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Research Article

MALDI-TOF Clinical Filamentous Fungi Identification. Bead Beating Versus Acid Solvent Protein Extraction Method

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Abstract

In recent years, Matrix-Assisted Laser Desorption Ionization–Time-Of-Flight Mass Spectrometry (MALDI-TOF) has changed clinical microbiology, allowing filamentous fungi identification. The aim of this paper is to validate a novel protein extraction method using bead beating to identified filamentous fungi and compare the fingerprinting spectra obtain to acid solvent method. A total of 50 clinical isolates, including *Trichophyton interdigitale*, *T.rubrum*, *T.tonsurans*, *Aspergillus terreus*, *Microsporum canis* were analyzed in this study. The MALDI-TOF spectra scores were identified correctly at the genus and species level for all. According to our results the fingerprinting spectra obtained by acid solvent extraction have, especially for *Trychophyton spp*, quantitative and qualitative poorly defined protein expression because of the similarities of the species. Our bead beating protein extraction methods allows to obtain a wider variability of protein pattern and huge defined number of peaks. Bead beating protocol could in this way implement a clinical in-house reference library.

Keywords: Bead beating; Filamentous fungi; MALDI-TOF; Protein extraction; Spectra

Introduction

In last year's fungal infections represent a huge and emergent worldwide public health problem with high financial costs for diagnosis, treatment and prevention [1,2]. It is estimated that around one billion people are affected by superficial fungal infections [3,4]. The high rate of isolation in the most affected site, skin and nails, making them one of the most frequent form of infection that also exercise a strong impact on people lives [5]. The severity of the disease varies from bland or asymptomatic skin to appendages or lethal systemic infections [6]. Considering that numerous cases are not correctly diagnosed and turnaround time of conventional diagnostic methods made superficial fungal infections probably underestimated. Therefore, an accurate etiological diagnosis is important for the prescription of the correct treatment and also for epidemiological purpose [7,8]. Fungal identification routine methods are mainly based on *in vitro* culture, morphological analysis of the colonies and microscopic characteristics of conidia and hyphae. The morphological similarities among some species made the conventional identification very complicated as a result requires high competence and experience [9]. In the last decades, Polymerase Chain Reaction (PCR) - based methods are performed for fungal strains identification, contributing to accelerate diagnosis and prescribe the right therapy. However, these approaches require high costs for the identification of fungal isolates in all clinical samples [10]. This underlines a novel fungal identification method, which is rapid and low cost. In recent years, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) has changed clinical microbiology, allowing fast and precise identification of microorganisms with simple procedure [11]. This method measures the mass-charge ratio of proteins from the composition of the microorganism assembles spectra, compared with MALDI-TOF MS database [12]. The widespread interest of this approach is the simple use and the fast microbial identification with reduced costs in the routine of clinical practice. MALDI-TOF MS approach is validated for discrimination of bacteria and yeast

[13]. In contrast, few studies have treated the use of MALDI-TOF MS to identify filamentous fungi, due to the simultaneous presence of different fungal structure (hyphe and/or conidia) in the same culture and mainly for their complex morphology [14]. Fungal cells are larger in size than the bacterial cell and they have a very tight cell wall. The cell wall of the fungi mainly consists of polysaccharides and by reduced quantities of proteins, lipids and polyphosphates [15,16]. This particular composition requires a protein extraction phase before the traditional MALDI-TOF fungal identification process. Between 2015 and 2019, eight studies have faced topic related to using of MALDI-TOF MS in the filamentous fungal discrimination [17]. Most of them report the success MALDI-TOF identification of different genus of filamentous fungi based on chemical acid solvent protein extraction protocol. The aim of this paper is validate "bead beating" protein extraction protocol to identify clinical filamentous fungi. A focus on exemplifying species spectra underlined how the MALDI-TOF identification rates and fingerprinting spectra varied between the bead disruption method and chemical acid solvents method in the protein extraction phase.

Materials and Methods

Fungal Culture

A total of 50 fungal strains have been isolated from clinical sample (skin, hair and nails). Strains belonging to 6 genera: *Trichophyton, Microsporum, Aspergillus, Fusarium*, *Rhizopus*, *Epidermophyton*. The strains were cultured on Sabouraud Dextrose Agar (SDA) and SDA with chloramphenicol and gentamicin added (Vacutest Kima, Italy) at 30˚C for 10-13 days. Isolated strains were identified according to National Mycology Reference Laboratory (NMRL). Macroscopic and microscopic phenotypic characterization, biochemical methods and different culture media were used. We report exemplifying fungal strains subjected to identification through MALDI-TOF MS included: *Trichophyton interdigitale, Trichophyton tonsurans, Trichophyton soudanense, Microsporum gypseum Aspergillus terreus*.

Negative control was obtained by directly transferring growing mycelium samples on Maldi target plate with the addition of a-cyano-4-hydroxycinnamic acid solution (Bruker Daltonics, Germany).

Protein Extraction from Fungal Strains Using Acidic Solvents

CColonies have been selected from SDA with chloramphenicol and gentamicin added, because of purer colonies than Sabouraud dextrose agar, and after 10-13 days growing inoculated into 8ml tubes of Sabouraud dextrose Broth (Vacutest Kima, Italy). The inoculum has been incubated at room temperature until growth is observed. The fungal has been left to settle for about 10 minutes. The sediments have been collected from the bottom of the tube (1,5 ml), transferred in Eppendorf tube and centrifuged for 2 minutes at 13000 g. After the removal the supernatant, the pellet had been resuspended in 1 ml of deionized water, and the centrifugation was repeated (13000 g for 2 minutes). After discard the supernatant 300 µl of deionized water was added. Subsequently, after mixed, throwed in 900 µl ethanol (100%) (Sigma-Aldrich, United States) and centrifuged at 13000 g for 2 minutes. In the next step, the supernatant had been removed and 50 µl of formic acid added to the pellet. The samples have been exhaustively mixed. After, 50 µl of acetonitrile (Sigma-Aldrich, United States) is added to the sample and centrifuged again. Than 1 µl of supernatant has been transferred on MALDI target plate (Bruker Daltonics, Germany) and dried at room temperature for 5 min. Finally, 1 µl of saturated a-cyano-4-hydroxycinnamic acid solution (Bruker Daltonics, Germany) overlay has been putted on witch target plate spot for MALDI-TOF MS analysis [18].

Protein Extraction from Fungal Strains Using Bead Beating

After 10-13 days of incubation on Sabouraud dextrose agar with chloramphenicol and gentamicin, colonies of clinical isolates pure culture were scraped and entered into 2 ml of bidistilled H_2O. To obtain complete protein extraction in the Eppendorf with solution is included a chromo steel lysis beads of 1.3-mm diameter (BioSpec, Bartlesville, OK, USA). The magnetic field generated by tissuelyser (TissueLyser II, Qiagen, Hilden, Germany) activated for 5 cycles of 1.30 minutes each with 30 oscillations per second. After added 900 µl of pure EtOH (ethanol) with 300 µl of surnatant, the solution was carefully resuspended and centrifuged 17000 rpm for 2 minutes. The protein material's pellet left for drying 10 minutes' Real Temperature (RT). Once the pellet is completely dried, was resuspended it in 50 µl of Formic Acid (70%) and left for 30 minutes RT. Before the last centrifugation at 17000 rpm for 2 minutes, was added 50 µl of Acetonitrile. The supernatant has been transferred on MALDI target plate (MSP 96 BC ground steel target; Bruker Daltonics) and dried. As the same of chemical extraction, 1 µl of saturated a-cyano-4-hydroxycinnamic acid solution (HCCA matrix; Bruker Daltonics) overlay has been putted on witch target plate spot for MALDI-TOF MS analysis.

MALDI-TOF MS Analysis

Analyses was performed using Bruker Microflex instrument, Biotyper software (Version 3.0), and database (Version 3.1.66; Bruker Daltonics). The identification species-related data generated by MALDI-TOF MS were classified following the manufacturer's instructions. The score values ≥ 2.0, 1.7-1.99, and < 1.7 indicate species identification, genus level detection, and Non-Reliable Identification (NRI), respectively. Spectra were baseline corrected and normalized to total positive ion current [19].

Results

MALDI-TOF MS fungal identification is mainly influenced by critical factors involved in the normal laboratory process [20]. The variability in the identification is principally due in critical step of protein extraction procedure, performed before the

discrimination process. The protein extraction with acid solvents is the simplest and most used approach. However, previous studies have shown that the spectral profiles obtained with acid solvents method have a quantitatively and qualitatively poorly defined protein expression [21]. Consequently, the detection of some fungal species is not reliable. To overcome this methodological limitation, we have introduced bead beating approach in order to improve the fingerprinting and filamentous fungi identification [22]. The beads are composed externally by a vitreous surface and the internal part is made up of a magnet which exploiting the formation of a magnetic field by a tissuelyser. Chains of cell wall, damages or completely breaks, allowing the extraction of proteins, DNA and RNA [23]. A total of 50 different clinical filamentous fungi isolates were analyzed with MALDI-TOF MS. According to the spectral scores recommend by MALDI-TOF all the isolates were correctly identified at the species and genus levels, respectively [24]. We reported exemplifying species focusing on the comparison between extraction techniques and not on species level identification by using MALDI - TOF MS Bruker System. The spectra obtained with our experimental method were compared to acid solvent extraction method. Acid solvent protein extractive method is the most used in dermatophytes diagnosis with MALDI-TOF technology [25]. The method has a good reproducibility, but not ensure accurate identification because of the incorrect ionization [26]. We analyzed 6 times (in duplicates) the same colony for dermatophyte strains and *Aspergillus spp.* with the standard method and obtained a non-homogeneous protein structure. In fact, the protein relation m/z have a variable and inconstant absorption peaks, so it was not possible to have a single reference spectrum. In particular, *Trichophyton interdigitale* chemical extraction (Figure 1) show some truncated proteins, this proteins quantitative variability is related to incomplete extraction by chemical agents. Therefore, the reproducibility of spectra illustrated in this method, are particularly different in terms of protein expression intensity.

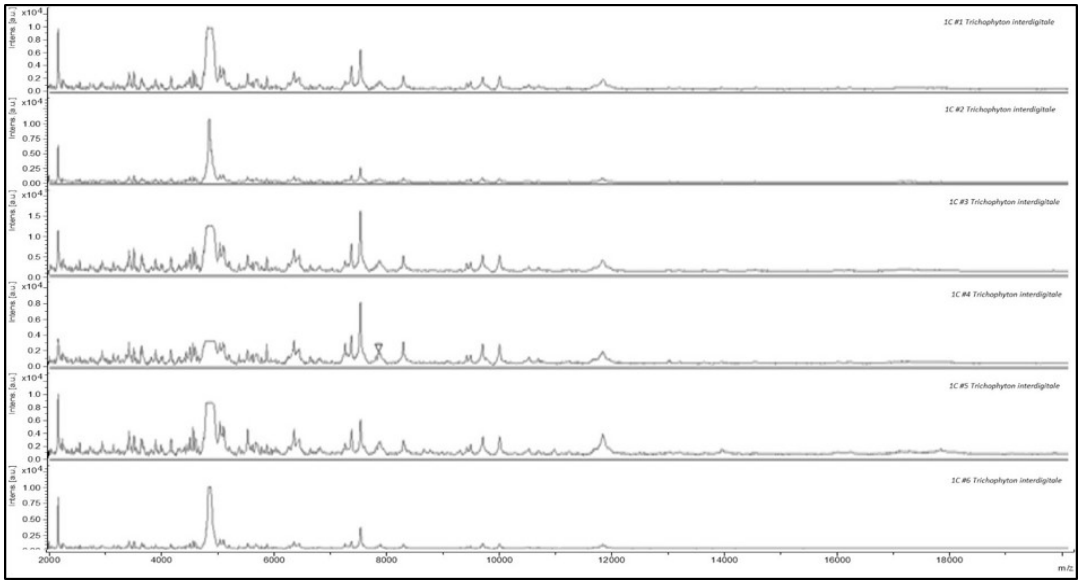

Figure 1: Spectra MALDI-TOF of *Trichophyton interdigitale* chemical extraction. The intensities and m/z refers to six single spectra of the same isolates in duplicate.

In this scenario powerful extraction method is required for repeatable and more reliable filamentous fungi fingerprinting mass spectra. Mechanical support (bead) was used to break the fungal cell wall that was incomplete with the exclusive use of acidic substances [27]. The bead, without altering the protein structure, totally destroyed polypeptide chains, thereby facilitating the acids action and matrix ionization. In Figure 2 spectra bead beating extraction have wider variability of protein pattern and it evidence more and better defined peaks. In fact, the bead beating spectra had a log-score values of 1,7-1,99 so there was a correct identification for genus but not species reliable. On the other hand, *Trichophyton interdigitale* isolates chemical extraction show the log-score values of <1,700. For all clinical isolates tested we obtained the same result about the improve of number and quality of spectra with bead beating extraction method. In particular, as shown in Figure 3, we reported same spectra of exemplifying species: *Trichophyton tonsurans, Trichophyton soudanense, Microsporum canis*, *Aspergillus terreus.* As known in literature, chemical extraction for *Aspergillus spp.* was validate instead of dermatophyte strains that could not be identified at the species level [28]. In our study a height identification was for *Aspergillus terreus*. The comparison between our experiment method, chemical method and a negative control, revealed however a better defined protein expression with bead beating Figure 4. It is evident that in chemical extraction the average spectrum is less precise and less linear average for interference peaks. Clinical isolates were confirmed for genus and species with score of ≥ 2.309.

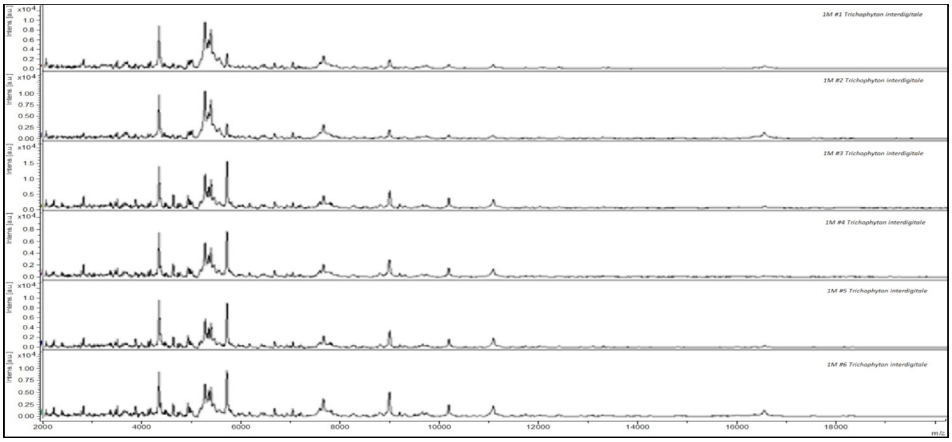

Figure 2: Spectra MALDI-TOF of *Trichophyton interdigitale* bead beating extraction. The intensities and m/z refers to six single spectra of the same isolates in duplicate.

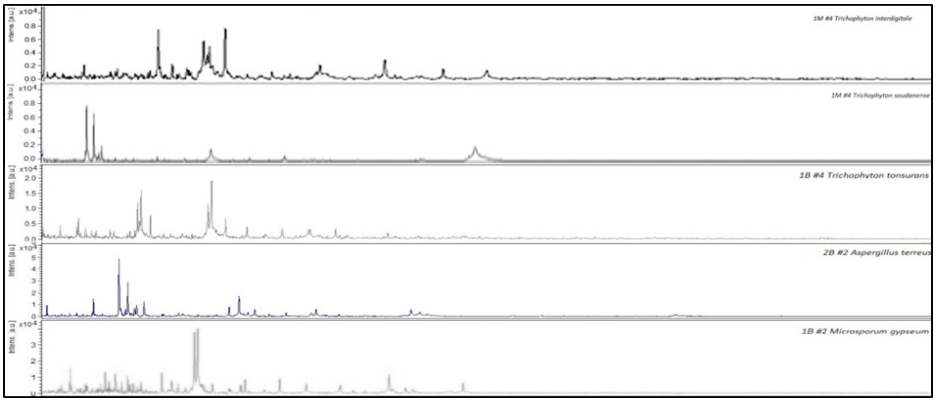

Figure 3: Clinical isolated spectra obtained with beat beating experimental method.

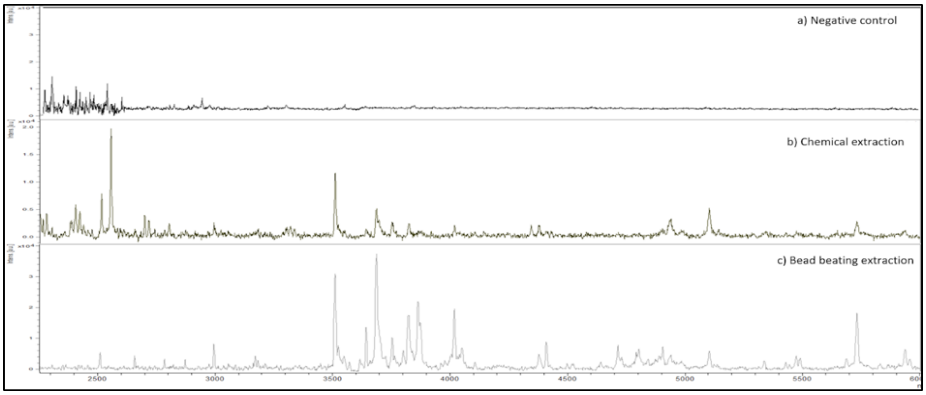

Figure 4: Comparison *Aspergillus terreus* fingerprinting spectra obtained with chemical (b) and bead beating (c) protein extraction protocol. Negative control spectra (a) was obtained by directly transferring growing mycelium samples on Maldi target plate with the addition of HCCA matrix.

In conclusion, it was found MALDI TOF MS based identification of filamentous fungi was easier than the traditional method and less expensive than the sequencing analysis, however could be a limitation about species and genus correctly identification. Hettick et al. present MALDI fingerprinting spectra of Aspergillus species using zirconium beads protein extraction method, our experimental method used the same principle but use different type of beads to improve the fingerprinting spectra and suppress the background noise. For example, the MALDI-TOF mass spectra of *T.rubrum* and *T. soudanense* show a lot of similarities to one another than to other species. Our experimental method is able improve the extraction protein by obtain more and defined fingerprinting spectra.

Discussion

Macroscopic observation and direct post-staining microscopic examination was conventional methods that are turn-around time and misidentify the fungi [28]. Rapid test (β 1,3- D glucan and galactomannan) was developed to detect a fungal infection but the specificities are limited because of false negative or positive result. MALDI-TOF MS represents an important and valuable tool to discriminate fungal species. This approach reduces the time to identified filamentous fungi by assessing microscopic and macroscopic morphology or by DNA sequencing [29]. In clinical laboratories practise, dermatophytes and other filamentous fungi identification has recently been explored compared to bacterial identification due to the high biological complexity fungal structure [30]. In addition, the identification may be influenced to sample preparation, incubation time and medium selection. MALDI TOF compared to molecular - based identification is rapid, accurate and cost effectiveness; however commercial libraries are incomplete [26]. We were able to demonstrate that MALDI- TOF mass spectrometry with bead beating protein extraction protocol, is reliable and comparable method for the identification of filamentous fungi to genus and species level. The aim of our study was to start from 50 clinical isolates, comparing current methods and identifying a standard protocol in laboratory practice [31-34].

Conclusion

In this vision we would implement the current database with more clinical and environmental filamentous fungi to construct a wider and representative database for assessing the intergenera and interspecies variability. Bead beating extraction method will widen the in-house reference libraries and support conventional identification by obtaining a fast, accurate and definitive result.

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