
Danilo Y. Thomaz,\textsuperscript{a} Rafaela C. Grenfell,\textsuperscript{b} Monica S. M. Vidal,\textsuperscript{a} Mauro C. Giudice,\textsuperscript{a} Gilda M. B. Del Negro,\textsuperscript{a} Luiz Juliano,\textsuperscript{b} Gil Benard,\textsuperscript{a} João N. de Almeida Júnior\textsuperscript{a,c}

Laboratory of Medical Mycology, LIM-53, Hospital das Clínicas FMUSP and Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, São Paulo, Brazil; Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil; Central Laboratory Division, LIM-03, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil

We described the impact of the capsule size for Cryptococcus neoformans and Cryptococcus gattii identification at the species level by Bruker matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). After experimental capsule size modulation, we observed that reducing the capsule size resulted in improved identification by Bruker MALDI-TOF MS across all of the reference strains analyzed.

Cryptococcus neoformans and Cryptococcus gattii are relevant species among the pathogenic basidiomycetous yeasts responsible for infection in humans (1). These organisms usually produce a polysaccharide capsule that acts as an important virulence factor against the host’s defenses (2). Besides its epidemiological importance, species differentiation in this genus has major clinical relevance since patients with central nervous system (CNS) infection by C. gattii have a higher risk of neurological complications, need a more prolonged course of induction antifungal therapy, and have poorer prognoses than those with C. neoformans infections (3, 4).

Recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been shown to be a precise technology for Cryptococcus species identification (ID), replacing conventional and time-consuming phenotypic methods and providing an alternative to expensive and labor-intensive molecular techniques (5, 6). However, during routine practice in our clinical microbiology laboratory, we observed that some Cryptococcus isolates with prominent capsule sizes had low discriminatory ID when using Bruker MALDI-TOF MS analysis. These isolates required the application of old phenotypic methods, which delayed the release of the final result. This led us to investigate the impact of cryptococcal capsule size in correct species ID by Bruker MALDI-TOF MS analysis.

For this purpose, reference strains of the eight genotypes of C. neoformans and C. gattii, WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM629 (serotype D, VNIIV), WM179 (serotype B, VG1), WM178 (serotype B, VGII), WM161 (serotype B, VGIII), and WM779 (serotype C, VGIV), were subjected to capsule size modulation according to previous described methods (7, 8). Briefly, 2 ml of capsule growth-inducing medium (CGIM) (Sabouraud dextrose broth [BD, Franklin Lakes, NJ, USA] diluted 10 times with sterile water, pH 7.3) containing $2 \times 10^6$ yeast cells was incubated at 37°C with shaking. In an attempt to increase the variability in capsule size, all strains were subjected to a prolonged incubation in CGIM (up to 28 days) and were evaluated simultaneously on days 2, 3, 7, 14, 21, and 28, giving a total of 48 capsule size measurements (six replicates for each strain of the two Cryptococcus species). Next, yeast cells collected from the CGIM were submitted to a progressive capsule reduction protocol with four consecutive initial seedings in Sabouraud dextrose agar (SDA; BD) and two more seedings in the capsule-reducing medium (CRM) (SDA plus 2.9% NaCl). During this reduction assay, strains were incubated at 30°C, and each seeding had its capsule size analysis after 48 h of incubation, giving a total of 48 capsule size measurements (six replicates for each strain of the two Cryptococcus species, with four from the SDA medium and two from the CRM). For the capsule size measurements, yeast cell suspensions were stained with India ink and examined in an optical microscope equipped with an AxioCam MRc digital camera and AxioVision release 4.8 software (Zeiss, Oberkochen, Germany). Different slide fields were randomly chosen, and 40 to 50 cells were measured to determine the mean value of the capsule sizes. Finally, the same yeast cell suspensions were analyzed by Bruker MALDI-TOF MS. For protein extraction, the suspension was washed twice with sterile water and was centrifuged at 13,000 rpm for 10 min; the pellet was resuspended in sterile water and mixed thoroughly. Subsequently, chemical extraction with ethanol and formic acid was carried out according to the manufacturer’s instructions. After the extraction protocol, 1.2 μl of the supernatant was spotted on each well of the steel target plate and was air dried and overlaid with 1.2 μl of matrix solution (saturated solution of α-cyano-4-hydroxy cinnamic acid in organic solvent [50% acetonitrile and 2.5% trifluoroacetic acid]; Sigma-Aldrich, St. Louis, MO, USA). Mass spectra were generated with the microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and were compared to the main spec-
tra (MSP) of *C. neoformans* and *C. gattii* from an extended database (Biotyper v3.1 [Bruker Daltonics] plus our in-house database with the MSPs of the aforementioned *Cryptococcus* reference strains). Mass spectrometry results were expressed in log-score (LS) values between 0 and 3.000, which is considered acceptable for species ID at values of ≥2.000 and for genus ID between values of 1.700 and 1.999. The Bruker MALDI-TOF MS results were correlated to the cryptococcal capsule size, and comparisons between groups were performed using Fisher’s exact or chi-square tests for categorical variables and Mann-Whitney and Kruskal-Wallis tests for continuous nonparametric variables (SPSS 18.0; SPSS, Inc., Chicago, IL, USA). The *P* value was set to 0.05.

The mean capsule size of the 48 replicates with capsule induction was 9.42 ± 6.12 μm, with 4.91 ± 2.94 μm and 13.93 ± 5.05 μm for *C. neoformans* and *C. gattii* (*P* < 0.0001, respectively). Successful species ID was obtained in 10 (20.8%) of the 48 replicates from the SDA, whereas all of the 24 replicates from the CRM identified the correct species assignment in 14 (58.3%) of 24 replicates (17). Some authors hypothesized that the capsule prevents efficient lysis, which results in poor spectral quality, while others addressed this issue by proposing removal of the extracellular matrix (e.g., washing steps, vortexing with beads) (17–18). However, modifications in the extraction protocol (e.g., nonimmunocompromised hosts). Our data should be further validated by analysis with a higher number of isolates in the routine clinical laboratory.

In conclusion, our results illustrate the negative impact of the cryptococcal capsule for ID by Bruker MALDI-TOF MS, with the species *C. gattii* being more susceptible to this phenomenon. Re-
ducing the capsule size may improve mass spectra quality, and consequently, Cryptococcus species ID using this technology may be achieved.

ACKNOWLEDGMENTS

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo for the maintenance of the Bruker MALDI-TOF instrument and Marcia S. C. Melhem from the Instituto Adolfo Lutz of São Paulo who kindly provided us with the Cryptococcus strains.

REFERENCES


FIG 1 Mass spectra (MS) of Cryptococcus gattii VGIII after capsule size modulation. (A) Poor quality MS with a log-score value of <2 from a replicate with a mean capsule size of 17.02 μm. (B) Higher quality MS from a replicate with a mean capsule size of 1.43 μm and showing a best match LS of 2.285.
