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Development and optimization of a new MALDI-TOF protocol for identification of the *Sporothrix* species complex

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Abstract

Accurate species identification of the *Sporothrix schenckii* complex is essential, since identification based only on phenotypic characteristics is often inconclusive due to phenotypic variability within the species. We used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for species identification of 70 environmental and clinical isolates of the *Sporothrix* complex. A reference database was established for MALDI-TOF MS-based species identification according to minor adjustments in the manufacturer's guidelines. The MALDI-TOF MS clearly distinguished strains of *Sporothrix brasiliensis, Sporothrix globosa, Sporothrix mexicana, S. schenckii, Sporothrix luriei* and *Sporothrix pallida*, enabling identification of all isolates at the species level, as confirmed by partial calmodulin gene sequence analyses. The present methodology is simple, reliable, rapid and highly suitable for routine identification in clinical mycology laboratories and culture collections, particularly for updating and reclassifying of deposited *Sporothrix* isolates.

Keywords: *Sporothrix* complex; Sporotrichosis; MALDI-TOF MS; Calmodulin gene

1. Introduction

Sporotrichosis is a globally distributed subcutaneous mycosis, with areas of high endemicity in Latin America, South Africa, India, China and Japan [1,2]. It is usually associated with puncture injuries in farmers, florists, leisure gardeners, nursery workers, landscapers and greenhouse workers [3]. Moreover, in several Brazilian states, especially in Rio de Janeiro, a hyperendemic region of sporotrichosis, transmission of the disease has been widely occurring in the populace via scratches and bites of cats naturally infected with *Sporothrix* spp. [3].

The diagnosis of sporotrichosis is classically attained by correlation of clinical, epidemiological and laboratory data, including culture and fungal phenotypic characteristics. Although studies in the past few years have considered *Sporothrix schenckii* as a single taxon, Liu et al. [4] reported the existence of genetic variation within isolates belonging to this species. In addition, other studies using different methodologies, such as M13 PCR fingerprinting, have demonstrated that *S. schenckii* isolates have different genetic characteristics, which suggests that they do not belong to the same species [1,5].

Through the association of phenotypic traits and sequencing data, Marimon et al. [6] recognized three new

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species, *Sporothrix brasiliensis*, *Sporothrix globosa* and *Sporothrix mexicana,* and proposed an identification key for the *Sporothrix* species complex. In addition to molecular data, species identification included conidial morphology, growth rates and auxonogram analysis using raffinose and sucrose as carbon sources. *S. globosa* has been defined as having worldwide distribution [1,7]. On the other hand, *S. brasiliensis* was believed to be restricted to Brazil [6,8,9] and *S. mexicana* to environmental samples from Mexico [6]. Recently, however, *S. mexicana* was found in a human patient in Portugal [10] and in re-identification of three clinical isolates maintained in fungal collections since 1955 [9]. Taxonomic revision of *S. schenckii* var. *luriei* as a new species, *Sporothrix luriei*, was also proposed [11]. Furthermore, phylogenetic analysis based on the rDNA and β-tubulin regions from *Sporothrix albicans*, *Sporothrix pallida* and *Sporothrix nivea* revealed significant similarity, with the proposal of designating all of these species as *S. pallida* [12]. Based on the latter study, Romeo and collaborators studied the molecular phylogeny and epidemiology of *S. schenckii* complex strains isolated in Italy, and demonstrated that 26 environmental strains co-clustered with *S. pallida* and two clinical strains grouped with *S. schenckii* stricto sensu [13]. Nonetheless, a recent report showed that *S. pallida* caused keratitis in a corneal transplant recipient [14].

The correlation between molecular data and phenotypic characteristics was described as fundamental to the identification of species of the *Sporothrix* complex [8]. Therefore, in recent years, taxonomists are seeking tools for rapid and reliable identification of this complex [15,16]. Nevertheless, research on the identification of species belonging to the *Sporothrix* complex has been neglected in the literature. Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an example of an emerging tool for microorganism characterization and differentiation at the species and, in some cases, strain level [17–20].

MALDI-TOF MS methodology requires a relatively small number (10^6–10^7) of microbial cells [21]. It has been applied in the field of mycology in order to generate a spectrum that is characteristic of each fungal taxon. Its remarkable reproducibility is based on measurement of proteins in a mass range between 2000 and 20,000 Da, where important ribosomal proteins could be used as biomarkers. The entire process is reviewed and described in detail for analysis of yeasts [19,22] and filamentous fungi [20,23]. At present, several important contributions have been published on MALDI-TOF MS for fungal identification [24–31]. However, there is a paucity of studies applying this technique to thermally dimorphic fungi such as *Histoplasma capsulatum*, *Coccidioides posadasii*, *Paracoccidioides brasiliensis* or the *Sporothrix* complex. In addition, reference spectra for these dimorphic fungi are scarce in commercially available databases.

The aim of this study was to develop and optimize a new MALDI-TOF protocol for the identification of clinical and environmental isolates of the *Sporothrix* species complex based on their proteomic profiles, by direct analysis of fungal colonies. Furthermore, construction and validation of a reference database, with development of a specific database for routine identification of the *S. schenckii* complex, was achieved. Our findings emphasize that MALDI-TOF MS is a reliable, fast and accurate method for routine identification of the *Sporothrix* complex.

2. Materials and methods

2.1. Strains, phenotypic identification and growth conditions

This study was carried out with 70 *Sporothrix* isolates (Table 1) previously characterized as *S. schenckii* by classical morphology [12] and divided into two groups of strains. The first group, used to build the reference database for the MALDI-TOF MS (Axima LNR system, Kratos Analytical, Shimadzu, UK), consisted of 6 reference strains belonging to the following clinically and environmentally relevant *Sporothrix* species: *S. brasiliensis* (CBS120339/IPEC16490), *S. schenckii* (IPEC27722), *S. globosa* (IPEC27135), *S. pallida* (SPA8), *S. mexicana* (MUM11.02) and *S. luriei* (CBS937.72) (Table 1). The second group, that included 64 clinical and environmental isolates obtained from different culture collections, was used to evaluate the new *Sporothrix* complex MALDI-TOF MS database engineered in this study.

Fungi were subcultured on Sabouraud dextrose agar and mycobiotic agar (both from Difco™ BD/Sparks, MD, USA) and then presumptively identified at the species level by phenotypic characteristics (macro and micromorphology, thermotolerance and carbohydrate assimilation) as previously described [8,32]. A standardized method was established for microbial growth for MALDI-TOF MS analysis. Each *Sporothrix* isolate was subcultured on the yeast-like form using brain-heart infusion (BHI) agar or yeast extract peptone dextrose (YEPD, peptone 2%, yeast extract 1%, dextrose 2%) and on the filamentous form using potato dextrose agar (PDA). Cultures were incubated for a period ranging from 3 to 8 days at 25, 34, 35.5 or 37 °C. *Escherichia coli* strain DH5α was obtained from the Portuguese Fungal Culture Collection, Micoteca da Universidade do Minho, MUM (WDCM816 and ISO 9001:2008 Certification N°: PT-2011/CEP.3911) and it was used for in situ extraction of proteins, which were used as standard for MALDI-TOF MS external calibration. *E. coli* DH5α cells were grown on Luria–Bertani medium agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37 °C for 20 h as previously described [17,19].

2.2. Molecular analysis

As the gold standard method for species designation in the *Sporothrix* complex, genomic DNA was obtained from the fungal mycelial phase and sequencing of the partial calmodulin (CAL) gene was performed at the sequencing platform of the Fundação Oswaldo Cruz (PDTIS/FIOCRUZ), Rio de Janeiro (Brazil) as previously described [8]. Sequences from both DNA strands were generated and edited