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Towards proteomic species barcoding of fungi – An example using *Scedosporium/Pseudallescheria* complex isolates

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ABSTRACT

MALDI-ToF mass spectrometry offers fast and reliable species identification for bacteria and yeasts under clinical routine conditions. Here, we produced mass spectra for identification of clinically important species of the *Pseudallescheria/Scedosporium* complex using the recently suggested new nomenclature and use this example to discuss to what extent the principle of DNA barcoding might be transferred to mass spectrometry.

Introduction

To date, six different potentially human pathogenic mould species have been recognized within the *Pseudallescheria/Scedosporium* genus, namely *Scedosporium apiospermum*, *Scedosporium aurantiacum*, *Scedosporium dehoogii*, *Lomentospora prolificans*, *Scedosporium boydii*, and *Pseudallescheria minutispora* (Zeng *et al.* 2007).

Infections due to moulds of this genus have been increasingly reported over the past decades. Originally observed in cases of near-drowning, these fungi are now also known to be common colonizers of lungs of cystic fibrosis patients. The six species significantly differ in their susceptibility patterns towards commonly used clinical drugs (Alastruey-Izquierdo *et al.* 2007; Lackner *et al.* 2012a). Fast and accurate species identification of these pathogens is therefore a clinical diagnostic necessity.

Although tests are available to amplify and differentiate DNA directly from clinical specimen [e.g. (Babady *et al.* 2011; Harun *et al.* 2011; Buelow *et al.* 2012; Lackner *et al.* 2012b)], culture is still necessary to obtain isolates for susceptibility testing procedures. With an isolate at hands, also microscopy or less cumbersome mass spectrometric species identification is possible. Indeed, MALDI-ToF mass spectrometry has

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previously been shown to be able to identify most of these organisms to the species level using both the MALDI BioTyper (Coulibaly *et al.* 2011) and the Andromas (Sitterle *et al.* 2014) systems.

Here, we contribute publicly available reference mass spectra for identification of clinically relevant *Scedosporium/Pseudallescheria* complex isolates using the liquid culture procedure according to the MALDI BioTyper Fungi library and use this example to discuss to what extent the principle of barcoding can be transferred to mass spectrometry.

Materials and methods

Strain set

As reference isolates we used *Pseudallescheria angusta* (CBS 245.72), *Scedosporium boydii* (CBS 117432, CBS 120157), *Pseudallescheria ellipsoidea* (CBS 301.79), *Scedosporium apiospermum* (CBS 117410), *Scedosporium aurantiacum* (CBS 116910, CBS 136046, CBS 136047, CBS 136049), and *Lomentospora prolificans* (IMMI-1647) (Steinmann *et al.* 2011). Strains were supplied as living cultures, and subsequently propagated on Sabouraud's agar at 25 °C. For local long-term storage the strains were placed at −80 °C in cryobank tubes (Mast Diagnostica, Reinfeld, Germany). Additionally we used a set of clinical testing isolates of *S. boydii* (IMMI-B-F90), *S. apiospermum* (IMMI-E-71 and IMMI-E-77), and *L. prolificans* (IMMI-1644, red; IMMI-1645, IMMI-1646, IMMI E78, and IMMI E79) (Steinmann *et al.* 2011).

Sample preparation and spectrum acquisition

Sample preparation followed the 'Fungi library' sample preparation protocol recommended by the manufacturer of the MALDI BioTyper (Bruker Daltonics, Bremen Germany). Briefly, a 5 ml liquid Sabouraud's medium culture (Oxoid, Wesel, Germany) was inoculated with a large amount of culture material scraped from an agar plate and grown on a turning wheel at 25 °C for 24 h yielding a high quantity of small mycelial balls. Mycelium from 1 ml was harvested by centrifugation at maximum speed in a conventional table top centrifuge, supernatant aspirated and the pellet washed with 70 % ethanol. After thorough drying, mycelium was suspended in 100 µl 70 % aqueous formic acid and incubated at room temperature for 30 min in a tube shaker at maximum shaking (180 rpm). To the lysate, 100 µl acetonitrile was added, the sample mixed thoroughly by vortexing and the debris removed by centrifugation. 1 µl of the supernatant was placed on a polished steel target (Bruker Daltonics, Bremen, Germany), air dried and overlaid with 1 µl of saturated HCCA matrix solution (Bruker Daltonics) spiked with recombinant human insulin (Sigma Aldrich, Taufkirchen, Germany) for internal calibration. Twelve mass spectra (technical replicates) of three biological replicates each were acquired using standard settings implemented in the MALDI BioTyper software on a Bruker Daltonics Autoflex III series instrument.

Generation of MALDI BioTyper database reference spectra

Low-quality mass spectra were eliminated manually and the remaining internally calibrated to the insulin mass peak (m/z = 5806 Da). MSPs were generated using the standard settings implemented in the BioTyper software (tophat baseline correction, 80 % peak presence required for MSP inclusion). The insulin mass signal was manually removed from the MSPs.

Results and discussion

MALDI-ToF mass spectrometry of whole cell lysates, also called 'intact cell mass spectrometry (ICMS)', has found its way into clinical routine species identification over the past decade, replacing biochemical methods to a very large degree. This method relies on the generation of species-specific fingerprints that can be looked up in a custom-made database. Easy sample preparation, high-speed lasers and modern computers have advanced this to be the most effective method currently available. Initially mainly developed on human pathogenic bacteria, identification of commonly occurring yeasts [reviewed in (Bader 2013)] is routinely possible. In contrast, the identification of moulds is not yet a standard procedure. In part this is due to the fact, that the understanding of mould phylogeny is a highly fluctuating field and there is no generally accepted database. Therefore, MALDI-ToF-based mould species identification currently resides in the hands of experts for the particular fungal genus, leading to a diverse set of custom databases [e.g. (Erhard *et al.* 2008; Cassagne *et al.* 2011; Alshawa *et al.* 2012; De Carolis *et al.* 2012; Schrodl *et al.* 2012)].

MALDI-ToF mass spectrometry has recently also been shown to be able to recognize and discriminate the most important species of the *Pseudallescheria/Scedosporium* complex using both the MALDI BioTyper (Coulibaly *et al.* 2011) or the Andromas (Sitterle *et al.* 2014) systems. Here, we have generated ten database references ('MSPs') for the species in the complex for use with the MALDI BioTyper adhering to the suggested novel nomenclature (Lackner *et al.* 2014). The references were internally validated using a jack-knife test and all correctly produced nearly 100 % score values >2.000 within their own species (*Lomentospora prolificans*, *Scedosporium aurantiacum*) and importantly no top-ranking false hits with the other respective four. In accordance with the proposed novel nomenclature, a clear distinction of isolates within the *S. apiospermum* complex (*S. apiospermum*, *S. boydii*, and potentially *Pseudallescheria ellipsoidea*, and *Pseudallescheria angusta*) was not possible using the MALDI BioTyper. Similarly, clustering of MSPs showed that *L. prolificans* was most distinct, while the MSPs of the other five species were more similar. This larger cluster could be further divided into two sub-clusters containing *Scedosporium boydii* and *P. ellipsoidea*, *Scedosporium apiospermum*, and *P. angusta* in one, *S. aurantiacum* in the other (Fig 1). The *Scedosporium/Pseudallescheria* identification ('SPID') mass spectrum dataset (distinguishing *L. prolificans*, *S. aurantiacum*, and the *S. apiospermum* complex) is available upon request for local integration into the MALDI BioTyper.

In analogy to DNA barcoding approaches where usually a divergent region of an rRNA gene is used for discrimination (e.g. the internal transcribed spacers separating the three different rRNAs), in bacteria the masses of highest intensity observed tend to correspond to ribosomal proteins as those are