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Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Differentiation of the Dimorphic Fungal Species *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*

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Isolates of *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*, previously characterized by molecular techniques, were identified for the first time by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). All isolates were correctly identified, with log score values of >2.0. Thus, MALDI-TOF MS is a new tool for differentiating species of the genus *Paracoccidioides*.

Two species of the genus *Paracoccidioides* are now considered the causal agents of paracoccidioidomycosis (PCM) (1), the most important systemic mycosis in nonimmunocompromised hosts in Latin America (2, 3). Epidemiological surveys in areas where the parasite is endemic suggest that >10 million people are infected by these fungi (3). *Paracoccidioides* spp. have been recovered from human clinical samples, soil, and tissues from certain armadillo species, such as *Dasypus novemcinctus* (4).

Early observations from our group (5, 6) and others (C. J. Fontes and A. P. Vicentini, unpublished data) have reported a lack of reactivity to *Paracoccidioides brasiliensis* antigens in routine serological assays by sera from PCM patients living in areas of Brazil outside the southern and southeastern regions endemic for the parasite. These observations suggest that fungi from different regions endemic for the parasite might present significant antigenic differences, and these differences have only recently been elucidated. Multilocus phylogenetic analysis showed that *P. brasiliensis*, which was previously considered the single causal agent of PCM, in fact comprises a complex of cryptic species (7, 8), one of which was separated into a very divergent phylogenetic branch and is currently being proposed as a new species, *Paracoccidioides lutzii* sp. nov. (9). This new species appears to occur in the central, southwestern, and northwestern regions of Brazil, but the ecology, boundaries, and specific clinical aspects of infections related to *P. lutzii* remain unclear (9). For example, a recent report identified a *P. lutzii*-like isolate in a PCM patient who lived only in the southern and southeastern areas endemic for the parasite, and the identification of this isolate required the utilization of molecular techniques (10). Although molecular techniques are able to accurately identify the two *Paracoccidioides* species, these methods are time-consuming and labor-intensive.

Thus, only technically accessible routine species differentiation can provide the proper tools to epidemiologists and clinicians to address the unresolved issues of PCM. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) has been successfully applied in clinical laboratories worldwide and has also provided a rapid and accurate alternative methodology for fungal identification (11–13). The aim of this

TABLE 1 *Paracoccidioides* strains and their identification by MALDI-TOF mass spectrometry according to the newly created MSP library

Strain name	Origin	Best match log score for main spectra of:	
		P. brasiliensis Pb18	*P. lutzii* Pb01
P. brasiliensis			
D03	Southeast Brazil	2.283	0.805
T15LN1	Southeast Brazil	2.502	0.949
Pb113	North Brazil	2.163	0.946
Pb339	Brazil	2.247	1.297
Pbdog	South Brazil	2.483	1.512
Pb262	Southeast Brazil	2.368	1.953
Pb927	Uruguay	2.537	1.643
Pb03	Southeast Brazil	2.287	1.685
BACR	Colombia	2.455	1.187
BAT	Southeast Brazil	2.470	1.722
CNH	Colombia	2.381	1.736
SMA	Southeast Brazil	2.382	1.487
CA	Colombia	2.114	0.718
DM	Southeast Brazil	2.353	1.637
192	Southeast Brazil	2.362	1.327
P. lutzii			
Pb8334	Central-West Brazil	1.452	2.107
1578	Central-West Brazil	1.936	2.641
Pb66	Central-West Brazil	1.556	2.590
EE1	Central-West Brazil	1.663	2.022
ED01	Central-West Brazil	1.722	2.419

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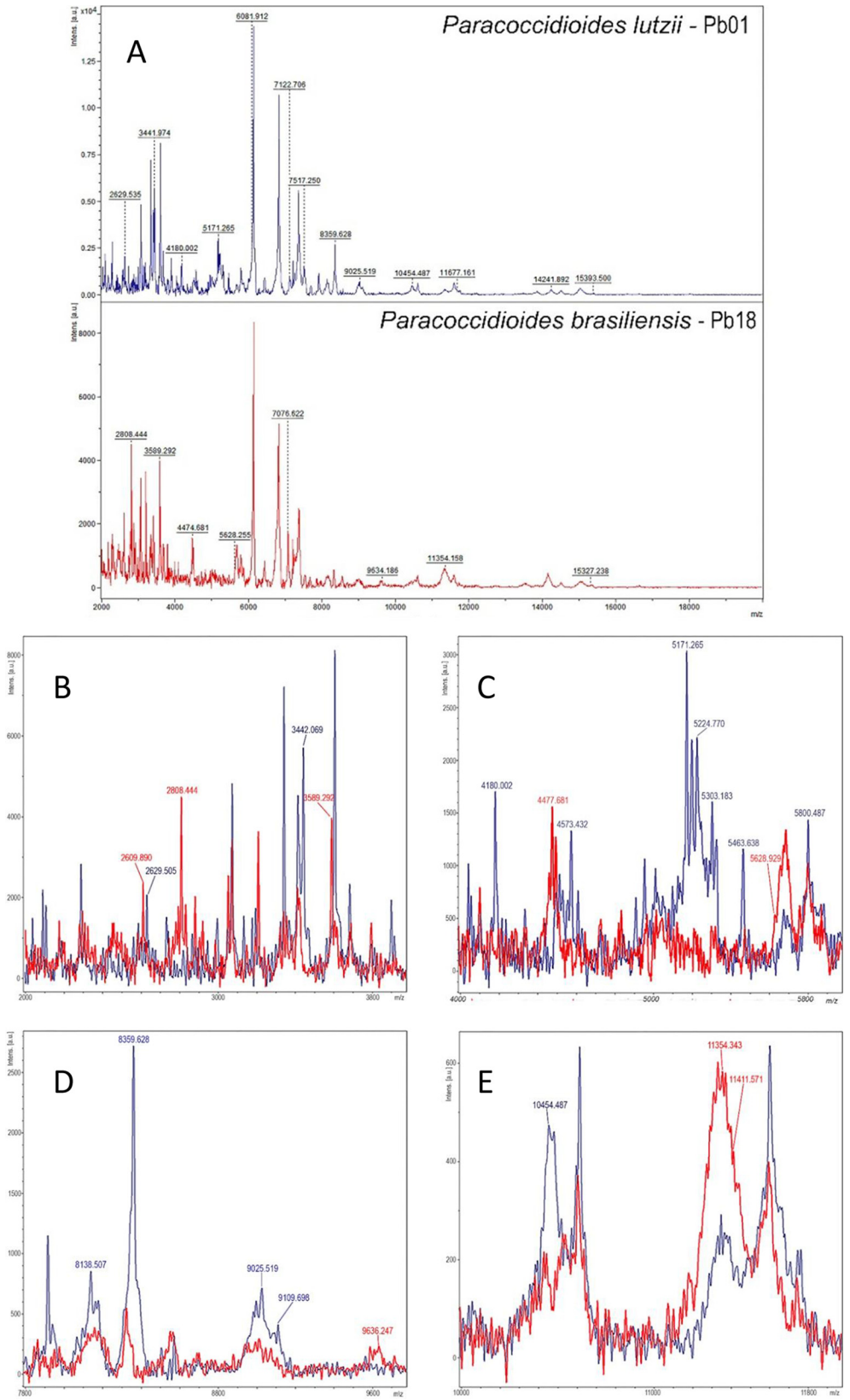

FIG 1 (A) Representative mass spectra (smoothed and baseline subtracted) from *P. lutzii* Pb01 (blue) and *P. brasiliensis* Pb18 (red) with potential species-specific ion peaks retrieved from the main spectra peak list. The absolute intensities of the ions are shown on the *y* axis, and the masses (*m/z*) of the ions are shown on the *x* axis. The *m/z* values represent the mass-to-charge ratio. Amplified images highlighting overlaying mass spectra of *P. lutzii* (blue) and *P. brasiliensis* (red) are shown at 2 to 4 kDa (B), 4 to 6 kDa (C), 8 to 10 kDa (D), and 10 to 12 kDa (E).

study was to differentiate between *P. lutzii* and *P. brasiliensis* using MALDI-TOF MS analysis.

A total of 22 strains representing the two species previously identified by molecular techniques, including multilocus sequence typing, PCR of the *hsp70* gene, and internal transcribed space (ITS) rRNA gene sequencing (7, 9, 14), were analyzed (Table 1). Due to biohazard issues, the protocol was performed with *Paracoccidioides* yeast cells, the noninfective parasitic form observed within patient lesions. The colonies were grown on Fava-Netto solid medium at 37°C (15) and subcultured once a week. A standard protein extraction protocol, with some modifications, was carried out (16). The yeast cell suspensions were heated for 30 min at 95°C in a dry bath, and absolute ethanol (Merck, Darmstadt, Germany) and glass beads were then added to the suspensions before vortexing. After centrifugation, extractions were performed using 85% formic acid and acetonitrile (Sigma, St. Louis, MO, USA), and the clear supernatants were spotted in quadruplicate onto the MALDI target. After air-drying, each sample was overlaid with 1.2 μl of cyano-4-hydroxycinnamic acid matrix solution (Sigma) and dried completely before MALDI-TOF MS measurement in an autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Calibration was performed before each experiment using a Bruker bacterial test standard (Bruker Daltonics GmbH).

More than 60 spectra were acquired for each of the two strains, *P. brasiliensis* Pb18 and *P. lutzii* Pb01, from three independent culture extracts (20 spectra per culture extract). The quality of each spectrum was assessed with the Flex analysis 3.5 software (Bruker Daltonics). Flat-liners and spectra with peak variations (outliers) were removed from the collection, and additional measurements were performed to complete the 60 spectra from each strain. The raw spectra were then loaded into Biotyper 3.1 (Bruker Daltonics), and the creation of main spectra (MSPs) was carried out with the default settings of the Biotyper software.

A total of six MSPs were created: three MSPs for Pb18, the standard type strain of *P. brasiliensis*, and three for Pb01, the standard type strain of *P. lutzii* (9). To check the specificity of the newly created MSPs, identifications were carried out against all MSPs available in the Bruker database. Finally, to evaluate the performances of the created MSPs, the remaining 15 strains of *P. brasiliensis* and five of *P. lutzii* were extracted and subjected to MALDI-TOF MS identification with the automated option in the Biotyper software. To ensure reproducibility, all tests were performed in triplicate. A selection of the specific mass spectra from the two species of *Paracoccidioides* delineates two different protein profiles (Fig. 1).

The created *Paracoccidioides* MSPs were found to be unique and suitable for MALDI-TOF MS-based identification, as no misidentifications (log score, ≥2.0) with the Biotyper database were observed. All strains had a correct species assignment, with best match log score values in the range of 2.022 to 2.641 for the *P. lutzii* strains and 2.114 to 2.537 for the *P. brasiliensis* strains. The final identifications of the strains by the new MSP library are summarized in Table 1.

Paracoccidioides is the second causal agent of a severe endemic deep mycosis to have its genus split in two different species through recent advances in phylogenetic analyses. More than 10 years ago, the causative agent of coccidioidomycosis was also split in two species: *Coccidioides immitis* and *Coccidioides posadasii* (17). It is thought that these species cause the same spectrum of clinical manifestations in humans, although there are few data regarding the features of the diseases caused by each species. Nonetheless, this division is of morphological and epidemiological relevance, because differences in phenotype and geographical distribution were found between the two species (18). In contrast, differences in virulence, response to chemotherapy, and the clinical and laboratorial characteristics of the illnesses caused by the two *Paracoccidioides* species have been suggested (9). However, these issues are still subject to debate, in part due of the lack of rapid and straightforward methods for species identification of patient isolates. Indeed, micromorphological differentiation between *P. brasiliensis* and *P. lutzii* is not reliable. Early studies of some isolates suggested differences in conidial morphology, such as size and shape (8); however, as more *P. lutzii* isolates became available, the variability in these characteristics increased and overlapped (1). Thus, differentiation based on these characteristics can lead to misinterpretations. MALDI-TOF MS for microorganism identification is a technique that does not require extensive training and is well adapted to routine laboratories. Our results demonstrate that it can be the method of choice for species differentiation of the genus *Paracoccidioides*, benefiting clinical and laboratorial studies aiming to determine possible differences between the diseases caused by these two species.

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The *Paracoccidioides* MSPs produced in this work are freely available by contacting the corresponding author.

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