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Short Communication

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MALDI-TOF MS for the identification of veterinary non-*C. neoformans*–*C. gattii Cryptococcus* spp. isolates from Italy

Patrizia Danesi[1,2,*], Ilenia Drigo[1], Roberta Iatta[2], Carolina Firacative[3,4], Gioia Capelli[1], Claudia Cafarchia[2] and Wieland Meyer[3]

[1]Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (Padua), Italy, [2]Department of Veterinary Medicine, University of Bari, Italy, [3]Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Sydney Medical School—Westmead Hospital, Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney, Westmead Millennium Institute, Sydney, Australia and [4]Grupo de Microbiología, Instituto Nacional de Salud, Bogotá, Colombia

*To whom correspondence should be addressed. Patrizia Danesi, IZS delle Venezie, Viale dell'Università 10, 35020, Legnaro (PD), Italy. Tel: 0039 049 8084166/374; Fax: +39 049 8084258; E-mail: pdanesi@izsvenezie.it

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers an effective alternative to phenotypic and molecular methods for the rapid identification of microorganisms. Our aim in this study was to create an in-house library for a set of strains of nine uncommonly reported human and animal cryptococcal species, including *Cryptococcus adeliensis*, *C. albidosimilis*, *C. albidus*, *C. aureus*, *C. carnescens*, *C. laurentii*, *C. magnus*, *C. victoriae* and *C. uniguttulatus*, and to use this library to make timely and correct identifications using MALDI-TOF MS for use in routine laboratory diagnostics. Protein extracts obtained via the formic acid extraction method of 62 veterinary non–*C. neoformans*–*C. gattii* cryptococcal isolates were studied. The obtained mass spectra correctly grouped all 62 studied isolates according to species identification previously obtained by internal transcribe spacer sequence analysis. The in-house database was than exported and successfully uploaded to the Microflex LT (Maldi Biotyper; Bruker Daltonics) instrument at a different diagnostic laboratory in Italy. Scores >2.7 obtained from isolates reanalyzed in the latter laboratory supported the high reproducibility of the method. The possibility of creating and transferring an in-house library adds to the usefulness MALDI-TOF MS an important tool for the rapid and inexpensive identification of pathogenic and saprophytic fungi as required for differential diagnosis of human and animal mycoses.

Key words: MALDI-TOF, non–*C. neoformans*–*C. gattii Cryptococcus* species, veterinary isolates.

Introduction

Cryptococcosis is a yeast infection that, in most instances, is caused by *Cryptococcus neoformans* and *C. gattii* in humans and domestic and wild animals [1–3]. In recent decades, noncommon cryptococcal species, generally considered to be saprophytes such as *C. laurentii*, *C. albidus*,

C. adeliensins, C. curvatus, C. humicolus, C. luteolus, C. macerans, and *C. uniguttulatus,* have also been reported to cause infections in humans and animals [4]. However, these species are usually misidentified because they share characteristics with *C. neoformans* and *C. gattii*. As a result, molecular identification methods, in addition to conventional techniques, are required for proper identification; however, these methods can be laborious, costly, and time consuming [5–10].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an important technique for the rapid identification of fungal [11] and bacterial pathogens [12] as well as *Prototheca* [13]. This methodology is used to rapidly determine particular mass spectral patterns of peptides and proteins that are then compared with those included in a reference spectral library, allowing for species identification [11,14]. Currently one limiting factor for yeast identification using this technology is that commercial reference libraries contain a limited number of spectra for non-*Candida* species, resulting in poor performance for such genera as *Aureobasidium*, *Cryptococcus*, *Geotrichum*, and *Pichia* [15–17]. Especially in the veterinary field, cultures from nonclinical specimens result in a very diverse detection of yeast and filamentous fungi that need to be identified in order to discriminate the role of a pathogen from that of a saprophyte. Relative to *Cryptococcus* spp., the Bruker spectral reference library, which was available at the time of this study, contained only *C. flavus*, *C. laurentii*, *C. macerans*, *C. albidus*, and *C. uniguttulatus* as reference strains, in addition to a few spectra from the *C. neoformans*–*C. gattii* species complex.

Based on this information, our aim was to create an in-house library that would include a set of strains of nine rarely reported human and animal cryptococcal species, including *C. adeliensis*, *C. albidosimilis*, *C. albidus*, *C. aureus*, *C. carnescens*, *C. laurentii*, *C. magnus*, *C. victoria*, and *C. uniguttulatus*. This resource was to be used for timely and correct identification of those species by MALDI-TOF MS in a diagnostic laboratory.

Materials and methods

An in-house database was prepared by the Molecular Mycology Research Laboratory (MMRL), University of Sydney at Westmead Hospital, Sydney, Australia, to include *Cryptococcus* species that were not available in the Bruker database. Here we identified 76 *Cryptococcus* spp. isolates, of which 62 were used for the in-house database creation (Table 1); the remaining 14 (Table 2) where used to evaluate the quality of the newly created database. All strains were isolated from veterinary samples submitted to the Parasitology Laboratory, Istituto Zooprofilattico delle Venezie (IZSVe), Legnaro (PD), Italy, between 2009 and 2012 and previously identified by sequence analysis of the internal transcribe spacer (ITS) region of the rDNA gene cluster. Isolates are maintained at −80°C at the culture collection of the MMRL, where MALDI-TOF MS analyses were initially performed using the Microflex LT instrument (MALDI Biotyper, Bruker Daltonics, Bremen, Germany).

Isolates were cultured on Sabouraud glucose agar and incubated at 27°C for 48 h. Protein extracts of each isolate were obtained via formic acid extraction, as previously reported [18]. Mass spectra were produced automatically in positive linear mode with a range from 2 to 20 KDa using the MALDI Biotyper Flex control software (version 2.0.43.8; Bruker Daltonics). For each spectrum, 240 laser shots in 40-shot steps from different positions of the sample spot were accumulated and analyzed using automatic mode and default settings. MALDI Biotyper software 3.0 (Bruker Daltonics) was used to identify the isolates and to create the mass spectrum (MSP) dendrogram.

For generation of reference spectra obtained from the different *Cryptococcus* spp. studied, each extract was analyzed in triplicate to generate a consensus MSP. For the establishment of the in-house database, 10 replicates for each isolate were generated from *C. albidus* (WM 773) and *C. laurentii* (WM 884). Three replicates of *C. uniguttulatus* (DSM 4652, DSM 70225, H05108ERL), two replicates of *C. gattii* (formerly classified as *C. bacillisporus* [RV490]), four of *C. neoformans* (29 PSB, American Type Culture Collection 14116 THL, CCM 8312, and RV07 0218), one each of *C. flavus* (DSM 70227T) and *C. macerans* (DSM 70822T) already present in the Bruker database (version DB4613) were also used as reference spectra.

The in-house database created at the MMRL (Westmead Hospital, Westmead, Australia) was then exported and uploaded to the Microflex LT instrument (Maldi Biotyper; Bruker Daltonics) at the Clinic Diagnostic and Serology Laboratory, Fontane di Villorba, Treviso, Italy. In order to evaluate interlaboratory reproducibility, 12 isolates (*C. albidosimilis*, $n = 1$; *C. albidus*, $n = 1$; *C. carnescens*, $n = 3$; *C. laurentii*, $n = 3$; *C. magnus*, $n = 2$; and *C. uniguttulatus*, $n = 2$; Table 1) were reanalyzed with a new culture of the same strain grown in the latter laboratory and compared with the MSP created in the first laboratory.

Results

MALDI-TOF MS grouped all 62 isolates according to the species level previously determined by ITS sequence analysis. The dendrogram generated with the mass spectra of the 62 isolates shows all isolates of the same species group in distinctly different clusters (Fig. 1).

Table 1. Veterinary *Cryptococcus* spp. isolates included in the study.

PD No.	WM No.	Host	Scientific name	Source	Accession No.†
C. adeliensis					
90	13.141	Cormorant	*Phalacrocorax carbo*	Cloaca	KF958204
1600	13.142	Cat	*Felis silvestris catus*	Nasal cavity	KF958205
C. albidosimilis					
208*	13.126	Pigeon	*Columba livia*	Cloaca	KJ439600
1264	13.150	Cat	*F. silvestris catus*	Nasal cavity	KF958206
1209	13.151	Cat	*F. silvestris catus*	Nasal cavity	KF958207
C. albidus					
2698*	13.122	Cat	*F. silvestris catus*	Nasal cavity	KF958193
2679	13.123	Cat	*F. silvestris catus*	Nasal cavity	KF958194
514	13.124	Shoveler	*Anas clypeata*	Cloaca	KF958195
574	13.125	Wigeon	*A. penelope*	Cloaca	KJ439601
1629	13.127	Cat	*F. silvestris catus*	Nasal cavity	KF958197
1548	13.129	Cat	*F. silvestris catus*	Nasal cavity	KF958199
1542	13.130	Cat	*F. silvestris catus*	Nasal cavity	KF958200
781	13.133	Cat	*F. silvestris catus*	Nasal cavity	KF958202
1766	13.134	Cat	*F. silvestris catus*	Nasal cavity	KF958196
C. aureus					
399	13.137	Gadwall	*A. strepera*	Oropharynx	KJ439602
1559	13.140	Wild duck	*A. platyrhynchos*	Oropharynx	KF958208
C. carnescens					
1597	-	Cat	*F. silvestris catus*	Nasal cavity	KF958229
599	13.105	Wild duck	*A. platyrhynchos*	Oropharynx	KF958230
500	13.106	Wigeon	*A. penelope*	Cloaca	KJ439603
420	13.108	Wigeon	*A. penelope*	Cloaca	KJ439604
1840	13.109	Cat	*F. silvestris catus*	Nasal cavity	KF958232
571*	13.110	Teal	*A. crecca*	Cloaca	KJ439605
2700	13.111	Cat	*F. silvestris catus*	Nasal cavity	KF958233
84	13.112	Cat	*F. silvestris catus*	Nasal cavity	KF958234
1591	13.113	Cat	*F. silvestris catus*	Nasal cavity	KF958235
572*	13.114	Teal	*A. crecca*	Cloaca	KJ439606
507*	13.115	Wigeon	*A. penelope*	Cloaca	KJ439607
1151	13.117	Cat	*F. silvestris catus*	Nasal cavity	KF958237
C. laurentii					
425*	13.143	Wild duck	*A. platyrhynchos*	Cloaca	KJ439608
75	13.144	Shoveler	*A. clypeata*	Oropharynx	KJ439609
358*	13.145	Teal	*A. crecca*	Oropharynx	KJ439611
269*	13.146	Wild duck	*A. platyrhynchos*	Oropharynx	KJ439610
C. magnus					
1550	13.86	Cat	*F. silvestris catus*	Nasal cavity	KF941325
1768*	13.87	Cat	*F. silvestris catus*	Nasal cavity	KF941326
1625	13.88	Cat	*F. silvestris catus*	Nasal cavity	KF941327
1537	13.89	Cat	*F. silvestris catus*	Nasal cavity	KF941328
1163	13.91	Pigeon	*C. livia*	Oropharynx	KF941337
2235	13.92	Cat	*F. silvestris catus*	Nasal cavity	KF941329
1419	13.93	Pigeon	*C. livia*	Oropharynx	KF941330
2273	13.94	Cat	*F. silvestris catus*	Nasal cavity	KF941331
1544	13.95	Cat	*F. silvestris catus*	Nasal cavity	KF941332
2467*	13.97	Cat	*F. silvestris catus*	Nasal cavity	KF941334
1592	13.98	Cat	*F. silvestris catus*	Nasal cavity	KF941335
1616	13.99	Cat	*F. silvestris catus*	Nasal cavity	KF941336
2570	13.101	Cat	*F. silvestris catus*	Nasal cavity	KF941339
46	13.102	Cat	*F. silvestris catus*	Nasal cavity	KF941343
2647	13.103	Cat	*F. silvestris catus*	Nasal cavity	KF941341
86	-	Cormorant	*P. carbo*	Oropharynx	KF941338

Table 1. (Continued)

PD No.	WM No.	Host	Scientific name	Source	Accession No.†
1545	13.104	Cat	*F. silvestris catus*	Nasal cavity	KF941342
C. uniguttulatus					
909	13.152	Parrot	Nonidentified	Cloaca	KF958239
981*	13.153	Eagle-Owl	*Bubo bubo*	Cloaca	KF958240
979*	13.154	Eagle-Owl	*B. bubo*	Cloaca	KF958241
209	13.156	Pigeon	*C. livia*	Cloaca	KJ439612
2676	13.158	Cat	*F. silvestris catus*	Nasal cavity	KF958242
1237	13.159	Magpie	*Pica pica*	Oropharynx	KF958243
411	13.160	Dog	*Canis lupus familiaris*	Auricular canal	KJ439613
982	13.161	Eagle-Owl	*B. bubo*	Oropharynx	KF958244
1210	13.163	Falcon	*Falco peregrinus*	Oropharynx	KF958245
1176	13.164	Pigeon	*C. livia*	Oropharynx	KF958246
1144	13.165	Pigeon	*C. livia*	Oropharynx	KF958247
364	13.166	Pigeon	*C. livia*	Cloaca	KJ439614
C. victoriae					
1889	-	Cat	*F. silvestris catus*	Nasal cavity	KF958211

PD, Italian collection number (IZSVe, Legnaro, Italy); WM, Australian collection number (Molecular Mycology Research Laboratory, Westmead Hospital, Westmead, Australia).

*Isolates reprocessed at the Italian laboratory used to attest the reproducibility of the method.

†Sequencing with similarity ranging from 99% to 100% when compared with the ISHAM-ITS database (http://www.mycologylab.org).

Table 2. Isolates used to verify the quality of the newly created database.

PD No.	WM No.	Host	Scientific name	Source	Accession No.
Cryptococcus albidus					
1538	13.135	Cat	*Felis silvestris catus*	Nasal cavity	KF958203
1766	13.134	Cat	*F. silvestris catus*	Nasal cavity	KF958196
208	13.126	Pigeon	*Columba livia*	Cloaca	KJ439600
1630	13.128	Cat	*F. silvestris catus*	Nasal swab	KF958198
33	13.131	Cat	*F. silvestris catus*	Nasal swab	KF958201
Cryptococcus carnescens					
2811	-	Cat	*F. silvestris catus*	Nasal cavity	KF958236
2701	-	Cat	*F. silvestris catus*	Nasal cavity	KF958238
Cryptococcus magnus					
2061	13.149	Cat	*F. silvestris catus*	Nasal cavity	KF958209
2678	13.96	Cat	*F. silvestris catus*	Nasal cavity	KF941333
726	13.100	Cat	*F. silvestris catus*	Nasal cavity	KF941340
45	-	Cat	*F. silvestris catus*	Nasal cavity	KF941344
Cryptococcus uniguttulatus					
1244	13.162	Magpie	*Pica pica*	Oropharynx	KF958249
Cryptococcus victoriae					
2750	-	Cat	*F. silvestris catus*	Nasal cavity	KF958212
1261	13.155	Magpie	*P. pica*	Oropharynx	KF958248

PD, Italian collection number (IZSVe, Legnaro, Italy); WM, Australian collection number (Molecular Mycology Research Laboratory, Westmead Hospital, Westmead, Australia).

Among the 14 isolates, 2 *C. carnescens*, 4 *C. magnus*, and 1 *C. victoriae* failed to achieve a reliable identification (scores <1.7) using the original Bruker database. Whereas when the spectra were rerun against the in-house library generated at the MMRL, all 14 *Cryptococcus* species were correctly identified, reaching scores >2.

The 12 isolates regrown, reextracted, and reanalyzed in Italy produced clear mass spectra (Fig. 2) and showed excellent identification when compared with the in-house database generated at the MMRL, reaching scores >2.7. This demonstrated that the method is highly reproducible.

Discussion

These results demonstrate that MALDI-TOF MS provides the correct identification of non–*C. neoformans*–*C. gattii*

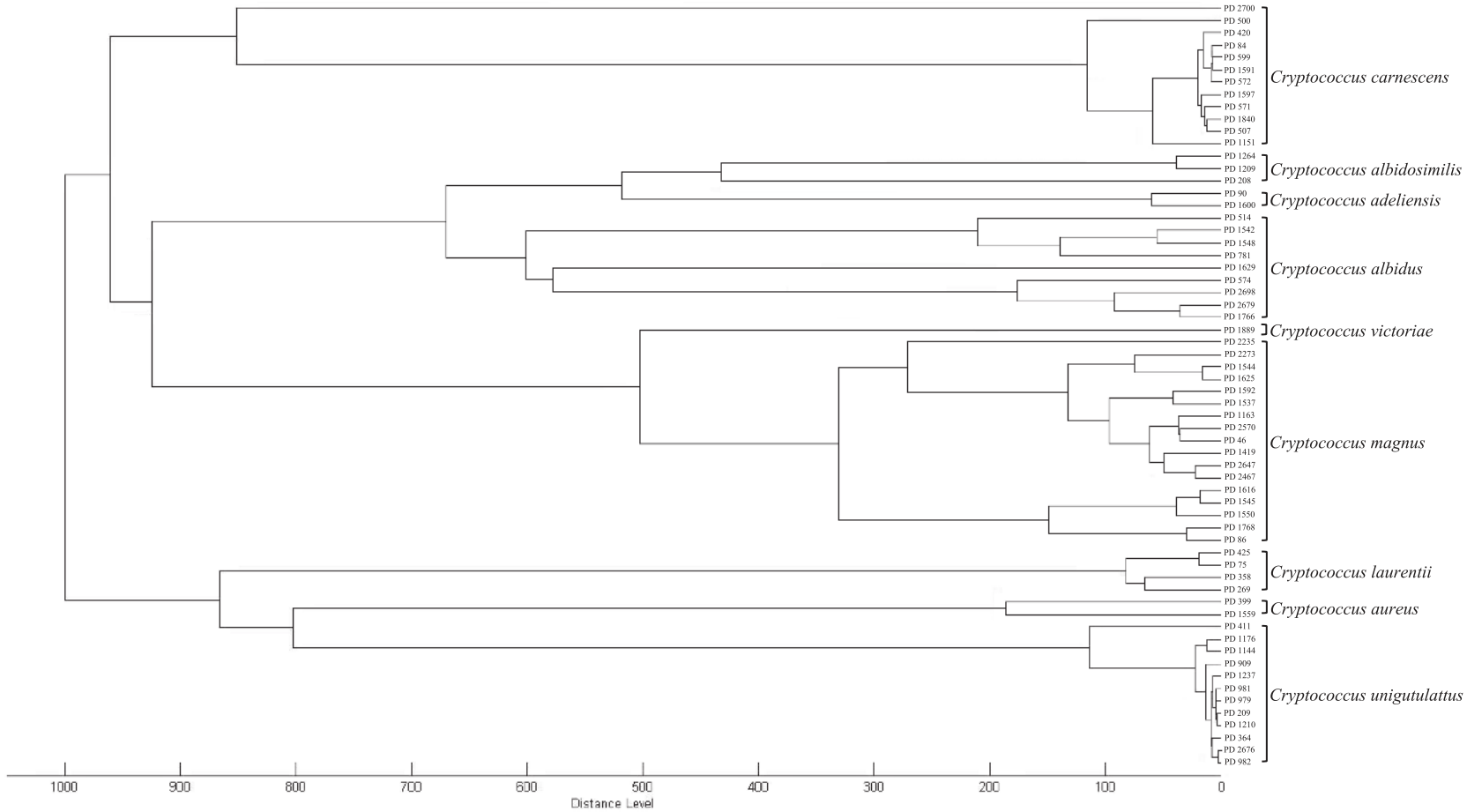

Figure 1. Mass spectrum dendrogram grouping *Cryptococcus adeliensis*, *C. albidosimilis*, *C. albidus*, *C. aureus*, *C. carnescens*, *C. laurentii*, *C. magnus*, *C. uniguttulatus*, and *C. victoriae* isolates to the species level.

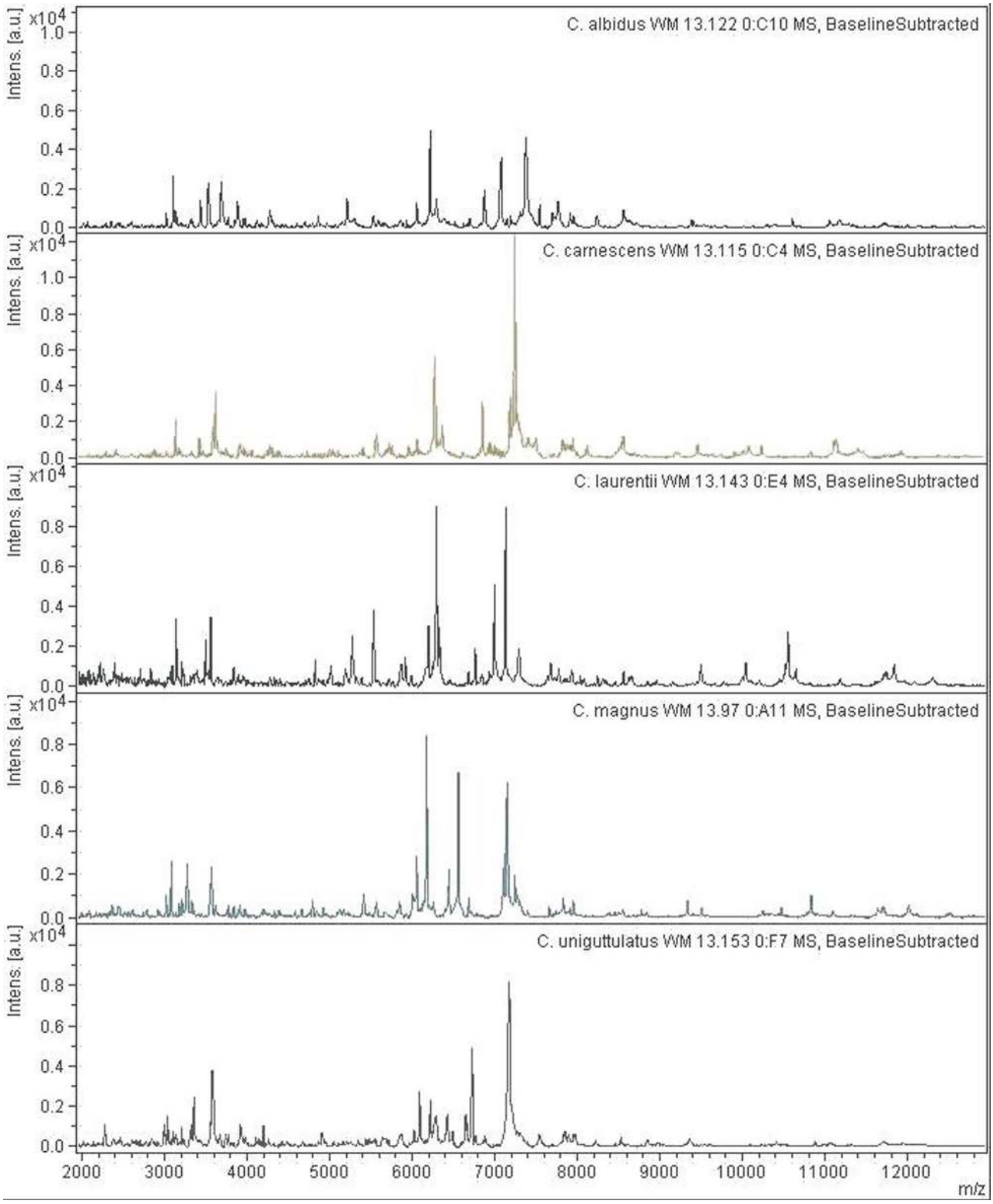

Figure 2. Profiles of five *Cryptococcus* species used to test the interlaboratory reproducibility of the method: *C. albidus, C. carnescens, C. laurentii, C. magnus,* and *C. uniguttulatus.*

Cryptococcus spp. However, the identification power of the Bruker MALDI-TOF Biotyper system is still limited by the robustness of the reference library being used. For instance, the better performances reported with *Candida* spp. [16] in comparison with non-*Candida* yeast-like isolates (ie, *Geotrichum* spp. and *Trichosporon* spp.) [15,19] reflect the absence of reference spectra for those genera or species in the Bruker MALDI-TOF MS library, not inadequate processing [15,19]. Similarly, the creation of supplementary libraries has successfully increased the identification performance for *Cryptococcus* spp. to species [20], subspecies, and even hybrid levels [19,21]. Supplementary library entries are relatively easy to generate using type or reference strains that have identities confirmed through other phenotypic and/or molecular methods, as in the present study, where the Biotyper 2.0 library was supplemented with 62 additional entries of *C. adeliensis*, *C. albidosimilis*, *C. albidus*, *C. aureus*, *C. carnescens*, *C. magnus*, *C. laurentii*, *C. uniguttulatus*, and *C. victoriae*.

Recent studies that focused on the identification tools for fungal and bacterial isolates show that the MALDI-TOF MS system and biochemical performances are comparable [15,22]. For analysis of isolates absent from the databases, MALDI-TOF MS systems recognized these as "unknown," whereas the classic approach misidentified them as closely related species [15]. Some *Cryptococcus* species that we investigated, such as *C. adeliensis*, *C. albidosimilis*, *C. aureus*, *C. carnescens*, *C. magnus*, and *C. victoria*, cannot be identified using commercial biochemical tools (ie, ID32C bioMérieux) and require time-consuming and costly molecular methods. Consequently, the reduction of false identifications through the use of MALDI-TOF MS clearly represents a clinically relevant advantage of the methodology for the early and correct identification of important human and animal fungal pathogens. The possibility of creating and transferring an in-house library adds to the usefulness of MALDI-TOF MS as an important tool for the rapid and inexpensive identification of pathogenic and saprophytic fungi that is required for differential diagnosis of human and animal mycoses. This study should encourage collaboration and exchange of data among diagnostic laboratories so that these laboratories can establish a shared global spectral reference library for human and animal pathogens and, in turn, achieve a highly standardized identification of fungal disease agents.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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