un pigment rouge dans la gélose Sabouraud Dextrose similaire aux autres membres du complexe. Les macroconidies sont produites par des phialides portées par des conidiophores multi-brancheds rassemblés en sporodochies. Elles sont falciformes, dorsiventrales, de stature robuste, 2–5-septées (surtout 5-septées), avec une cellule apicale en pointe arrondie et une cellule...
Introduction

The genus *Fusarium* includes more than 200 species of which 73 have been isolated from human infections [1]. The majority of the identified *Fusarium* pathogens belong to the *Fusarium solani* species complex (FSSC) [2]. *Fusarium* spp. cause a broad spectrum of infections in humans. Immunologically competent hosts show mainly localized skin infections, whereas disseminated fusariosis occurs almost exclusively in immunocompromised patients. Invasive fungal rhinosinusitis is an uncommon disease with high mortality rates in patients with hematological malignancies. There is currently no consensus on the best treatment timing [3]. Fungal rhinosinusitis can be categorized into non-invasive and invasive groups. While non-invasive fungal rhinosinusitis does not exhibit the penetration of mucosa by hyphae, in invasive fungal sinusitis hyphae do invade the mucosa. Acute invasive fungal rhinosinusitis (AIFRS) is considered the most aggressive form of fungal sinusitis [4,5].

The aim of our study is to describe a new species of FSSC causing invasive rhinosinusitis in an immunocompromised patient, featuring its morphological and molecular aspects and susceptibility profile against the main antifungal agents currently used to treat this type of infection.

Case report

A 11-year-old man child presented to our facility with a 2-week history of fever, epiphora and bilateral nasal obstruction with the presence of hyaline rhinorrhea in August 2014. The patient was diagnosed with acute lymphoblastic leukemia, precursor B-cell type in 2011. The patient completed induction chemotherapy with Hyper-CVAD protocol (cyclophosphamide, vincristine, doxorubicin, and dexamethasone), maintenance treatment with methotrexate, vincristine, and prednisone lasted until September 2013. On physical examination, a black-crusted lesion was observed in right nasal fossa. Blood tests on admission revealed neutropenia with a neutrophil count of 300/mm³. Biopsy of the nasal lesion revealed on fresh microscopic examination (KOH 10%) the presence of septate hyaline hyphae. The histopathologic examination using hematoxylin and eosin (HE) (Fig. 1) and Gomori’s methenamine silver staining revealed presence of invasive hyphae of the nasal tissue. Paranasal sinus and chest Computed tomography (CT) were normal. The culture of the biopsy fragment was inoculated on Sabouraud dextrose agar (SDA; Oxoid®; UK) with chloramphenicol (50 μg/mL) (Sigma-Aldrich, USA) in 9 cm plastic Petridishes for morphological identification. SDA plates were incubated at 25 °C and 35 °C for 1 week and were examined daily. Cultures showed whitish colonies, which became dirty brown. Microscopic examination (KOH 10%) have shown robust and sickle-shaped macroconidia, ellipsoid microconidia and chlamydospores. The preliminary morphological identification of this fungus was Fusarium sp. (HCF3). Patient was started treatment with intravenous voriconazole (20 mL, 10 mg/mL), IV of 12/12 h for 10 days, and replaced by voriconazole oral tablet 200 mg in 12/12 h for 12 weeks. The patient was successfully treated under this therapeutic regimen with complete regression of the lesion.

Materials and methods

Fungal isolate

Fungal isolate (HCF3) was deposited in the Collection of Microorganisms, DNA and Cells of Minas Gerais Federal University (A Coleção de Micro-organismos, DNA e Células da Universidade Federal de Minas Gerais, UFMG, http://www2.icb.ufmg.br/cmufmg/) under accession number UFMG-CM F12570, and at the mycology collection of Pernambuco Federal University (Micoteca URM da Universidade Federal de Pernambuco, UFPE, http://www.ufpe.br/micoteca) under accession number URM-7361. Isolates were grown on potato-dextrose agar (PDA; Liofilchem, Italy) carnation leaf agar (CLA), synthetic nutrient-poor agar (SNA) and on SDA with chloramphenicol (50 μg/mL) (Sigma-Aldrich, USA) at 25 °C in the dark by 7 to 10 days’ growth [6]. The description of the morphological characteristic was according to with Aoki et al. [7]. All microcultures were incubated at 25 °C in dark environment for 10 days. Measurements of minimal and maximal sizes of conidia, chlamydospores, and phialides were performed, and characteristic morphological traits were photographed using a ZEISS PALM MicroBeam microscope, digital camera and PALMRobo 4.6 Pro software (Table 1).

DNA extraction and MLST

Genomic DNA was extracted by using Pure link™ Genomic DNA Mini kit (USA) followed by DNA quantification in NanoDrop 2000 (Thermo Scientific, Wilmington, USA). A multi-locus sequence typing was performed in order to classify HCF3 in the FSSC clade. The procedure was used according to the *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium/). The Internal Transcribed Spacer (ITS) region and Large Subunit (LSU) rDNA, a portion of the translation elongation factor 1-alpha (TEF-1α) gene, and a second largest subunit of RNA polymerase (RPB2) were amplified according to O’Donnell et al. [8]. PCR products were purified and sequenced using an ABI3730xl DNA analyzer and an ABI3500
Table 1
Macroscopic and microscopic characteristics of Fusarium riograndense isolated from a nasal lesion of a patient with acute lymphoblastic leukemia.

<table>
<thead>
<tr>
<th>Morphological characterization</th>
<th>FSSC</th>
<th>SNA</th>
<th>PDA</th>
<th>SDA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Colonial characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colony colour</td>
<td>Soft mycelium, almost transparent, smooth</td>
<td>Spacemycelium grows quite rapidly and smooth</td>
<td>Grows quite rapidly with a dense aerial mycelium</td>
<td>Slower growth</td>
</tr>
<tr>
<td>Colony color reverse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigmentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth rate (mm/day)</td>
<td>3.57 ± 1.54</td>
<td>9.36 ± 0.99</td>
<td>7.36 ± 1.75</td>
<td>67</td>
</tr>
<tr>
<td>Growth total (mm)</td>
<td>80</td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>Microscopic characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean length of macroconidia</td>
<td>41.78 ± 5.89</td>
<td>37.93 ± 5.88</td>
<td>43.55 ± 4.14</td>
<td></td>
</tr>
<tr>
<td>Mean width of macroconidia (µm)</td>
<td>4.29 ± 0.48</td>
<td>5.39 ± 0.88</td>
<td>6.01 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Number of septa in macroconidia</td>
<td>2 to 5</td>
<td>2 to 5</td>
<td>2 to 5</td>
<td></td>
</tr>
<tr>
<td>Mean length of microconidia (µm)</td>
<td>17.19 ± 3.56</td>
<td>15.47 ± 3.37</td>
<td>17.68 ± 3.29</td>
<td></td>
</tr>
<tr>
<td>Mean width of microconidia (µm)</td>
<td>4.43 ± 0.62</td>
<td>4.06 ± 0.69</td>
<td>4.40 ± 0.79</td>
<td>0 to 1</td>
</tr>
<tr>
<td>Number of septa in microconidia</td>
<td>0 to 1</td>
<td>0 to 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean values of length and width of 50 randomly picked macroconidia ± standard deviation.

* After 7 days of incubation at 25°C.

** After 10 days of incubation at 25°C.

Genetic analyzer (Applied Biosystems, USA). Raw sequences were assembled and manually edited. After, the consensus sequence was compared with reference sequences deposited in Fusarium-ID (http://isolate.fusariumdb.org/index.php), FUSARIUM MLST (http://www.cbs.knaw.nl/fusarium/) and via BLAST to sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The obtained sequences were deposited in the Genbank database under the accession numbers KT18636 (ITS), KX534001 (LSU), KX534002 (TEF-1α), and KX534003 (RPB2).

Phylogenetic analysis

Multiple sequences alignments of TEF-1α and RPB2 genes were performed and manually edited for 231 strain sequences. Phylogenetic analyses were conducted using the combined genes through Bayesian inference analysis (BI) (Beast v1.8.3 and featured Beauty v1.8.3 software). Each locus was aligned separately in MEGA version 7 using the CLUSTALW algorithm and analyzed separately before analyzing the combined two-loci. The best substitution model for all gene matrices was estimated by JmodelTest v2.1.3. Parameters were fixed in the BI using 500000 Markov chain Monte Carlo (MCMC) generations and samples were recorded every 1000 generations. The TreeAnnotator v1.8.3 software was used to select the 50% majority-rule consensus tree and we discarded the first 25% of the samples. Posterior probability values (PP) were calculated and P values > 0.95 were considered significant. The final tree was plotted and edited in Figtree v1.4.2 software. Rect fusarium robinianum CBS 830.85 was used as out-group. Aligned datasets were deposited in TreeBASE (accession number: 20164).

Antifungal susceptibility

The clinical isolates were evaluated for susceptibility to voriconazole (VRC; Sigma-Aldrich, USA), itraconazole (ITC; Sigma-Aldrich, USA), amphotericin B (AMB; Sigma-Aldrich, USA), and fluconazole (FLC; Sigma-Aldrich, USA). Minimal inhibitory concentrations (MICs) were determined according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method for filamentous fungi, M38-A2 [9]. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth as compared to the drug-free control well. The quality control (QC) isolates Candida krusei (ATCC 6258) and Candida parapsilosis (ATCC 22019), were included to monitor the general performance of the broth microdilution test.

Results

Molecular analysis

The four loci of the HCF3 isolate (ITS, LSU, TEF-1α, and RPB2) were compared using BLAST with Fusarium-ID, FUSARIUM MLST and GenBank databases, and the identity permitted identify the genus Fusarium, inside the F. solani complex (FSSC). The Genbank identity with individual sequences was low (97%). We repeated the BLAST of these four sequences using the “Fusarium MLST database” (www.cbs.knaw.nl/fusarium/BiolomICS.aspx) and showed 98.62% of identity with cured sequences of FSSC. These results suggested that HCF3 is a novel species within the F. solani species complex (Fig. 2). Multilocus DNA sequence data was used to assess the phylogenetic relationships and species limits of a comprehensive collection of clinical and environmental important isolates within the FSSC (Fig. 2). The aligned TEF-1α and RPB2 genes consisted of 724 bp, and 1838 bp characters, respectively, totalling 2562 bp of aligned DNA sequence per isolate. All sequences used for comparison were retrieved from Fusarium MLST or GenBank databases. A Multilocus Sequence Typing (MLST) analysis was used to confirm the independent BLAST comparisons and to find the genotype inside the FSSC. A phylogenetic tree was constructed using 231 Fusarium sequences from TEF-1α and RPB2 two-loci datasets and FUSARIUM MLST or GenBank databases, and contained three highly supported clusters. The combined TEF-1α and RPB2 genes consisted of 2562 bp (724 bp, and 1838 bp characters, respectively) of aligned DNA sequence per isolate. The new phylogenetic species identified in this study, named as F. riograndense (HCF3), was supported using Bayesian inference (BI) analysis, located at the base of the clade 3, closely related to four phytopathogenic strains of Fusarium: NRRL 22178, isolated from dicot tree in Venezuela; NRRL 22153 and 22098, Fusarium sp. f. sp. Cucurbitae (non-formal species names), isolated from cucumber in California, USA; and NRRL 22570, isolated from Piper nigrum in Brazil (Fig. 2). The number of nucleotide substitutions varied in a range of 23–33 for TEF1α gene, 35–66 for RPB2 gene, 10–35 for ITS region and 5–16 for LSU region, which suggests a variability greater than 1% for all the sequences analyzed in this clade (Table 2). The pairwise comparisons of nucleotides, in addition to the HCF3 with NRRL numbers, were examined for NRRL numbers to have a better view of the sequences divergences between the 5 isolates. Considering that NRRL numbers were placed in different phylogenetic species, this could be additional information to strengthen the description of a new species for HCF3. In this clade, the number
of nucleotide substitutions varied between 22 and 109 nucleotides, depending on the locus assessed for all five sequences. When we looked a pairwise comparison between HCF3 and other NRRL strains, the variability was 23-33 for TEF1a gene, 35-66 for RPB2 gene, 10-35 for ITS region and 5-16 for LSU region. These results demonstrated good variability (greater than 1%) for all the sequences analyzed in this clade and support the hypotheses that at least two strains, including HCF3, belong to different species.

### Table 2

Pairwise comparisons of nucleotide variability between HCF3 and closely related sequences in the tree (Fig. 2).

<table>
<thead>
<tr>
<th>Strains analyzed</th>
<th>TEF1a (724bp)</th>
<th>RPB2 (1838bp)</th>
<th>ITS (626bp)</th>
<th>LSU (515bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCF3-NRRL 22178</td>
<td>23</td>
<td>35</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>HCF3-NRRL 22098</td>
<td>26</td>
<td>66</td>
<td>31</td>
<td>16</td>
</tr>
<tr>
<td>HCF3-NRRL 22153</td>
<td>27</td>
<td>66</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>HCF3-NRRL 22570</td>
<td>33</td>
<td>45</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>HCF3-all NRRL</td>
<td>62</td>
<td>109</td>
<td>47</td>
<td>22</td>
</tr>
<tr>
<td>Between NRRL (excluding HCF3)</td>
<td>57</td>
<td>95</td>
<td>43</td>
<td>21</td>
</tr>
</tbody>
</table>

**Morphological analysis**

Colonies on PDA showed an average radial mycelial growth of 7.4 ± 1.75 mm per day at 25 °C in the dark, and presented a diameter of 8 cm after 10 days of incubation. Aerial mycelia was cream and mostly floccose. The mycelium grew much sparser in the SNA and CLA media (Fig. 3). Microscopically, the microconidia were oval or elliptical, usually 0-septate, and aerial conidia hyaline had
15.5 ± 3.4–17.7 ± 3.3 μm in length, and 4.1 ± 0.7–4.43 ± 0.6 μm in width (Fig. 4). Macroconidia was falciform and robust, with 2- to 5-septate. Apical cell morphology was blunt and rounded, and basal cell morphology cylindrical, usually with a notched or a rounded end. The wall of the macroconidia acquired a more globular form between one septum and another in the SNA medium. Sporodochial conidia were 37.9 ± 5.8–43.5 ± 4.1 μm (range length SNA, CLA and PDA) × 5.4 ± 0.8–6.0 ± 0.4 μm (width), branched or unbranched. Monophialides were often quite long, generally 40 μm. Chlamydospores, single or in chains, were observed daily for growth up to 10 days (Fig. 4).

This study used the GCPSR-based MLST schemes for the two bootstrapped single-locus genealogies (TEF + RPB2). In addition, bootstrapping revealed that none of the individual genealogies contradicted the monophyly of the species. GCPSR-based analyses indicated that HCF3 comprises a phylogenetically distinct species, strongly supported using Bayesian inference (BI) analysis, located at the base of the clade 3.

**Taxonomy**

*Fusarium riograndense* Rosa, Ramírez-Castrillón, Valente, Fuentefría, van Diepeningen, Goldani, sp. nov. The type strain is UFMG-CM F12570, deposited at the Microorganisms, DNA and Cells Collection of Minas Gerais Federal University. Also, was deposited at the mycology collection of Pernambuco Federal University under accession number URM-7361. The Mycobank number is MB 814515.

**Etymology**

The name of the new species is related to the geographical location of the type strain (Rio Grande do Sul state, Brazil), causing a human infection.

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*Fig. 3.* Colony surface (A and C) and reverse of the colony (B) on SDA. Colony surface (D) on SNA. Colony surface (E) and reverse of the colony (F) on PDA, all cultures were incubated at 25 °C for 1 to 2 weeks.
Antifungal susceptibility

In vitro studies demonstrated that AMB was the most active drug, followed by VRC, ITC and FLC. The values of minimum inhibitory concentrations were 1 µg/mL, 8 µg/mL, > 64 µg/mL and > 64 µg/mL respectively. Antifungal activity is similar to the species of its complex, showing low sensitivity to the first generation triazole, and good sensitivity to the second generation triazole and polyene (Figs. 3 and 4, Table 1) antifungal agents.

Discussion

Fusarium species may cause invasive sinusitis in immunocompromised host. The clinical manifestations of fusarial sinusitis are indistinguishable from those caused by Aspergillus or Mucors species. Necrosis of the mucosa is a hallmark and is a consequence of the angioinvasive nature of these mycoses [10]. Risk factors for severe fusariosis include prolonged neutropenia and T-cell immunodeficiency. The principal portal of entry for Fusarium spp. is the airways, followed by the skin at site of tissue breakdown and possibly the mucosal membranes.

The present study identified and describes F. riograndense sp. nov, a novel species pathogenic to humans in a patient with leukemia. We combined morphological and molecular characters to describe the new species, including MSLT with RPB2, TEF-1α and ITS-LSU regions. All queries showed similarity below 98%, indicating the queries sequences are from a novel species within the F. solani species complex. TEF-1α and RPB2 genes were used for phylogenetic reconstruction because they are the most informative genes for Fusarium [11]. Furthermore, several studies have shown that TEF-1α and RPB2 have the highest discriminatory power in delineating FSSC using comparative sequence analyses [12–14]. These techniques were used to delimit species and to estimate the genetic and phylogenetic relatedness of the isolate, avoiding the ITS or LSU regions [11,15].

Traditionally, plant pathogens within FSSC were named after their host plant as Forma specialis (f.sp.) on that host. These forma especiales in FSSC are assumed to correspond to biologically and phylogenetically distinct species [16]. Some species of FSSC are known to be weak plant pathogens that cause fruit rot of cucurbits, and are equivalent to the group known historically as “Fusarium sp. f. sp. cucurbitae race 2”, along with the species “Fusarium sp. f. sp. cucurbitae Race 1”, which is an unrelated species that infects roots, stems and fruits [17]. Interestingly, plant pathogenicity experiments have established that FSSC isolates from human infections are often pathogenic to cucurbits [18]. Therefore, considering the conservative criteria of genealogical concordance for phylogenetic species recognition and its importance as a human pathogen, we formally describe a new Fusarium species, and designated as Fusarium riograndense. This new species belongs to clade 3 of the FSSC, phylogenetic analyses provide clear evidence for an ingroup relationship of F. riograndense (HCF3) with FSSC 10, a and b (= NRRL 22153 and 22098), which were previously described as pathogenic fungi to humans and plants [19]. The phylogenetic tree was constructed using 231 Fusarium sequences and two-loci datasets, TEF-1α and RPB2, and formed three highly supported clusters, designated 1, 2, and 3. The clade 3 comprises several clinically important species, such as F. falciforme, F. petroliphilum, F. keratoplasticum, and F. lichenicola, as reported in other studies [12,19,20]. Zhang et al. found that all the FSSC isolates from humans were members of a previously defined major clade within the complex (“clade 3”) [12]. Only two species within clade 3 have been previously shown to be pathogenic to both humans and plants, and these include FSSC 1 (informally known the Fusarium sp. f. sp Cucurbitae race 2) and FSSC 8 (Fusarium neocosmospori- lum = Neocosmospora vasinfecta) [19].

After the 2011 meeting of the Nomenclature Session of the Botanical Congress in Melbourne, it was decided that there should be one name for each fungus, with the banishment of the dual nomenclatural system for anamorph and teleomorph fungi [21]. We decided to follow the arguments by Geiser et al. to maintain Fusarium as the sole name for the genus. Therefore, the new species is described as F. riograndense, and it belongs to the FSSC [22].

The shared characteristics of the new species with those of the FSSC consist of the development of conidia on long monophialides of the aerial mycelium. This microscopic characteristic is routinely used in order to identify fungi isolated from clinical samples, but the overall shortage of diagnostic morphology characters complicates the separation of similar species and the description of new species in the FSSC based on morphology alone [23].

Considering all these aspects, description of a novel species in complex F. solani including F. riograndense relies on a combination of characteristics including, phenotypic and molecular characteristics (Table 1). F. riograndense produced conidia and conidiophores with shapes and dimensions typical for the classic morphological concept of F. solani [24]. Other members of the F. solani family were shown to have several characteristics in...
common, such as the growth rate of the colony at 24 h and after 72 h of cultivation in PDA, as well as the macroscopic characteristics in SDA [25]. *F. riograndense* and other *Fusarium* spp. synthesizes a red pigment, which was previously described as bikaverin in *F. fujikuroi*, aurofusarin in *F. graminearum*, and in other FSSC members [24].

Invasive *Fusarium* infections are extremely difficult to treat, with high mortality rates. Amphotericin B and voriconazole are the drugs of choice recommended for treatment of deep and disseminated *Fusarium infections* [26–28]. In general, *Fusarium* spp. shows high in vitro resistance to available antifungal drugs, not only azoles, but also echinocandins and polyenes. In our study, we have observed high MICs for itracanazole and floconazole. Amphotericin B followed by voriconazole were the most active antifungal agent against our new species of *Fusarium*, consistent with previous studies for most of the *Fusarium* species. However, previous studies have shown that species- and strain-specific differences in antifungal susceptibility exist within *Fusarium*. Susceptibility testing is important in this setting and may improve the prognosis of these infections [29].

In summary, we described phenotypic and molecular aspects, and susceptibility profile of a new pathogenic *Fusarium* species isolated from a clinical sample of an immunocompromised host. Further assessment of the ecologic, epidemiologic, and clinical features of infections caused by this new species is required to facilitate its distinction from other known infections caused by different *Fusarium* species.

**Disclosure of interest**

The authors declare that they have no competing interest.

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