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Identification of *Aspergillus tubingensis* in a primary skin infection

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ABSTRACT

Objective. – *Aspergillus* section *Nigri* comprises a group of related species that include *Aspergillus niger*, *A. welwitschiae*, *A. carbonarius*, *A. brasiliensis* and *A. tubingensis*. Some of these species are morphologically very similar to *A. niger* but exhibit different patterns of susceptibility to antifungal agents; such is the case for *A. tubingensis*. Therefore, when diagnosing aspergillosis, it is important to identify the pathogen at the species level. This study aimed to identify the species of an *Aspergillus* spp. isolate (MM-82) obtained from a patient with a dermatosis localized to the right leg.
Materials and methods. – The MM-82 isolate was examined for macro- and microscopic morphology, conidia size and thermotolerance, and a phylogenetic analysis of a *benA* gene segment was performed for molecular identification. Susceptibility to antifungals was determined using antifungal microdilution according to the methodology of European Society of Clinical Microbiology and Infectious Diseases (EUCAST).
Results. – Based on its phenotypic characteristics and the phylogenetic analysis of the sequence of a *benA* gene segment, the MM-82 isolate was identified as *A. tubingensis*. This fungus did not show resistance to antifungal agents commonly used for treatment.
Conclusion. – This study demonstrated that *A. tubingensis* can cause skin infection; this constitutes the first report of a case of aspergillosis caused by *A. tubingensis* in Mexico.

1. Introduction

Invasive aspergillosis (IA) is caused by *Aspergillus fumigatus* in most cases [1]; however, the emergence of IA caused by non-*fumigatus* species, such as *A. niger*, has been reported since 2000 [2,3]. *A. niger* has been considered to have low virulence compared to other species of this genus, despite its frequent isolation [3]. Although the taxonomy of *Aspergillus* section *Nigri* is not well defined, it has been shown that in addition to *A. niger* (the main species of this section that causes aspergillosis), other species within section *Nigri* are capable of causing infections in humans, such as *A. tubingensis* [4–7]. Specifically, a study involving clinical isolates identified as *Aspergillus* section *Nigri* revealed the presence of other species of this section, mainly *A. awamori* (55.6%) and *A. tubingensis* (17.8%) [8]. In another study, molecular typing was used to identify isolates obtained from 19 patients with IA as *Aspergillus* section *Nigri*; the species identified were *A. niger* (68.4%) and *A. tubingensis* (31.6%) [9]. In addition, a retrospective study by Vermeulen et al. [10] focused on the incidence of species of section *Nigri* in 16 patients with invasive disease in a Belgian university hospital from 2005 to 2011. Through molecular typing, five of the six isolates were identified as *A. tubingensis*, and these isolates also exhibited high minimum inhibitory concentration (MIC) values for triazoles. It was concluded that in addition to *A. niger*, *A. tubingensis* is an etiologic agent causing otomycosis [4,11]. It is important to consider the difficulty of classifying the species included in section *Nigri*; in clinical laboratories, they are identified at the section level only through phenotypic methods [12]. Currently, other methodologies have been used at in-hospital laboratories, such as the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) for the identification of *Aspergillus*

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species [13–15]; however, in some cases it has had problems when trying to phylogenetically identify related species [16,17]. Likewise, molecular methods based on amplification of gene segments are successfully used to identify species within section *Nigri*; in particular, the *benA* gene, which encodes β-tubulin, can differentiate amongst all of the species in this section [12,18,19].

The aim of this study was to characterize an isolate of *Aspergillus* spp. (MM-82) obtained from a patient with an abscess located on the right leg. The characterization was based on phenotypic characteristics of the isolate (macro- and micromorphology, size of conidia, thermotolerance and susceptibility to antifungals) and the sequence of a *benA* gene fragment.

2. Material and Methods

2.1. Fungal culture

The MM-82 *Aspergillus* spp. isolate was kindly provided by Dr Carlos Atoche of the Dr Fernando Latapí Dermatological Center in Yucatan, Mexico. This fungus was isolated from a 56-year-old female patient from the state of Yucatan, Mexico. She presented type 2 diabetes mellitus and periodic loss of metabolic control with blood sugar levels from 90 to 180 mg/dL. She used to take antidiabetic drugs as sulfonylureas (glibenclamide 5 mg) and biguanides (metformin 500 mg) irregularly. The patient presented with chronic asymptomatic dermatological disease located on the right leg, which consisted of a fluctuating abscess approximately 10 cm in diameter with slight pigmentation and a purulent exudate (Fig. 1a). After fungal isolation the patient did not come back to the skin disease control dermatological centre.

2.2. Macro- and micromorphology, size of conidia, thermotolerance and antifungal susceptibility

MM-82 monosporic isolate was obtained and seeded onto Sabouraud (Bioxon®, Mexico city, MX) agar and was incubated for 7 days at 37 °C to identify its macro- and micromorphology, size of conidia and thermotolerance, as described by Frías-De-León et al. [20]. Susceptibility to amphotericin B (AMB), terbinafine (TER), itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ), caspofungin (CAS) and anidulafungin (AN) was tested following the methodology reported by European Society of Clinical Microbiology and Infectious Diseases (EUCAST) in version 9.2 of its document published in August 2014 [21].

2.3. Molecular identification

DNA extraction was performed as described by Refojo et al. [22]. To identify *Aspergillus* spp., amplification of a partial DNA sequence of the *benA* gene was performed according to Glass and Donaldson [23]. The PCR product from the MM-82 isolate was sequenced in both directions at the High-Throughput Genomics Center of Washington University (http://www.htseq.org/). The sequence was edited and deposited in the GenBank database (accession number KU674384). Maximum likelihood and maximum parsimony analyses [24] were performed to compare the sequence of the MM-82 isolate to the sequences of the following pathogenic *Aspergillus* section *Nigri* species deposited in GenBank: *Aspergillus aculeatus* EF661107, *A. uvarum* KM276889, *A. heteromorphus* AY585529, *A. sclerotioniger* AY819996, *A. carbonarius* KF434634, *A. brasiliensis* EF661094, *A. tubingensis* KJ136082, *A. vadensis* AY585531, *A. costaricaensis* AY820014, *A. awamori* KY416565, *A. lacticoffeatus* AY819998, *A. foetidus* FJ629280, *A. ellipticus* AY585530, *A. ibericus* AM419748, *A. niger* AY585536, and *A. piperis* AY820013. The sequence of *Penicillium flavigenum* (AY495995) was used as the outgroup. The maximum likelihood analysis was performed with the MEGA 6 program [25] using the GTR substitution model with gamma distribution. The maximum parsimony analysis was performed with the TNT program [http://www.zmuc.dk/public/phylogeny/TNT/] through a heuristic TBR search. A bootstrap analysis with 1000 replicates was used to estimate the robustness of the tree branches [26].

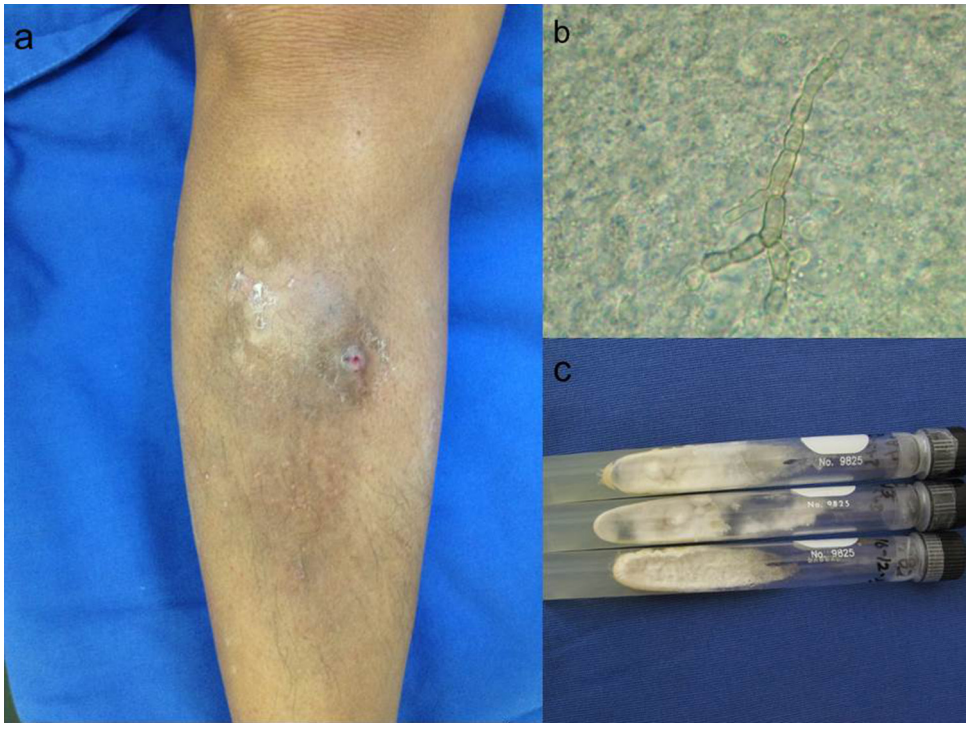

Fig. 1. a: localized skin abscess; b: direct examination with pigmented filaments (KOH 40x); c: Sabouraud agar cultures.

3. Results

3.1. Macro- and micromorphology, size of conidia, thermotolerance and antifungal susceptibility

The macromorphology of the MM-82 isolate was shown to be compatible with that described for the *Nigri* section [12]: the colony formed was black with a halo of white mycelium and a "ground pepper" appearance (Fig. 1b). The micromorphologic analysis revealed aspergillus heads with vesicles completely surrounded by phialides, black conidia and hyaline hyphae (Fig. 1c). The vesicles had an average diameter of 21.8 μm, and the microconidia had an average diameter of 3.2 μm, which correspond to measurements reported in the literature for the *Nigri* section. The MM-82 isolate was not thermotolerant; it grew only at 28 °C. The MM-82 isolate had the following MIC values: 0.25 mg/L for VCZ, 0.12 mg/L for PCZ, 0.5 mg/L for ITZ, 0.6 mg/L for TER, 0.015 mg/L for AN, 0.25 mg/L for AMB and 0.15 mg/L for CAS. These data demonstrated that this isolate did not show resistance to these antifungals.

3.2. Molecular identification

The tree constructed using the maximum likelihood method showed that the MM-82 isolate was associated with the reference sequence *A. tubingensis* (SousseC33) with a bootstrap value of 97% (Fig. 2a). The maximum parsimony analysis resulted in 1077 trees. The chosen consensus tree showed a consistency index of 0.648 and a retention index of 0.687, and it also revealed that the MM-82 isolate was associated with the reference sequence

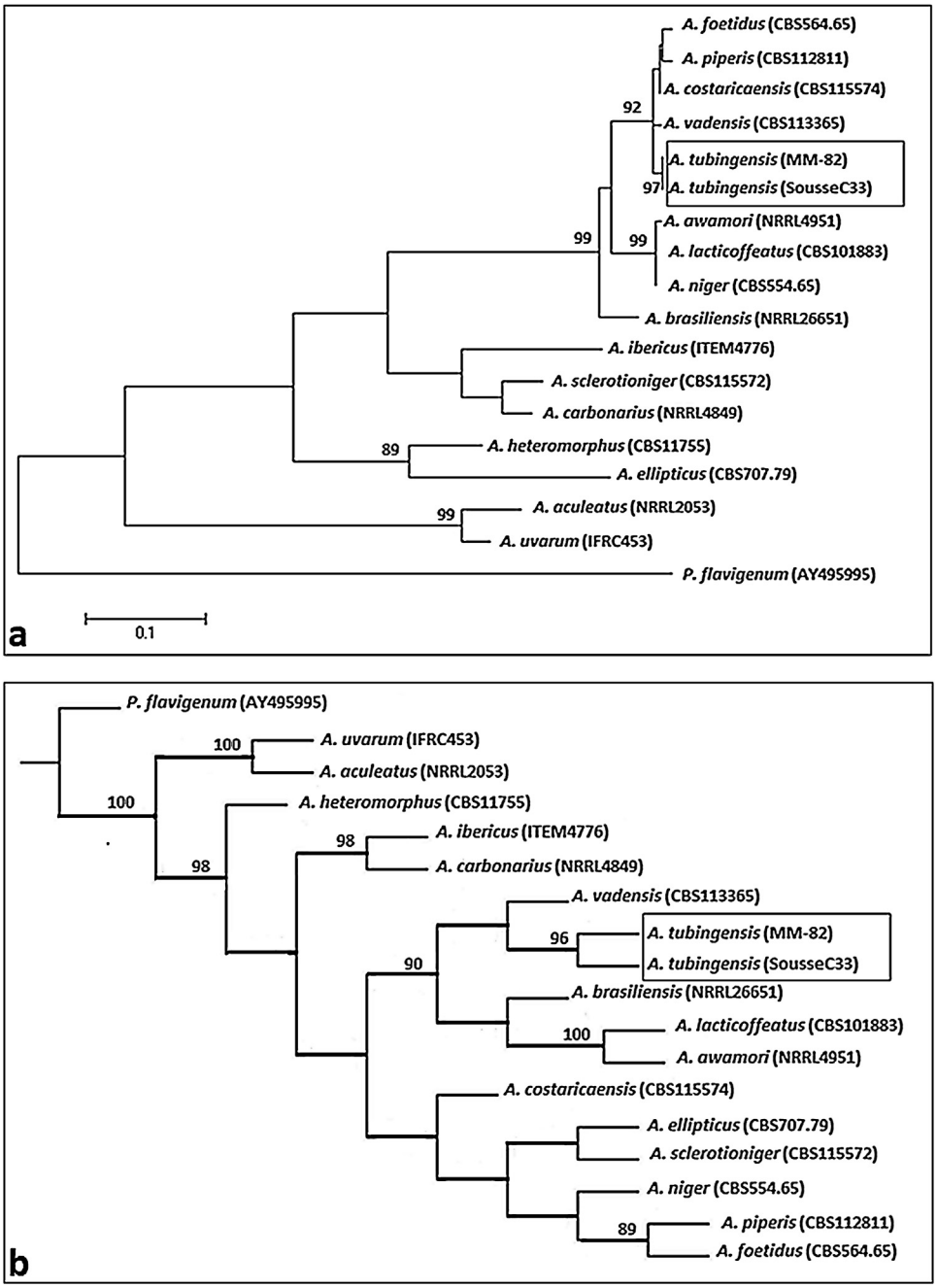

Fig. 2. Dendrograms constructed using the methods of maximum likelihood and maximum parsimony, based on the sequences of the *benA* gene fragment from the MM-32 isolate and representative sequences of *Aspergillus* section *Nigri* obtained from the GenBank database. The numbers in the branches represent bootstrap values.

A. tubingensis (SousseC33), with a bootstrap value of 96% (Fig. 2b). Thus, the maximum likelihood and maximum parsimony methods produced concordant results regarding phylogenetic trees constructed from the sequence of a *benA* gene fragment from the MM-82 isolate and other species of *Aspergillus* section *Nigri*; these phylogenetic trees showed the same topology.

4. Discussion

The accurate identification of the causative agent of aspergillosis is critical for diagnosis early in disease progression. Currently, most clinical laboratories routinely diagnose aspergillosis based on traditional methods, such as culture and microscopic identification, however, this methods has certain limitations. Therefore, molecular techniques have been an important tool for the identification of species within the *Aspergillus* genus. This is the case for section *Nigri* because in this section, *A. brasiliensis*, *A. acidus*, *A. awamori*, *A. niger*, and *A. tubingensis* have very similar morphological characteristics. It has also been shown that *A. niger* and *A. tubingensis* are often isolated from both the environment and clinical specimens, and they are involved in different pathologies, such as keratitis and IA [9,27–29]. On the other hand, Hendrickx et al. [6] conducted a genetic re-identification of isolates originally classified as *Aspergillus* section *Nigri* and found eight different species, amongst which the most common were *A. niger* and *A. tubingensis*. In addition, a study involving 43 clinical and environmental *Aspergillus* section *Nigri* isolates from China identified 20 isolates as *A. niger* and 23 as *A. tubingensis*. Within the environmental isolates, five were identified as *A. niger* and 11 as *A. tubingensis*. Likewise, within the clinical isolates (ear and respiratory tract), 15 isolates were identified as *A. niger* and 12 as *A. tubingensis*. These results suggest that *A. niger* and *A. tubingensis* are the main species present in clinical and environmental samples [30].

In addition to the previously described pathologies caused by *A. tubingensis*, this paper describes the discovery of this fungus as a causative agent of dermatosis in a patient with type 2 diabetes mellitus in Mexico, representing the first report of a case of aspergillosis caused by *A. tubingensis*. The phenotypic identification of this fungus allowed it to be classified at the section level only, as a member of section *Nigri*, which confirms that morphological structures are insufficient for the precise identification of *Aspergillus* species and that the use of molecular tools is essential to distinguish closely related species [31]. The topologies of the trees obtained using maximum likelihood and maximum parsimony analyses were shown to be correlated and demonstrated that the MM-82 isolate was associated with the sequence from *A. tubingensis* with bootstrap values of 97% based on maximum likelihood and 96% based on maximum parsimony. These findings suggest that phylogenetic reconstruction is ideal for the proper identification of species of section *Nigri* because sequence analyses using the maximum likelihood and maximum parsimony methods are based on optimization criteria, i.e., they focus on searching for the tree with optimal topology. Furthermore, a tree constructed using maximum parsimony indicates the alignment site that contributes to the length of each branch at each mutational step and identifies the topology with the shortest total length for a given tree, i.e., the topology that requires the smallest number of evolutionary changes (changes in character states) to explain the observed differences between OTUs. On the other hand, one of the main advantages of using the maximum likelihood method in phylogenetic research is its statistical power and the use of a surrogate model that specifies how the evolution of characters can occur between states and describes the rates relative to different types of evolutionary change [32]. Moreover, the misidentification of *A. niger* and *A. tubingensis* in clinical cases could have important implications regarding treatment due to differences in susceptibility to the antifungals VCZ and ITZ [5]. *A. tubingensis* isolates have higher VCZ and ITZ resistance, indicating that a higher dose is required for the effective treatment of aspergillosis caused by this pathogen compared with *A. niger*. For this reason, antifungal susceptibility testing was conducted on the isolate obtained from the patient in the present study, and no antifungal resistance was observed, not even against VCZ or ITZ. However, it is important to note that isolates of *A. tubingensis* have exhibited resistance in other studies [5,30]. Therefore, the lack of resistance of the MM-82 isolate to the antifungals tested in this work may be because the patient was never administered any antifungals.

This work is the first report of a case of aspergillosis caused by *A. tubingensis* in Mexico. Moreover, it has been shown that this species can also cause localized primary cutaneous infection with abscess in diabetic patients. In addition, this fungus did not show resistance to antifungals commonly used in the treatment of aspergillosis.

Disclosure of interest

The authors declare that they have no conflicts of interest.

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