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Short Communication

MALDI-TOF MS PROFILING OF PISCINE *ACINETOBACTER* SPECIES FROM WASTEWATER-RELATED WATERS

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Abstract

Acinetobacter species are common inhabitants of freshwater and marine ecosystems with a capacity to induce disease in affected fish. To facilitate their rapid and reliable identification, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), was applied to *Acinetobacter* from fish. The aims of the study were, thus, to identify and profile the *Acinetobacter* species from tissues of fish living in a vulnerable environment impacted by wastewaters, and to assess the potential of MALDI-TOF MS as a method for discriminating these acinetobacters. Fish were sampled from waterways impacted by the activity of a wastewater treatment plant. Samples of gills, spleen, kidney and liver were streaked onto general-purpose media to purity. The profiling and identification of acinetobacters was conducted with MALDI-TOF MS, with the samples prepared by ethanol/formic acid extraction.The identified acinetobacters were retrieved from gills (68.96 %), kidney (13.79 %), liver (10.34 %), and spleen (6.89 %). The *Acinetobacter* species isolated from all tested fish tissues were *A. johnsonii* (79.31 %), *A. pittii* (10.34 %), *A. tandoii* (3.44 %), *A. guilouiae* (3.44 %), and *A. gerneri* (3.44 %). Highly probable and probable species identifications were obtained for 48.27 % of all acinetobacters tested, indicating fully reliable identification. MALDI-TOF MS gave excellent identification and profiling results for piscine *Acinetobacter* species from the wastewater-affected waterways. It is a recommendable technique for future *Acinetobacter* species discrimination, as accurate and rapid identification of these

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bacteria is critical in both environmental pollution management and human/veterinary clinical diagnostics.

Key Words: *Acinetobacter*, fish, MALDI-TOF MS, wastewater

INTRODUCTION

Gram-negative *Acinetobacter* rod-shaped bacteria are common inhabitants of freshwater and marine ecosystems. They often populate skin and gills of fish, and the microorganisms are likely in continuous contact with fish. However, any break in the integument of the host may lead to colonization of the tissues and start a disease cycle (Austin and Austin, 2007). Fish exhibiting clinical signs of disease often harbor *Acinetobacter* species as co-infectious agents (Anjur et al., 2021). Clinically, depigmentation of the skin, loss of scales, exophthalmia with congestion of the eye, gill petechiae, hemorrhages in the skin, and intestinal inflammation are observed in diseased fish, accompanied by mortality rates up to 20% (Pękala-Safińska, 2018). Emerging *Acinetobacter* threats for fish include *A. baumanii* and *A. johnsonii* (Kozińska et al., 2014), highly pathogenic and virulent *A. pittii* (Wang et al., 2020), and *A. lwoffii* as a causal agent for red head disease in cultured freshwater fish (Cao et al., 2016). Furthermore, several *Acinetobacter* species have high zoonotic potential (Wang et al., 2020).

To facilitate rapid and reliable identification of aquatic and piscine bacteria, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been introduced as a vital tool. It allows high throughput, sensitive and specific applications in clinical diagnostics and profiling of bacteria to the genus, species and even strain-level (Topić Popović et al., 2021). In short, protein mass spectra containing m/z peaks relating to ribosomal proteins in bacterial cells are detected. For ionization of protein samples, the matrix solution is mixed with the sample to be analyzed, enabling formation of protein mass spectra with specific molecular weight ranges. Measured mass signals are compared with mass spectra from reference bacterial strains collected in a mass spectra library (Topić Popović et al., 2017). With respect to *Acinetobacter*, the technique can distinguish between 22 species (Table 1).

In this work, we used MALDI-TOF MS for identification of *Acinetobacter* species retrieved from fish living in wastewater-related waters. One of the characteristics of *Acinetobacter* species is their ability to survive in adverse environmental conditions. They have previously been isolated from raw sewage, wastewater treatment plants, and activated sludge (Adewoyin and Okoh, 2020; Topić Popović et al., 2015a; Doughari et al., 2011). The aims of this study were, thus, to identify and profile the *Acinetobacter* species from tissues of fish living in a highly vulnerable environment impacted by wastewaters and to assess the potential of the MALDI-TOF MS method used to discriminate these bacteria.

Table 1. *Acinetobacter* species covered in the MALDI Biotyper 3.0 database (Bruker Daltonik). Species commonly isolated from fish (Xie et al, 2020; Austin and Austin, 2007) are in boldface type.

Species
Acinetobacter baumanii
Acinetobacter bayiyi
Acinetobacter berezinіae
Acinetobacter bouvetii
Acinetobacter calcoaceticus
Acinetobacter gerneri
Acinetobacter guillouiae
Acinetobacter haemolyticus
Acinetobacter johnsonii
Acinetobacter junii
Acinetobacter lwoffii
Acinetobacter nectaris
Acinetobacter nosocomialis
Acinetobacter parvus
Acinetobacter pittii
Acinetobacter radioresistens
Acinetobacter schindleri
***Acinetobacter* sp.**
Acinetobacter tandoii
Acinetobacter tjernbergiae
Acinetobacter towneri
Acinetobacter ursingii

MATERIALS AND METHODS

The study was carried out throughout the treatment process of the Virovitica (Croatia) wastewater treatment plant (WWTP). Fish were sampled from the waterways impacted by the activity of the WWTP. The study was conducted in accordance with the EC Directive 86/609/EEC for animal experiments, complying with the provisions of national legislation. Prussian carp (*Carassius gibelio*) were caught by nets and angling by registered anglers, while competent licensed veterinarians manipulated the fish. Prussian carp (n = 69) had mean weight of 313.30 g ± 164.68, and mean length of 191.84 mm ± 55.13. Fish were rapidly sacrificed by overdose of tricaine methanesulfonate (MS-222, Sigma, St. Louis, Missouri, USA).

Samples of fish gills, spleen, kidney, and liver were streaked onto Tryptone Soya Agar, MacConkey Agar (both Oxoid Ltd, Basingstoke, England, UK) and Blood Agar

(Certifikat doo, Osijek, Croatia). Representative colonies from up to 72h incubation at 22°C were selected and restreaked on fresh media until purity. Pure cultures were subcultured and maintained in the above conditions. The taxonomic profiling of the isolates was determined by MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany) (Figure 1). Samples for MALDI-TOF MS were prepared by ethanol/formic acid extraction (full extraction) as described in Topić Popović et al. (2021) (Figure 2).

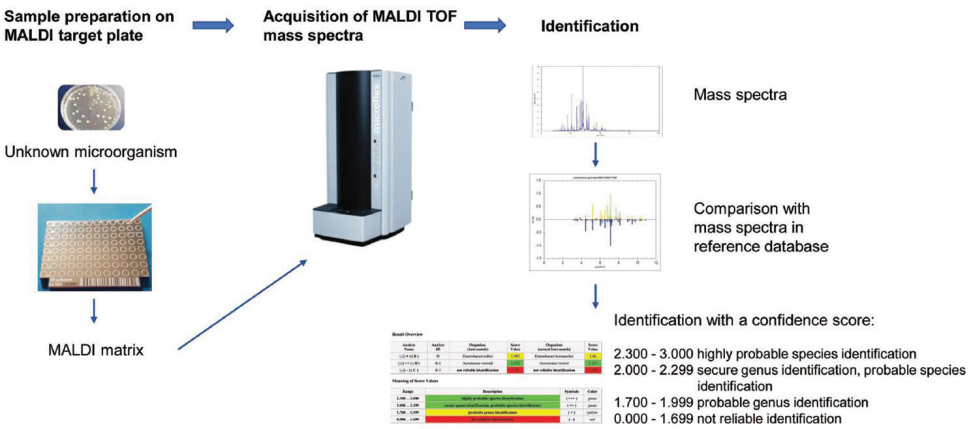

Figure 1. Schematic representation of the principles of a typical workflow for MALDI-TOF MS bacterial identification. For ionization of protein samples, the matrix solution is mixed with the sample to be analyzed, enabling formation of protein mass spectra with specific molecular weight ranges. Measured mass signals are compared with mass spectra from reference bacterial strains collected in a dedicated mass spectra library or with publicly available proteomics/genomics data (Adopted with permission from Topić Popović et al., 2021).

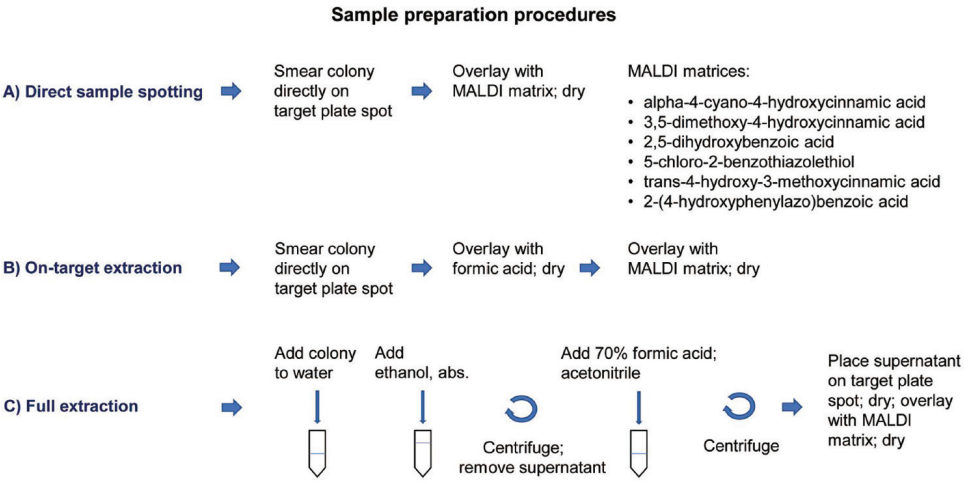

Figure 2. Schematic summary of sample preparation procedures for MALDI-TOF MS bacterial identification: A) Direct sample spotting, B) On-target extraction, C) Full extraction. The most commonly used matrices are listed (Adopted with permission from Topić Popović et al., 2021).

Obtained mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker Daltonik), using standard settings. The identification criteria were following: a log score of 2.300 to 3.000 equals highly probable species level identification, a log score of 2.000 to 2.299 equals probable species identification, a log score 1.700 to 1.999 equals probable identification to the genus level, while a log score of < 1.700 equals unreliable identification.

RESULTS AND DISCUSSION

Of all the bacteria retrieved from the fish living in wastewater-related waters and identified by MALDI-TOF MS, *Acinetobacter* species comprised 15.18 %. Of these, the majority were from gills (68.96 %), and the rest were from kidney (13.79 %), liver (10.34 %), and spleen (6.89 %). The following *Acinetobacter* species were isolated from all tested fish tissues: *A. johnsonii* (79.31 %), *A. pittii* (10.34 %), *A. tandoii* (3.44 %), *A. guilouiae* (3.44 %), and *A. gerneri* (3.44 %). Highly probable and probable species identifications were obtained for 48.27 % of all acinetobacters tested, indicating fully reliable identification. The probable genus was identified for 41.38 %, and unreliable identification was signified for 3.44 % of isolates. For 89.66 % of acinetobacters examined, the second-best matches were also acinetobacters (Adewoyin and Okoh, 2020; Doughari et al., 2011).

Although health status examinations of fish bearing acinetobacters revealed only minor opercular bleedings and hemorrhages at the base of the fins, the predisposing features for disease outbreaks involve multiple stress factors such as adverse water quality problems, temperature shock, low oxygen level, and high ammonia and heavy metal levels (Plumb and Hanson, 2010), all attributable to wastewaters and related watercourses. Overall, MALDI-TOF MS identification and profiling of *Acinetobacter* species allowed rapid characterization of acinetobacters at the genus and species levels. The method has repeatedly proved its potential for speciating the unknown environmental bacterial isolates (Topić Popović et al., 2015b), as various species of *Acinetobacter* were separated by hierarchical clustering.

However, since sample preparation methods, matrix solutions, and cultivation conditions play a role in MALDI-TOF MS identification to the species level (Topić Popović et al., 2021), that should be taken into account when identifying environmental bacteria such as *Acinetobacter* species in future. This is particularly important since, aside from being piscine disease agents, *Acinetobacter* species are also carriers of multidrug resistance genes (Santos and Ramos, 2018), and they can spread drug resistance in the environment by various mechanisms (Pękala-Safińska, 2018; Bošnjak et al., 2014). In addition, *A. calcoaceticus, A. baumannii, A. pittii, A.nosocomialis, A. seifertii* and *A. lactucae* are opportunistic pathogens in nosocomial infections, have extreme drug resistance to many chemotherapeutics, and have become an ongoing public health threat globally (Wang et al., 2020; Kozińska et al., 2014). Moreover, *Acinetobacter* species with zoonotic potential isolated from fish may indicate fish are important zoonotic reservoirs for the

transmission of this pathogen to humans (Wang et al., 2020). Notably, this pathogen can cause severe infections in fish that do not exhibit any symptoms (Preena et al., 2019). The public health risk is, thus, a significant concern when handling fish from wastewater-related waters. Reassuringly, selected strains of *Acinetobacter* species have shown ecological and economic importance in bioremediation of industrial effluents (Adewoyin and Okoh, 2020). However, MALDI-TOF MS reference databases, although fairly adequate for the majority of routine medical and veterinary clinical bacteria isolates (Topić Popović et al., 2021), need continuous updating for environmental species such as *Acinetobacter*.

CONCLUSION

MALDI-TOF MS gave excellent identification and profiling results for piscine *Acinetobacter* species related to the wastewater-affected waterways. Highly probable and probable species identifications were obtained for nearly half of all acinetobacters tested. In order to obtain more secure-level identification of *Acinetobacter* species, MALDI-TOF MS reference databases need constant upgrading. Regarding the importance of *Acinetobacter* species as fish disease and zoonotic agents, carriers of multidrug resistance, and sources of nosocomial infections, accurate and rapid identification of these bacteria is critical in environmental pollution management as well as in human and veterinary clinical diagnostics.

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Authors' contributions

NTP conceived the study, carried out the fieldwork, isolated the bacteria, and wrote the paper. SK conducted MALDI-TOF MS analyses and interpreted the results. RČR participated in design and coordination of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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MALDI-TOF MS PROFILI ACINETOBACTER SPP. IZ RIBA U VODOTOCIMA POD UTICAJEM PROČIŠĆENIH OTPADNIH VODA

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Kratak sadržaj

Bakterije iz roda *Acinetobacter* uobičajeni su stanovnici slatkovodnih i morskih ekosistema i mogu izazvati bolesti u riba. Radi ispitivanja njihove brze i pouzdane identifikacije, koristili smo spektrometriju masa – matricom potpomognutu jonizaciju uz desorpciju laserskim zračenjem i analizatorom vremena leta (MALDI-TOF MS). Cilj ovog rada je bio identifikovati i profilisati vrste iz roda *Acinetobacter* iz tkiva riba koje žive u narušenoj sredini pod uticajem pročišćenih otpadnih voda i proceniti potencijal koji MALDI-TOF MS metoda ima u njihovoj diferencijaciji. Ribe su uzorkovane iz vodotoka koji su pod uticajem aktivnosti postrojenja za prečišćavanje otpadnih voda. Materijal škrga, slezine, bubrega i jetre je inokulisan na ploče sa standardnim hranjivim podlogama te je presejavan do dobijanja čistih kultura. Profilisanje i identifikacija acinetobakterija sprovedena je korišćenjem MALDI-TOF MS metode, pripremom uzoraka ekstrakcijom u etanolu/mravljoj kiselini. Identifikovane acinetobakterije dobijene su iz materijala škrga (68.96 %), bubrega (13.79 %), jetre (10.34 %) i slezine (6.89 %). Vrste iz roda *Acinetobacter* iz svih tkiva uključivale su *A. johnsonii* (79.31 %), *A. pittii* (10.34 %), *A. tandoii* (3.44 %), *A. guilouiae* (3.44 %) i *A. gerneri* (3.44 %). Vrlo verovatna i verovatna identifikacija vrste dobijena je za 48.27 % od svih testiranih acinetobakterija, ukazujući na pouzdanu identifikaciju. MALDI-TOF MS metoda dala je izvrsne rezultate identifikacije i profilisanja ribljih *Acinetobacter* spp. bakterija povezanih sa vodotocima pod uticajem pročišćenih otpadnih voda. MALDI-TOF MS može se preporučiti za buduće razlikovanje vrsta iz roda *Acinetobacter* jer je njihova brza i precizna identifikacija ključna kako za strategiju upravljanja zaštitom životne sredine, tako i za dijagnostiku kliničkih slučajeva u humanoj i veterinarskoj medicini.

Ključne reči: *Acinetobacter* spp., ribe, MALDI-TOF MS, otpadne vode