Original

Laura Ferreira[1,2§]
Fernando Sánchez-Juanes[1§]
Silvia Vega[3]
Magdalena González[3]
Mª Inmaculada García[2,3]
Silvia Rodríguez[1]
José Manuel González-Buitrago[1,2,4*]
Juan Luis Muñoz-Bellido[2,3,5*]

Identification of fungal clinical isolates by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry

[1]Unidad de Investigación. Hospital Universitario de Salamanca. Spain.
[2]Grupo de Investigación Reconocido MICRAPE. Universidad de Salamanca. Spain
[3]Departamento de Microbiología. Hospital Universitario de Salamanca. Spain
[4]Departamento de Bioquímica y Biología Molecular. Universidad de Salamanca. Spain
[5]Departamento de Medicina Preventiva, Salud Pública y Microbiología Médica. Universidad de Salamanca. Spain

ABSTRACT

Background. Recently, bacterial identification by MALDI-TOF MS has acquired a high relevance in terms of speed and reliability. Conventional mycological identification has some disadvantages: it is frequently slow, reliability is sometimes low, and an extensive experience is required. The risk population for fungal infections, and therefore their clinical significance has progressively increased in recent years.

Methods. 153 yeast and mould clinical isolates were analyzed by MALDI-TOF MS and conventional identification. When both methods were discrepant to the genus or species level, ITS-2 sequencing was performed.

Results. The correlation in yeasts identification between conventional identification methods and MALDI-TOF MS was extremely high (99.2% to the species level and 100% to the genus level). The only discrepancy was checked by ITS-2 sequencing and confirmed the MALDI-TOF identification. The correlation in moulds identification was more heterogeneous. 68.7% of the isolates showed correlation at least to the genus level and 40.6% to the species level. Therefore, the correlation between conventional identification and MALDI-TOF MS in fungal identification was, in whole, 87% to the species level, and 93.5% to the genus level.

Conclusions. Identification of fungi by MALDI-TOF MS is reliable and shows potential advantages over conventional identification methods.

KEYWORDS: Moulds, yeasts, identification, mass spectrometry, MALDI-TOF.

Correspondence:
Juan Luis Muñoz Bellido
Servicio de Microbiología. Hospital Universitario de Salamanca.
Paseo de San Vicente 58-182. 37007 Salamanca. Spain.
E-mail: jlmubel@usal.es.
§ These authors share first authorship.
* These authors share senior authorship.

Identificación de aislamientos clínicos de hongos mediante espectrometría de masas MALDI-TOF

RESUMEN

Introducción. En los últimos años, la identificación bacteriana mediante espectrometría de masas (EM) MALDI-TOF ha adquirido progresivamente importancia, dada su rapidez y fiabilidad. La identificación micológica convencional tiene algunos inconvenientes, como son su lentitud, su baja fiabilidad en algunos casos, y el hecho de que para algunos procedimientos es imprescindible la intervención de personas con amplia experiencia. Sin embargo, la población de riesgo para infecciones fúngicas, y por tanto su importancia clínica, han aumentado en los últimos años.

Material y métodos. Se identificaron 153 aislamientos de hongos filamentosos y de levaduras mediante métodos convencionales y mediante EM MALDI-TOF. Cuando se dieron discrepancias entre ambos, éstas fueron dilucidadas mediante secuenciación del segmento ITS-2.

Resultados. La correlación entre los métodos convencionales y la EM MALDI-TOF para la identificación de levaduras fue muy alta (99,2% a nivel de especie y 100% al de género). La única discrepancia que debió ser comprobada mediante secuenciación, corroboró el resultado obtenido con la EM. La correlación fue más heterogénea en el caso de los hongos filamentosos. 68,7% de los aislamientos mostraron correlación al menos al nivel de género, y 40,6% al nivel de especie. En conjunto, la correlación entre identificación convencional y MALDI-TOF para la identificación de hongos fue del 87% al nivel de especie, y del 93,5% a nivel de género.

Conclusiones. La identificación de hongos mediante EM MALDI-TOF es fiable, y presenta ventajas potenciales con respecto a los métodos convencionales.

PALABRAS CLAVE: Hongos, levaduras, identificación, espectrometría de masas, MALDI-TOF.

INTRODUCTION

MALDI-TOF mass spectrometry (MS) has emerged in recent years as a revolutionary technique in clinical microbiology, especially in terms of speed and reliability[1-4]. Most studies have been carried out with bacteria, and available data on yeasts and moulds identification are scarce, but suggest that identification based on this technology might be an important contribution to this area[5-7]. Conventional mycological identification have some disadvantages: it is frequently slow, biochemical methods reliability is sometimes low, and an extensive experience is frequently needed for identifying fungi correctly, based on morphological (macro and microscopic) features. Although there are alternative methods based on other technologies, such as PCR-based methods, MALDI-TOF MS has the advantages of its easy implementation and the low cost of consumable items.

The clinical significance of fungal infections has progressively increased in recent years, as well as the risk population (immunosuppression, broad-spectrum antimicrobial treatments, invasive procedures, etc.)[8]. Several studies have shown that the emergence of fungal infections is associated to an increase of mortality in these patients[9]. *Candida albicans* is still the yeast species most commonly isolated. However, *Candida* species other than *C. albicans*, some of them more resistant to antifungal agents, such as *C. glabrata* and *C. krusei*, are becoming more common[10]. Moreover, moulds are also increasingly found in these patients, especially species belonging to the genus *Aspergillus*. This study compares conventional identification methods and MALDI-TOF MS-based identification, in order to know its usefulness for identifying both yeast and moulds, including dermatophytes.

MATERIAL AND METHODS

Strains, culture conditions and conventional identification. The study included 153 yeast and moulds clinical isolates, obtained at the Microbiology Department in the Hospital Universitario de Salamanca (Spain). All isolates were cultured on diagnostic fungal media. Yeasts were presumptively identified on chromogenic agar (CAND agar, Oxoid, ThermoFisher Scientific, UK), and their identification was confirmed by using RapId Yeast Plus System (Remel, ThermoFisher Scientific, UK). Moulds were identified based on their morphological features on Sabouraud agar (Becton Dickinson, USA) and their microscopic features after lactophenol blue staining.

MALDI-TOF MS ANALYSIS

1. Sample preparation. Fresh cells were transferred from plates to a 1.5 mL tube (Eppendorf, Germany) with a pipette tip and mixed thoroughly in 300 µL of water. Then, 900 µL of absolute ethanol was added and the mixture was centrifuged at 15,500 g for 2 min and the supernatant was discarded. The pellet was air-dried at room temperature for 1 hour. Subsequently, 50 µL of formic acid (70% v/v) were added to the pellet and mixed thoroughly before the addition of 50 µL of acetonitrile to the mixture. The mixture was centrifuged again at 15,500 g for 2 min. One microliter of the supernatant was placed onto a spot of the steel target and air-dried at room temperature. Each sample was overlaid with 1 µL of matrix solution (saturated solution of HCCA (alpha-cyano-4-hydroxy cinnamic acid)) in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried.

2. Mass spectrometry. Measurements were performed on an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany). Spectra were recorded in the linear positive mode at a laser frequency of 200 Hz within a mass range from 2,000 to 20,000 Da. The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.6 kV, the lens voltage was 6 kV, and the extraction delay time was 40 ns. For each spectrum, 500 laser shots were collected and analyzed (10 x 50 laser shots from different positions of the target spot). The spectra were calibrated externally using the standard calibrant mixture (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin, Bruker Daltonics). Calibration masses were as follows: RL36, 4364.3 Da; RS22, 5095.8 Da; RL34, 5380.4 Da; RL33meth, 6254.4 Da; RL32, 6315 Da; RL29, 7273.5 Da; RS19, 10299.1 Da; RNase A, 13682.2 Da; myoglobin, 16952.5 Da.

3. Spectrum generation and data analysis. For automated data analysis, raw spectra were processed using the MALDI Biotyper 2.0 software (Bruker Daltonics, Leipzig, Germany) at default settings. The software performs normalization, smoothing, baseline subtraction, and peak picking, creating a list of the most significant peaks of the spectrum (m/z values with a given intensity, with the threshold set to a minimum of 1% of the highest peak and a maximum of 100 peaks). To identify unknown bacteria, each peak list generated was matched directly against reference libraries (3,476 species) using the integrated patterns matching algorithm of the Biotyper 2.0 software (Bruker Daltonics, GmbH, Germany). The unknown spectra were compared with a library of reference spectra based on a pattern recognition algorithm using peak position, peak intensity distributions and peak frequencies. Once a spectrum has been generated and captured by the software, the whole identification process is performed automatically, without any user intervention.

4. Assessment of results. MALDI-TOF identifications were classified using modified score values proposed by the manufacturer: a score ≥ 2 indicated species identification; a score between 1.7 and 1.9 indicated genus identification, and a score < 1.7 indicated no identification. When identifications obtained by MALDI-TOF and conventional methods were coincident, these identifications were considered as reliable.

Sequence data. When MALDI-TOF and conventional methods were discrepant at the genus or species level, ITS-2 sequencing was performed according to previously described methods[11], and its results considered as the reference identification.

RESULTS

We analyzed the MS fingerprint patterns obtained from 153 yeast and filamentous fungi strains. Table 1 shows the results obtained. In general, the correlation in yeast identification was extremely high (99.2% to the species level and 100% to the genus level). There was only one discrepancy in one isolate identified as *C. parapsilosis* by conventional methods, and identified as *C. orthopsilosis* by MALDI-TOF MS. ITS-2 sequencing confirmed the identification as *C. orthopsilosis*. Thus, MALDI-TOF MS yeasts identification was correct, to the species level, in 100% of isolates.

On the other hand, the correlation between MALDI-TOF MS and conventional methods in moulds identification was more heterogeneous. In 10 cases, 6 of them belonging to the same genus, the identification obtained with MALDI-TOF MS was not reliable (31,3%). 28% of the mould isolates showed correlation only to the genus level and 40,7% to the species level. In *Microsporum canis* and *Trichophyton rubrum* isolates whose identifications were considered reliable only to the genus level, the identification provided as the most likely one by the BioTyper software was correct to the species level, although with a score value <2 (scores ranging between 1.68 and 1.86).

Therefore, the correlation between conventional identification and MALDI-TOF MS in fungal identification (yeasts and moulds) was 87% to the species level, and 93.5% to the genus level. MALDI-TOF MS identification was unreliable in only 6.5% of cases.

DISCUSSION

The main objective of the microbiological diagnostic techniques is to obtain accurate and clinically useful results in the shortest time. In the field of mycology, some techniques, mostly microscopic, allow a quick presumptive diagnosis. However, definitive diagnosis requires in most cases the growth of fungi on culture media and biochemical or morphological identification. This methodology can delay considerably the diagnosis. Molecular techniques have been shown very specific, but they are mostly homemade techniques, still poorly introduced in diagnostic mycology laboratories, and restricted largely to research or reference laboratories.

Nowadays, fast and reliable identification techniques development is an essential objective in the field of mycology. This might advance several days the diagnosis and, on the other hand, a reliable identification is crucial to therapeutic decisions-making, when species belonging to the same genus have

Table 1 **Correlation between conventional and MALDI-TOF MS identification of yeasts and moulds.**

Conventional identification (No. isolates)	MALDI ID. unreliable[1]	Correlation genus level	Correlation species level	Id. MALDI discrepancy
Candida albicans (62)	-	-	62 (100%)	-
Candida glabrata (15)	-	-	15 (100%)	-
Candida parapsilosis (30)	-	1 (3.3%)	29 (96.7%)	*Candida orthopsilosis*[2]
Candida krusei (5)	-	-	5 (100%)	-
Candida tropicalis (5)	-	-	5 (100%)	-
Clavispora lusitaniae (3)	-	-	3 (100%)	-
Aureobasidium pullulans (1)	-	-	1 (100%)	-
Microsporum canis (4)	-	2 (50%)[3]	2 (50%)	-
Trichophyton tonsurans (7)	6 (85.7%)	-	1 (14.3%)	-
Trichophyton mentagrophytes (5)	1 (20%)	1 (20%)[4]	3 (60%)	-
Trichophyton rubrum (3)	-	2 (66.7%)[5]	1 (33.3%)	-
Scopulariopsis spp. (3)	1 (33.3%)	2 (66.7%)	0 (%)	*Scopulariopsis brevicaulis*
Aspergillus fumigatus (10)	2 (20%)	2 (20%)[6]	6 (60%)	-
TOTAL 153	10 (6.5%)	10 (6.5%)	133 (87.0%)	

[1]MALDI-TOF score < 1.7

[2]Id. rRNA ITS-2: *Candida orthopsilosis*

[3]MALDI-TOF Id.: *Microsporum canis*. Score: 1.70-1.79.

[4]MALDI-TOF Id.: *Trichophyton mentagrophytes*. Score: 1.73-1.82.

[5]MALDI-TOF Id.: *Trichophyton rubrum*. Score: 1.79-1.86.

[6]MALDI-TOF Id.: *Aspergillus fumigatus*. Score: 1.81-1.85.

different antifungal susceptibility profiles. In this way, reliable species identification of *Candida* spp. is becoming increasingly important, due to the growing frequency of *Candida* species other than *C. albicans* as human pathogens, and the heterogeneity of their susceptibility to antifungal agents[12].

Mass spectrometry has been recently described as an useful and reliable method for bacterial identification, both directly from colonies growing on culture media[1-4], or even directly from certain samples (blood, urine)[13-16], and it is becoming a diagnostic resource that probably will change permanently procedures for bacterial identification in clinical microbiology laboratories in the near future.

Though the experience of MALDI-TOF MS in mycology is lower, some studies, mainly on yeasts, suggest that its usefulness for fungi identification would be similar to bacterial identification, at least working with colonies growing on agar plates[5,6]. Direct identification from blood cultures is more controversial, since some studies show acceptable results[17], while others get a very low sensitivity in direct identification from blood cultures in candidemias[13].

This study confirms the usefulness of MALDI-TOF MS as a good alternative to conventional methods for the identification of the most frequently isolated yeast species, including both *C. albicans* and different species of *Candida* non-*albicans*. Among the 121 yeast clinical isolates tested, only one discrepancy was found with conventional methodology. One isolate was identified as *C. parapsilosis* by conventional methods. However, MALDI-TOF MS identified it as *C. orthopsilosis*, which was subsequently corroborated by molecular methods. It must be noted that the correct identification of this group has interest both from the epidemiological as well as the antifungal susceptibility point of view, since some studies have shown that *C. orthopsilosis* may have a decreased susceptibility to echinocandins and amphotericin B[18].

Therefore, MALDI-TOF yeast identification allows high reliability with low time consumption, and avoids both the use of biochemical identification systems and, eventually, the chromogenic agar media, significantly more expensive than the most common selective media, such as Sabouraud agar.

Studies on MALDI-TOF MS performance in other species of fungi are more limited, and there are limited data published concerning dermatophytes[19]. The results obtained in this study are more heterogeneous than with yeasts. In some species belonging to the genus *Trychophyton*, such as *T. rubrum*, MALDI-TOF MS identified all isolates at least to the genus level. Even in these cases, the identification suggested as the most likely was correct, although the score obtained did not allow identification to the species level according to the criteria stablished. However, concerning other species of the same genus, such as *T. tonsurans*, protein profiles were very poor, and therefore the identification results presented very low scores, hindering a reliable identification even to the genus level. Other studies confirm a good behavior for *T. rubrum*, but data concerning *T. tonsuran*s are more difficult to compare since they identified just one isolate, which was correctly identified[19]. This heterogeneity, even within the same genus, has been associated to the complex protein profile of moulds. Protein profile can change significantly between different mould parts, and depending on the age of the culture. Therefore, wider databases comprising all these possibilities probably would improve identification efficacy. Other, extraction methods probably shall be also optimized.

Otherwise, we should remark identification reliability obtained by MALDI-TOF MS. In all cases in which MALDI-TOF yielded a reliable identification at least to the genus level, genus identification was coincident with genus identification obtained by conventional methods. This means that, while there is a certain probability, mainly in moulds, of getting no reliable identification by using MALDI-TOF MS, when it is obtained, the level of reliability is high.

In whole, the identification of fungi by using MALDI-TOF MS has potential advantages over conventional identification methods: chromogenic agar plates and biochemical identification systems are not necessary, and results are obtained earlier, with a high reliability, and with a very low spent on consumables. Yeast identification is 100% reliable, while results are more heterogeneous with filamentous fungi, probably because common extraction methods are not enough in this case, and more efficient extraction methods are needed. However, when the score is higher than 1.7, the identification is correct in all cases, whether to the genus or species level. In addition, MALDI-TOF MS allows identification as soon as the colonies are visible on plate, even before the colonies reach the morphological characteristics used for conventional identification.

CONFLICT OF INTERESTS

None to declare.

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