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PP-061 [Bee Health]

VF UNIVERSITY OF LJUBLJANA Veterinary Faculty

Application of MALDI-TOF Mass Spectrometry for the Identification of the Fungus *Ascosphaera apis*

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Background/Purpose

Ascosphaera apis is a fungus responsible for chalkbrood disease in bees, which occurs sporadically in Slovenia and worldwide. Although it usually does not lead to colony collapse, chalkbrood weakens bee colonies, reduces their resistance to other infections, and decreases honey production, making it economically significant. Understanding the pathogen and using reliable diagnostic methods are essential for managing and preventing its spread. Diagnostic methods for chalkbrood disease and the influence of different *A. apis* isolates on the incidence and severity of the disease remain poorly researched. Traditional mycological diagnostics have primarily relied on the morphological and biochemical properties of fungi. However, the MALDI-TOF system (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) has been used for fungal identification. Many fungal species can be identified based on specific protein profiles, but the current manufacturer's database does not yet contain corresponding reference profiles for *A. apis*.

FIGURE 1: We sampled diseased larvae (a), incubated them on SDA medium and isolated pure cultures (b). The sample was stained with cotton blue in lactophenol, allowing observation of reproductive structures (c). The mycelium was then processed using a specialized protocol for further analysis with MALDI-TOF.

Methodology

Reference and Clinical Isolates

Four *Ascosphaera apis* and six other *Ascosphaera* species reference strains were obtained from the ARSEF (USDA) collection. Thirty clinical isolates were isolated from mummified honeybee larvae showing chalkbrood-like symptoms in Slovenian apiaries.

Cultivation and Sample Preparation

Isolates were cultured on Sabouraud dextrose agar at 30 °C. Three protein extraction protocols were evaluated for MALDI-TOF MS analysis:
Liquid Cultivation Protocol – Mycelium grown in broth was extracted using a modified commercial protocol.
Ethanol/Formic Acid Extraction – Mycelium was directly harvested from solid media and processed.
Mycelium Transfer (MyT) – Fresh mycelium was applied directly to the MALDI target plate with formic acid and matrix overlay.

MALDI-TOF MS Analysis

Protein spectra were acquired using a Bruker Microflex LT/SH instrument. A reference library was generated from ten *Ascosphaera* reference strains and validated using spectra from the clinical isolates. Identification scores were interpreted according to manufacturer guidelines.

Molecular Confirmation

DNA was extracted from selected isolates and reference strains. Whole-genome sequencing (WGS) was performed to confirm species identity.

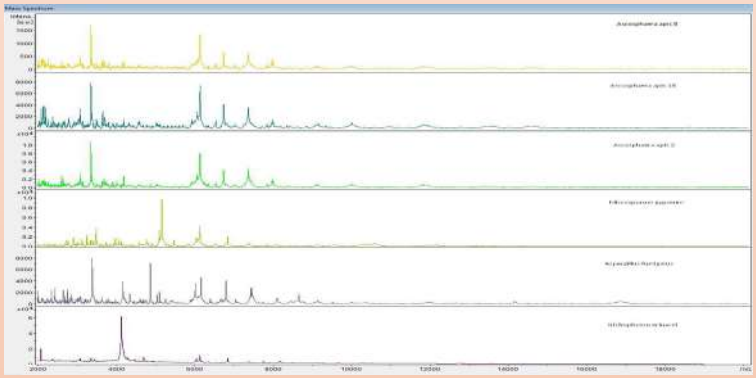

FIGURE 2: Protein profile graphs of *A. apis*. Comparison of curves indicates a similar profile among *A. apis* isolates, which significantly differ from those of other fungi

Results and Conclusion

Our findings demonstrate that MALDI-TOF MS is a rapid and reliable tool for species-level identification of *Ascosphaera*. Both the liquid cultivation and ethanol/formic acid extraction methods showed excellent performance in terms of identification accuracy under the tested conditions. While the direct mycelium transfer (MyT) method proved somewhat less consistent, it still demonstrated promising results and may serve as a useful rapid alternative.

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Problem Description
Diagnostic methods for chalkbrood disease and the influence of different A. apis isolates on the incidence and severity of the disease remain poorly researched. Traditional mycological diagnostics have primarily relied on the morphological and biochemical properties of fungi. However, the MALDI-TOF system (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) has been used for fungal identification. Many fungal species can be identified based on specific protein profiles, but the current manufacturer's database does not yet contain corresponding reference profiles for A. apis.

Methodology
To apply MALDI-TOF MS for diagnosing A. apis isolates, a reference spectra database was first required. Reference strains (AFSEF 7405 and ARSEF 7406) were analyzed to create specific protein spectra for comparison with spectra obtained from field samples. Our isolates were obtained from mummified bee larvae collected from bee colonies with chalkbrood signs in apiaries across Slovenia. Samples were inoculated on SDA medium at 30°C, where white, fluffy mycelium developed. Initial identification was conducted through macroscopic and microscopic examination. Cytoplasmic proteins of A. apis were extracted from mycelium grown in liquid SDA medium and processed according to the manufacturer's instructions.

Results and CONCLUSION: MALDI -TOF MS efficiently identified A. apis isolates by detecting distinct protein spectra. An internal database for A. apis and related fungi was created for the first time, confirming MALDI-TOF MS as a rapid and reliable tool for A. apis identification. The newly established database offers great potential for expanding the monitoring of bee pathogens.

Practical Implications for Beekeepers
By understanding the biology and symptoms of A. apis and utilizing MALDI-TOF MS, beekeepers can adopt proactive measures to protect their colonies, reduce economic losses, and improve overall beekeeping practices.