Journal of Clinical Microbiology

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry-Based Method for Discrimination between Molecular Types of Cryptococcus neoformans and Cryptococcus gattii

Brunella Posteraro, Antonietta Vella, Massimo Cogliati, Elena De Carolis, Ada Rita Florio, Patrizia Posteraro, Maurizio Sanguinetti and Anna Maria Tortorano

J. Clin. Microbiol. **2012, 50(7):2472. DOI: 10.1128/JCM.00737-12.**
Published Ahead of Print 9 May 2012.

JCM
Journals.ASM.org

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry-Based Method for Discrimination between Molecular Types of *Cryptococcus neoformans* and *Cryptococcus gattii*

Brunella Posteraro,[a] Antonietta Vella,[b] Massimo Cogliati,[c] Elena De Carolis,[b] Ada Rita Florio,[b] Patrizia Posteraro,[d] Maurizio Sanguinetti,[b] and Anna Maria Tortorano[c]

Institute of Hygiene, Università Cattolica del Sacro Cuore, Rome, Italy[a]; Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy[b]; Department of Public Health-Microbiology-Virology, Università degli Studi di Milano, Milan, Italy[c]; and Clinical Laboratory, Ospedale San Carlo, Rome, Italy[d]

We evaluated the usefulness of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for *Cryptococcus* identification at the species and subspecies levels by using an in-house database of 25 reference cryptococcal spectra. Eighty-one out of the 82 *Cryptococcus* isolates (72 *Cryptococcus neoformans* and 10 *Cryptococcus gattii*) tested were correctly identified with respect to their molecular type designations. We showed that MALDI-TOF MS is a practicable alternative to conventional mycology or DNA-based methods.

Two pathogenic basidiomycetous yeasts, *Cryptococcus neoformans* and *Cryptococcus gattii*, are known to cause meningoencephalitis in immunocompromised and apparently immunocompetent human hosts, respectively. *Cryptococcus neoformans* and *C. gattii* are considered two separate species (20), with the former including two varieties, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (15), as well as the intravarietal serotype AD hybrids (4). By grouping >2,000 cryptococcal isolates collected globally, eight major molecular types of *C. neoformans* (VNI to VNIV) and (VGI to VGIV) have been identified by means of two main typing systems, namely, PCR fingerprinting (9, 28) and amplified fragment length polymorphism (AFLP) analysis (3). The molecular types VNI and VNII correspond to *C. neoformans* var. *grubii*, type VNIII corresponds to AD hybrids, and type VNIV corresponds to *C. neoformans* var. *neoformans*, whereas types VGI, VGII, VGIII, and VGIV correspond to *C. gattii* (3, 28). Also, multilocus sequence typing (MLST), which is becoming the method of choice for *Cryptococcus* strain genotyping (27), allowed the identification of another cluster, VNB, among a set of *C. neoformans* var. *grubii* isolates from Botswana (21). However, these techniques are generally laborious, time-consuming, and expensive.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), through the generation of characteristic fingerprints of intact microbial cells, has been successfully applied for the rapid characterization and identification of bacteria and filamentous fungi (2, 16). With regard to pathogenic yeasts, a simple and fast protein extraction step is still required to obtain reliable results (35, 36). Lower identification scores were obtained with *C. neoformans* isolates (25, 30) than with *Candida* species isolates, leading to the claim that reduction of the scores required for species level identification may improve the diagnostic usefulness of MALDI-TOF MS.

In the present study, an in-house database of MALDI-TOF MS reference *Cryptococcus* spectra was established and evaluated for the capability to provide species or subspecies level identification of a number of *C. neoformans* and *C. gattii* challenge isolates. Results were compared with those obtained using DNA-based typing methods.

A total of 107 *Cryptococcus* isolates, including 89 of *C. neoformans* and 18 of *C. gattii*, were studied. Among the clinical isolates, 56 were collected in the mycology laboratory of the Università degli Studi di Milano (Milan, Italy) from 1991 to 2001 and 34 were collected in the mycology laboratory of the Università Cattolica del Sacro Cuore (Rome, Italy) from 1990 to 2009. Additional isolates were obtained from the National Institutes of Health (Bethesda, MD) (three isolates), the National Institute of Mental Health and Neurosciences (Bangalore, Karnataka, India) (two isolates), the Westmead Millennium Institute (Sydney, Australia) (four isolates), and the Institute Pasteur (IP, Paris, France) (one isolate). Seven type or reference strains were purchased from the American Type Culture Collection (Manassas, VA). For all *C. neoformans* isolates, the mating-type allelic pattern was determined by multiplex PCR (14), whereas molecular types were identified using PCR fingerprinting with the minisatellite $(GACA)_4$-specific primer (9). All of the *C. gattii* isolates and most of the *C. neoformans* isolates have been previously characterized at the molecular level by DNA-based typing methods (9, 10, 38).

For MALDI-TOF MS analysis, protein extracts were prepared from cryptococcal isolates grown on Sabouraud dextrose agar (Kima, Padua, Italy) for 48 h at 30°C and suspended in 10% formic acid (Sigma-Aldrich, Milan, Italy). One microliter of the mixture was spotted onto a polished steel target plate (Bruker Daltonics, Bremen, Germany), air dried, and overlaid with 1 μl of absolute ethanol (Sigma-Aldrich). After air dehydration, 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid (Bruker Daltonics) was

Received 19 March 2012 Returned for modification 16 April 2012
Accepted 1 May 2012
Published ahead of print 9 May 2012
Address correspondence to Maurizio Sanguinetti, msanguinetti@rm.unicatt.it.
B.P. and A.V. contributed equally to this work.
Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.00737-12

TABLE 1 Comparison of identification results obtained by MALDI-TOF MS analysis and DNA-based methods for 82 challenge and 25 reference isolates of *C. neoformans* and *C. gattii*

Isolate	Molecular characterization			MALDI-TOF MS			
						Log(score)[a]	
	Species	Mating-type allele	Molecular type	Species	Molecular type	First match	Second match
IUM[b] 97-4877	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.559	2.401
IUM 98-3592	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.355	2.137
IUM 97-4515	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.308	2.088
IUM 98-0977	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.437	2.345
IUM 98-2450	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.489	2.116
IUM 98-4519	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.237	2.175
IUM 98-4640	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.350	2.314
IUM 99-1838	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.271	2.260
IUM 99-5678	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.468	2.172
IUM 98-5021	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.255	2.209
IUM 99-5690	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.198	2.178
IUM 99-5719	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.237	2.204
IUM 01-4726	*C. neoformans*	αA	VNII	*C. neoformans*	VNII	2.217	2.205
IUM 98-4520	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.146	2.020
IUM 94-5982	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.297	2.262
IUM 94-4725	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.232	2.214
IUM 94-3443	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.381	2.330
CR[c] 15-422	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.647	2.505
CR 16-423	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.443	2.291
CR 28	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.361	2.252
CR 37	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.130	2.115
CR 38	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.664	2.471
CR 40	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.498	2.490
CR 42	*C. neoformans*	αA	VNI	*C. neoformans*	VNI	2.509	2.208
IUM 93-3233	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.520	2.355
IUM 94-2361	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.613	2.475
IUM 93-3922	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.444	2.278
IUM 93-1656	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.332	2.208
IUM 93-2095	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.430	2.423
IUM 93-0631/2	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.063	2.033
IUM 93-0333	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.264	2.005
IUM 93-0323	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.121	2.070
IUM 92-4211	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.234	2.202
IUM 92-0891	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.273	2.221
IUM 92-0160	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.114	2.100
IUM 92-6093	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.059	1.992
IUM 96-4739	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.046	1.996
IUM 93-4941	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.173	2.125
CR 2-415	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.358	2.314
CR 3-416	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.381	2.293
CR 10-417	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.488	2.391
CR 11-418	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.577	2.448
CR 12-419	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.552	2.371
CR 26	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.329	2.327
CR 27	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.274	2.274
CR 29	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.331	2.322
CR 31	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.503	2.494
CR 32	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.534	2.422
CR 33	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.382	2.341
CR 35	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.568	2.486
IUM 93-4941	*C. neoformans*	αD	VNIV	*C. neoformans*	VNIV	2.173	2.157
IUM 91-1871	*C. neoformans*	αAαD	VNIII	*C. neoformans*	VNIII	2.155	2.117
IUM 99-3615	*C. neoformans*	αAαD	VNIII	*C. neoformans*	VNIII	2.421	2.405
IUM 94-5754	*C. neoformans*	αAαD	VNIII	*C. neoformans*	VNIII	2.231	2.196
IUM 93-1666	*C. neoformans*	αAαD	VNIII	*C. neoformans*	VNIII	2.368	2.349
IUM 92-2562	*C. neoformans*	αAαD	VNIII	*C. neoformans*	VNIII	2.271	2.033
IUM 92-4734	*C. neoformans*	αAαD	VNIII	*C. neoformans*	VNIII	2.224	2.117

(Continued on following page)

TABLE 1 (Continued)

Isolate	Molecular characterization			MALDI-TOF MS			
	Species	Mating-type allele	Molecular type	Species	Molecular type	Log(score)[a]	
						First match	Second match
CR 14-421	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.551	2.446
CR 17-424	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.448	2.328
CR 18-425	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.359	2.292
CR 19-426	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.437	2.430
CR 20-427	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.458	2.419
CR 36	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.549	2.487
CR 39	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.281	2.220
CR 25	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.457	2.286
CR 41	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.503	2.483
CR 43	*C. neoformans*	αAaD	VNIII	*C. neoformans*	VNIII	2.600	2.430
IUM 92-6198	*C. neoformans*	aAαD	VNIII	*C. neoformans*	VNIII	2.432	2.360
IUM 92-4686	*C. neoformans*	aAαD	VNIII	*C. neoformans*	VNIII	2.133	2.125
CR 22	*C. neoformans*	aAαD	VNIII	*C. neoformans*	VNIII	2.453	2.322
CR 23	*C. neoformans*	aAαD	VNIII	*C. neoformans*	VNIII	2.474	2.404
CR 24	*C. neoformans*	aAaD	VNIII	*C. neoformans*	VNIII	2.482	2.423
IUM 92-6682[d]	*C. gattii*	αB	VGII	*C. gattii*	VGI	2.120	2.078 (VGIII)
IUM 91-6492	*C. gattii*	αB	VGI	*C. gattii*	VGI	2.298	2.067
WM[e] 163	*C. gattii*	αB	VGI	*C. gattii*	VGI	2.009	1.712 (VGIV)
IUM 92-6957	*C. gattii*	αB	VGI	*C. gattii*	VGI	2.159	1.821 (VGIII)
IUM 94-6315	*C. gattii*	αB	VGI	*C. gattii*	VGI	2.040	1.943 (VGIV)
IP[f] 189	*C. gattii*	αB	VGIII	*C. gattii*	VGIII	2.199	2.199
WM 137	*C. gattii*	αC	VGIII	*C. gattii*	VGIII	2.438	2.058
NIMH[g] 155	*C. gattii*	αC	VGIV	*C. gattii*	VGIV	2.240	2.188
WM 779	*C. gattii*	αC	VGIV	*C. gattii*	VGIV	2.495	2.294
NIMH 103	*C. gattii*	αC	VGIV	*C. gattii*	VGIV	2.145	2.064

[a] Log(score) values resulting from the second match gave correct identification, with the exception of four *C. gattii* isolates, for which the corresponding molecular types are indicated in parentheses.
[b] IUM, Universita degli Studi di Milano, Milan, Italy.
[c] CR, Universita Cattolica del Sacro Cuore, Rome, Italy.
[d] The only isolate with discordant results.
[e] WM, Westmead Millennium Institute, Sydney, Australia.
[f] IP, Institute Pasteur, Paris, France.
[g] NIMH, National Institute of Mental Health and Neurosciences, Bangalore, Karnataka, India.

added and the mixture was allowed to cocrystallize at room temperature. Measurements were performed with a microflex LT mass spectrometer (Bruker Daltonics), and spectra were recorded in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 16.7 kV; lens voltage, 8.5 kV) (11). Seventeen *C. neoformans* and eight *C. gattii* isolates representative of the eight known molecular types (see Table S1 in the supplemental material) were selected to generate MALDI-TOF MS reference spectra at *m*/*z* ratios of 2,000 to 20,000. These spectral data were added to the Bruker Daltonics BioTyper 3.0 library database (containing spectra of 3,740 microorganisms), which already included the spectra of four *C. neoformans* and two *C. gattii* isolates. Each database entry was generated as a composite of 10 to 12 spectra, resulting in a main spectrum (MSP) that contains the average mass, the average intensity, and the frequencies of the most significant peaks (11).

Raw spectra from a set of challenge isolates (72 of *C. neoformans* and 10 of *C. gattii*) were used for pattern matching (with default parameter settings) against the extended BioTyper 3.0 database using the BioTyper 3.0 software (Bruker Daltonics). Results of this process were expressed with log(score) values as proposed by the manufacturer; i.e., values of ≥2.0 are rated as identification at the species level, values of ≥1.7 and <2.0 are rated as identification at least at the genus level, and values of <1.7 are rated as unsuitable for identification. Samples from two biological replicates, i.e., separate cultivations of the same strain (11), or eight technical replicates of a given sample analyzed at different times (1) yielded results that were reproducible (data not shown). Also, hierarchical cluster analysis was conducted with the integrated statistical tool Matlab 7.1 of the BioTyper 3.0 software package using default settings. Briefly, a dendrogram was generated by similarity scoring of a set of MSPs to obtain graphical distance values between the cryptococcal isolates, which were calculated by a correlation function, through the use of an average statistical algorithm as implemented in the BioTyper 3.0 software. Species and subspecies with distance levels of <500 are reliably classified.

All 82 *Cryptococcus* isolates were correctly identified at the species level, as both *C. neoformans* and *C. gattii* isolates displayed log(score) values of spectra of >2.0 (Table 1), whereas nonidentification or misidentification with the 3,734 spectra from the other microorganisms contained in the database did not occur. In agreement with the DNA-based typing results, 81 (98.8%) of the 82 isolates were unambiguously assigned to a molecular type on the basis of the MALDI spectrum matching the expected one in the reference database (Table 1). The *C. gattii* isolate (IUM 93-6682) showing discordant results was identified as VGI instead as

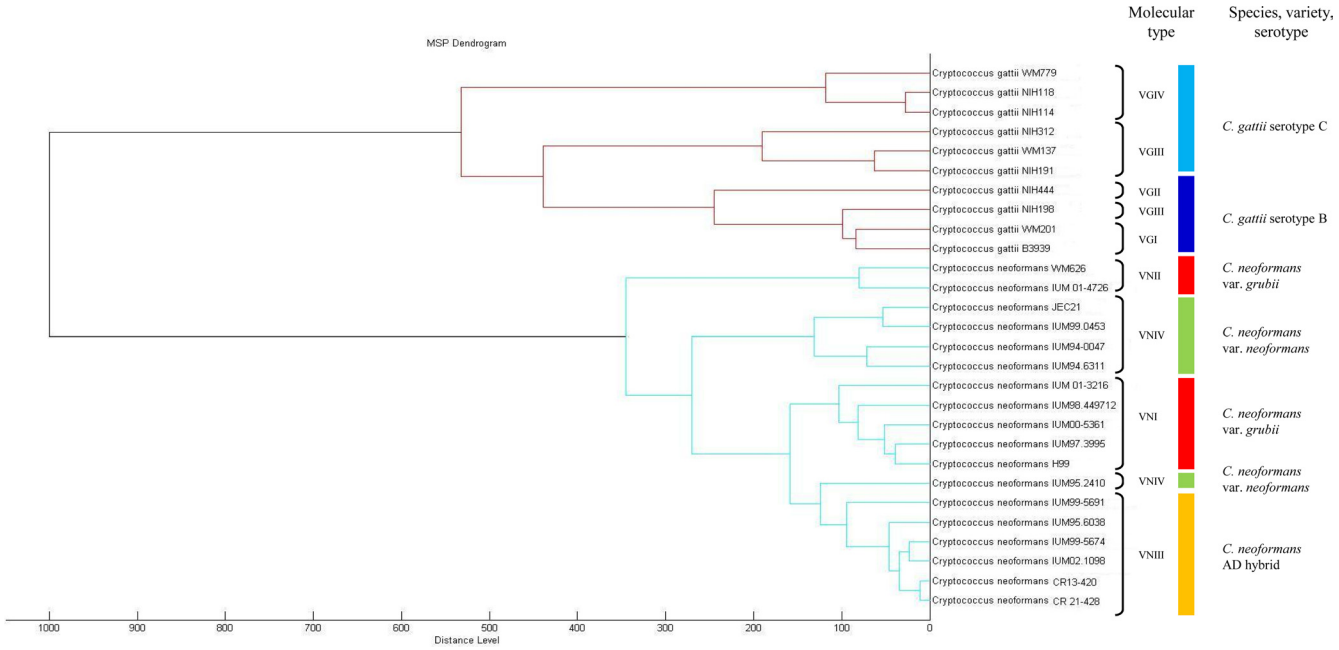

FIG 1 Cluster analysis of MALDI-TOF MS spectra of selected reference or challenge isolates of *C. neoformans* and *C. gattii*. Distance is displayed in relative units.

VGII (Table 1), suggesting that more *C. gattii* strains need to be analyzed to firmly establish the discriminatory power of the MALDI-TOF method. A cluster analysis with reference and challenge isolates based on a pairwise correlation matrix was performed for both the *C. neoformans* and *C. gattii* species, in order to assess the ability of the method to display the phylogenetic relationships of the strains. As depicted in Fig. 1, the resulting dendrogram for all cryptococcal isolates showed separate clusters corresponding to the eight molecular types of the two *Cryptococcus* species. However, the *C. neoformans* var. *neoformans* VNIV strains did not form a single cluster but did partially group (one isolate) within the *C. neoformans* var. *grubii* VNI cluster, showing that the cluster analysis mostly, but not fully, resolved *C. neoformans* var. *neoformans*. On the other hand, the *C. gattii* VGIII strains did cluster together with the other *C. gattii* strains of molecular types VGI and VGII, but they were completely separate according to their designated serotypes, perhaps implying a lack of discriminatory power of the molecular method.

Our results confirm the role of MALDI-TOF for species level differentiation of clinical fungi (11, 17, 24, 36; for a review, see reference 31). In addition, the current data mirror what has already been demonstrated in certain bacteria (13, 32, 34, 37) and *Cryptococcus* (25) and show that MALDI-TOF MS has the potential to differentiate not only between two closely related (sibling) species, *C. neoformans* and *C. gattii*, but also to discriminate *C. neoformans* at the subspecies level (i.e., to discriminate *C. neoformans* var. *neoformans* from *C. neoformans* var. *grubii* or, in this study, also from the AD hybrid). Furthermore, here we show for the first time and with a good level of reliability that MALDI-TOF can be applied for the rapid recognition of cryptococcal genotypes and, by extension, for fungal strain typing (unpublished data).

Misidentification of molecular genotypes within the *C. neoformans-C. gattii* complex has epidemiological and, more importantly, clinical repercussions (33). For example, *C. gattii* VGII was considered to be rare until it had been linked with the Vancouver Island outbreak of cryptococcosis (19), whose range has dramatically expanded into the Pacific Northwest of the United States (5). Differences exist among the molecular types of *C. neoformans* and *C. gattii* with regard to the *in vitro* susceptibility to antifungal agents, especially azoles (8, 18). Also, the emergence of highly virulent *C. gattii* strains (6, 7, 23) has positioned causative species, genotype, and geographic origin as important considerations when deciding on treatment options for cryptococcosis.

Whereas MLST or AFLP is usually employed for the molecular subtyping of *Cryptococcus* species (27), the use of a single target (i.e., the intergenic spacer) or "serotype-associated" allele (i.e., *CAP59*) through DNA sequencing or PCR amplification effectively distinguishes the *C. neoformans* varieties and *C. gattii* (12, 22). Although some inconsistency occurs with these genomic techniques, this limitation is minimal compared to those of conventional serotyping or biochemical methods. Thus, McTaggart et al. (26) developed a rapid identification algorithm that incorporates commercial biochemical tests, differential media, and DNA sequence analysis to distinguish clinically relevant *Cryptococcus* species. In contrast, the large spectrum of proteins detected by MALDI-TOF MS should enable it to discriminate between closely related species and to classify organisms at the subspecies level (29). Our study demonstrates the applicability of this approach to *Cryptococcus* by virtue of complexity at the species, variety, hybrid, serotype, and genotype levels.

In conclusion, MALDI-TOF MS has the potential to become a useful tool for the routine identification and typing of pathogenic fungi, providing the clinician with timely and reliable results.

ACKNOWLEDGMENT

This work was supported by a grant from the Università Cattolica del Sacro Cuore (Fondi Linea D1, 2011).

REFERENCES

1. **Albrethsen J.** 2007. Reproducibility in protein profiling by MALDI-TOF mass spectrometry. Clin. Chem. **53**:852–858.
2. **Bizzini A, Greub G.** 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin. Microbiol. Infect. **16**:1614–1619.
3. **Boekhout T, et al.** 2001. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology **147**:891–907.
4. **Bovers M, Hagen F, Boekhout T.** 2008. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex. Rev. Iberoam. Micol. **25**: S4–S12.
5. **Byrnes EJ, et al.** 2009. Molecular evidence that the range of the Vancouver Island outbreak of *Cryptococcus gattii* infection has expanded into the Pacific Northwest in the United States. J. Infect. Dis. **199**:1081–1086.
6. **Byrnes EJ, et al.** 2010. Emergence and pathogenicity of highly virulent *Cryptococcus gattii* genotypes in the northwest United States. PLoS Pathog. **6**:e1000850. doi:10.1371/journal.ppat.1000850.
7. **Chaturvedi V, Chaturvedi S.** 2011. *Cryptococcus gattii*: a resurgent fungal pathogen. Trends Microbiol. **19**:564–571.
8. **Chong HS, Dagg R, Malik R, Chen S, Carter D.** 2010. *In vitro* susceptibility of the yeast pathogen *Cryptococcus* to fluconazole and other azoles varies with molecular genotype. J. Clin. Microbiol. **48**:4115–4120.
9. **Cogliati M, Allaria M, Tortorano AM, Viviani MA.** 2000. Genotyping *Cryptococcus neoformans* var. *neoformans* with specific primers designed from PCR-fingerprinting bands sequenced using a modified PCR-based strategy. Med. Mycol. **38**:97–103.
10. **Cogliati M, et al.** 2012. *Cryptococcus gattii* serotype-C strains isolated in Bangalore, Karnataka, India. Mycoses **55**(3):262–268.
11. **De Carolis E, et al.** 2012. Species identification of *Aspergillus*, *Fusarium* and *Mucorales* with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Microbiol. Infect. **18**(5):475–484.
12. **Diaz MR, Boekhout T, Kiesling T, Fell JW.** 2005. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. FEMS Yeast Res. **5**:1129–1140.
13. **Dieckmann R, Helmuth R, Erhard M, Malorny B.** 2008. Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. **74**:7767–7778.
14. **Esposto MC, Cogliati M, Tortorano AM, Viviani MA.** 2004. Determination of *Cryptococcus neoformans* var. *neoformans* mating type by multiplex PCR. Clin. Microbiol. Infect. **10**:1092–1094.
15. **Franzot SP, Salkin IF, Casadevall A.** 1999. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J. Clin. Microbiol. **37**:838–840.
16. **Giebel R, et al.** 2010. Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) applications and challenges. Adv. Appl. Microbiol. **71**:149–184.
17. **Hettick JM, et al.** 2008. Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Anal. Biochem. **380**:276–281.
18. **Iqbal N, et al.** 2010. Correlation of genotype and *in vitro* susceptibilities of *Cryptococcus gattii* strains from the Pacific Northwest of the United States. J. Clin. Microbiol. **48**:539–544.
19. **Kidd SE, et al.** 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. U. S. A. **101**:17258–17263.
20. **Kwon-Chung KJ, Varma A.** 2006. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? FEMS Yeast Res. **6**:574–587.
21. **Litvintseva AP, Thakur R, Vilgalys R, Mitchell TG.** 2006. Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana. Genetics **172**:2223–2238.
22. **Lucas S, et al.** 2010. Differentiation of *Cryptococcus neoformans* varieties and *Cryptococcus gattii* using CAP59-based loop-mediated isothermal DNA amplification. Clin. Microbiol. Infect. **16**:711–714.
23. **Ma H, et al.** 2009. The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation. Proc. Natl. Acad. Sci. U. S. A. **106**:12980–12985.
24. **Marinach-Patrice C, et al.** 2009. Use of mass spectrometry to identify clinical *Fusarium* isolates. Clin. Microbiol. Infect. **15**:634–642.
25. **McTaggart LR, et al.** 2011. Rapid identification of *Cryptococcus neoformans* and *Cryptococcus gattii* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. **49**:3050–3053.
26. **McTaggart L, et al.** 2011. Rapid identification of *Cryptococcus neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* by use of rapid biochemical tests, differential media, and DNA sequencing. J. Clin. Microbiol. **49**:2522–2527.
27. **Meyer W, et al.** 2009. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med. Mycol. **47**:561–570.
28. **Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E.** 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg. Infect. Dis. **9**:189–195.
29. **Murray PR.** 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: usefulness for taxonomy and epidemiology. Clin. Microbiol. Infect. **16**:1626–1630.
30. **Pinto A, et al.** 2011. Matrix-assisted laser desorption ionization-time of flight mass spectrometry identification of yeasts is contingent on robust reference spectra. PLoS One **6**:e25712. doi:10.1371/journal.pone.0025712.
31. **Santos C, Paterson RR, Venâncio A, Lima N.** 2010. Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Appl. Microbiol. **108**:375–385.
32. **Seibold E, Maier T, Kostrzewa M, Zeman E, Splettstoesser W.** 2010. Identification of *Francisella tularensis* by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and subspecies levels. J. Clin. Microbiol. **48**:1061–1069.
33. **Sidrim JJ, et al.** 2010. Molecular methods for the diagnosis and characterization of *Cryptococcus*: a review. Can. J. Microbiol. **56**:445–458.
34. **Spanu T, et al.** 2011. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to *rpoB* gene sequencing for species identification of bloodstream infection staphylococcal isolates. Clin. Microbiol. Infect. **17**:44–49.
35. **Spanu T, et al.** 2012. Direct MALDI-TOF mass spectrometry assay of blood culture broths for rapid identification of *Candida* species causing bloodstream infections: an observational study in two large microbiology laboratories. J. Clin. Microbiol. **50**:176–179.
36. **Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR.** 2010. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically important yeast species. J. Clin. Microbiol. **48**:3482–3486.
37. **Teramoto K, et al.** 2007. Phylogenetic classification of *Pseudomonas putida* strains by MALDI-MS using ribosomal subunit proteins as biomarkers. Anal. Chem. **79**:8712–8719.
38. **Viviani MA, et al.** 2006. Molecular analysis of 311 *Cryptococcus neoformans* isolates from a 30-month ECMM survey of cryptococcosis in Europe. FEMS Yeast Res. **6**:614–619.