MALDI-TOF MS: a diagnostic tool for identification of bacterial fish pathogens

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Abstract

Traditional methods to identify bacterial pathogens involved in fish diseases are often slow and/or costly. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS) is an established, fast, accurate and cost effective protein-based technique for identification of bacterial pathogens in human and veterinary laboratories, and has now also been developed for identification of bacterial fish pathogens. Based on analysis of colonies sampled directly from agar plate cultures, the MALDI-TOF technique generates a protein spectrum which is then compared against spectra, called reference spectra, stored in the MALDI-TOF databases. Since not all bacterial fish pathogens are covered in commercial databases, database-libraries are usually constructed locally for relevant reference strains/species. In this study, results from two collaborative projects, involving four European veterinary medicine institutes, are presented, focusing on supplementation of available spectra and validation of the MALDI-TOF MS technique for identification of several bacterial fish pathogens. *Flavobacterium psychrophilum*, *F. columnare*, *Pseudomonas anguilliseptica*, *Renibacterum salmoninarum*, *Vibrio anguillarum* and *Yersinia ruckeri* are examples of important bacterial fish pathogens that may be successfully identified to the species level by MALDI-TOF MS. On the contrary, identification of *Aeromonas salmonicida* to species level requires additional phenotypic or genetic analysis to arrive at a correct identification. In conclusion, MALDI-TOF MS has proven a fast, cost effective and accurate identification method for use in fish bacteriology, with a potential for further improvements.

Introduction

Until recently, most clinical diagnostic laboratories used conventional phenotypic methods, such as biochemical tests, for bacterial identification. These techniques are laborious and time-consuming. Additionally, genotyping techniques were and are used, which are fast and accurate, but cannot replace cultivation, as there is often also a need for determination of the bacterium´s sensitivity to antimicrobials. Over the last decade, a new protein fingerprint-based mass spec-

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trometric technique has been introduced in many human and veterinary laboratories for identification of bacteria and fungi: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Croxatto et al., 2012; Lévesque et al., 2015). MALDI-TOF MS is an easy, accurate, rapid, high throughput and cost effective identification technique for Gram-positive and Gram-negative bacteria and fungi. Normally, the method readily identifies bacteria to the species level, and there are also examples of successful identification to the subspecies level (Croxatto et al., 2012; Sauget et al. 2017; Bridel et al., 2020; Giraud-Gatineau et al. 2020). Two commercial MALDI-TOF systems available are: Bruker Daltonic GmbH, Bremen, Germany, https://www.bruker.com/products/mass-spectrometry-and-separations/maldi-biotyper-for-microbiological-research.html and bioMérieux, Marcy-l´Étoile, France, https://www.biomerieux-diagnostics.com/vitek-ms-0, (Lévesque et al., 2015). Following the initial hardware and software investment, the running costs and time consumption per sample are low. MALDI-TOF has been successfully used for identification of several fish pathogens (Dieckmann et al 2010; Assis et al 2017; Fernández-Álvarez et al. 2017). The network of "Collaborating Veterinary Laboratories" (CoVet Lab) is a European communication and collaboration network, here represented by DTU (Denmark), WBVR (the Netherlands) and SVA (Sweden). The aim of this CoVetLab initiative, in cooperation with the Norwegian Veterinary institute (NVI), was to validate and improve the MALDI-TOF technique for identification of bacterial pathogens of fish, for implementation in our diagnostic laboratories.

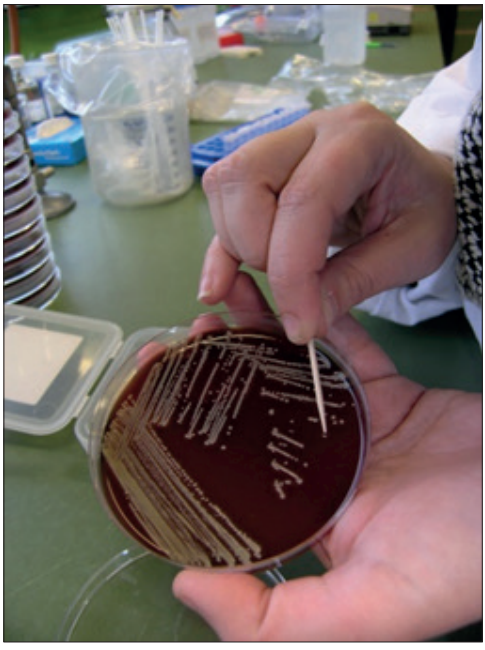

Figure 1. With a wooden stick, material from a single colony is taken for MALDI-TOF testing.

Method

The principle of MALDI-TOF MS is that the sample, i.e. the bacterial colony, is smeared onto a spot on a metal target plate, covered with a laser energy absorbing solution, the "matrix" (Figure 1 and 2). The target plate (MSP 96 target polished steel BC art no. 828000) has up to 96 spots for individual samples including a control bacterial test standard (BTS; Bruker Daltonic, art. no. 255343). The BTS standard is regularly used for calibration of the instrument. Fresh "matrix" is prepared by dissolving α-cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonic, art no 255344) in a standard solvent consisting of acetonitrile (50%) and trifluoroacetic acid (2.5%) in LC-MS water according to the manufacturer´s instructions. The sample and the "matrix" co-crystallize on the target plate prior to insertion of the plate into the MALDI-TOF device. When

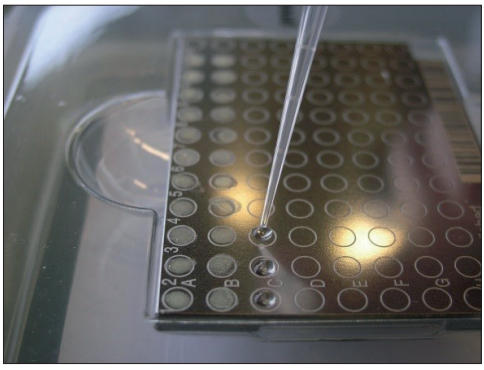

Figure 2. MALDI-TOF testing. After the bacterial colony material is smeared on a spot at the metal target plate, it is covered with an energy absorbing solution, the " matrix", after which the plate is dried and put into the MALDI-TOF device for identification of the bacterium.

vacuum is achieved, a laser beam irradiates each spot and the matrix molecules adsorb the energy, become protonated, and transfer the charge to the bacterial proteins, resulting in protonated molecules. The voltage difference between the target plate and the grid wire causes acceleration of the charged molecules into a vacuum tube. Their "time of flight" across the distance of the tube is variable, depending on the mass to charge ratio (m/z) of the proteins, which usually results in a unique mass spectrum for each bacterial species. Using a bio-statistical algorithm, this mass spectrum, generated from proteins with masses between 2000 to 20000 Daltons, is compared with the ten closest reference spectra in the database. The result is depicted in the MALDI Biotyper RTC software (Bruker®) as a log (score) value between 0.00 and 3.00. Interpretation is made easy by the use of a 'traffic light' color scheme: 2.0-3.00 gives a green color, indicating a high confidence of identification up to species level when morphology and growth characteristics is in agreement, 1.7-1.999 gives a yellow color, with a likely safe confidence to genus level, and < 1.70 gives a red color, with no organism identification possible (www.bruker.com).

Although highly cost- and time-efficient, the MALDI-TOF MS method is only capable of identifying bacterial species for which pre-established reference spectra are already present in the databases. While the technique was initially introduced in fish diagnostic laboratories in 2012, many bacterial fish pathogens were not present in the standard databases supplied by the companies at that time. Therefore, in 2013, a project to improve the MALDI-TOF MS system for identification of bacteria of veterinary interest was initiated by the CoVet Lab of the United Kingdom (APHA), the Netherlands (WBVR), Denmark (DTU) and Sweden (SVA). The National Reference Laboratories for Fish diseases at WBVR, DTU and SVA produced main spectra profiles (MSPs) for important fish pathogens according to instructions by Bruker. In short, strains from the laboratories own collections of verified identity were cultured on agar and a loopful of the bacterial colonies was suspended in 75 % ethanol for a formic acid extraction of the proteins: Bacteria were pelleted, air dried and resuspended in 20 µL of 70% formic acid. A similar volume of acetonitrile was added after 2 min. Following centrifugation of this mixture, the supernatant was spotted on the target plate. Clinical isolates and reference bacterial strains from each lab were subsequently MALDI-TOF tested against the Bruker® database supplemented with the new MSPs. The identifications were compared with the classical phenotypical identification using API systems (Biomerieux®: https://www.biomerieux-usa.com/clinical/api) or PCR, according to the standard procedures of each laboratory.

Several serotypes of *Vibrio anguillarum* and *Yersinia ruckeri* exist, as well as two different biotypes of *Y. ruckeri* with different grades of virulence. In the context of intervention against bacterial diseases, identification of serotype and biotype may be important. The intention in a follow-up CoVetLab project in 2015-2016, was therefore to test whether different serotypes and biotypes could be differentiated by identification of unique MALDI-TOF peaks. The bacteria involved were: *Aeromonas salmonicida* and biotypes and/or serotypes of *V. anguillarum, V. vulnificus*, and *Y. ruckeri*. The MALDI-TOF results were compared with those generated by biochemical or molecular methodologies. The project focused on minor spectral differences between subspecies, according to different MALDI-TOF subtyping techniques kindly provided by Bruker®. Spectra were produced from ten *A. salmonicida* isolates recovered from diseased Arctic char, brown trout, Atlantic salmon and rainbow trout. Spectra were also produced from reference strains of *A. salmonicida* (Table 1). Differences in MALDI-TOF spectra were compared with biochemical profiles and A-layer (*vap*A) genotyping (Gulla et al., 2019).

Results

Initial testing of identification of important fish pathogenic bacteria by MALDI-TOF MS, identified missing profiles for *Flavobacterium columnare, F. psychrophilum* and *Renibacterium salmoninarum* in the manufacturer´s database. Since there was also a goal to identify *V. anguillarum*, *V. vulnificus* and *Y. ruckeri* into serotypes and biotypes by MALDI-TOF MS technique, new reference MSPs were produced also for these, as a complement to the manufacturer´s database. MSPs were produced for *Aeromonas salmonicida*, *Flavobacterium columnare*, *F. psychrophilum*, *R. salmoninarum*, *Vibrio anguillarum, V. ichthyoenteri*, *V. splendidus, V. vulnificus* and *Yersinia ruckeri,* Table 1. Introduction of MSPs for *F. columnare, F. psychrophilum* and *R. salmoninarum* were all successful, and *F. columnare, F. psychrophilum, R. salmoninarum, V. anguillarum* and *Yersinia ruckeri* could all be successfully identified to species level. Several publications describe the extensive sequence diversity within *F. columnare* and up to six genomovars have been described (LaFrentz et al. 2018; García et al. 2018). Five of the *F. columnare* isolates used for production of MSPs in this study, have recently been classified by Runtuvuori-Salmela et al. (2020) with restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene according to LaFrentz (LaFrentz et al., 2014) to belong to genomovars I or I/II. The study by Runtuvuori-Salmela et al. demonstrates that genomovar I is dominating in Finland and Sweden, as all isolates from Finland (n=111) and 12 out of 15 from Sweden were classified as genomovar I. It remains to be investigated if the remaining four genomovars of *F. columnare* (II, II-A, II-B and III) are similarly successfully identified by MALDI-TOF MS.

Vibrios other than *V. anguillarum* were also often identified to species level using *Vibriobase* (Erler et al., 2015) as an extra uploaded library in the MALDI-TOF software. Analysis of *A. salmonicida* subsp. *salmonicida,* the non-motile bacterium (Colquhoun and Cunningham, 2002) responsible for furunculosis, resulted several times in identification as "*Aeromonas bestiarum*", which is a motile relative (Ali et al., 1996).
Three biochemical variants (reactions in maltose, arginine and lysine) and four A-layer types (I, IX, XI and XI) were identified amongst the clini-

Table 1. Produced MSPs (Main Spectra Profiles) of fish pathogenic bacteria, produced in the present project to be used together with the custom database for MALDI-TOF, for identification of fish bacteria.

Bacterial species	Strain/isolate no.	Host	Year
Aeromonas salmonicida subsp. *masoucida*	ATCC27013		
Aeromonas salmonicida subsp. *salmonicida*	ATCC33658		
Aeromonas salmonicida subsp. *achromogenes*	ATCC33659		
Aeromonas salmonicida subsp. *salmonicida* (vapI)	SW ASS170	Rainbow trout	1999
Aeromonas salmonicida subsp. *salmonicida* (vapI)	SW ASS182	Arctic char	2006
Aeromonas salmonicida subsp. *salmonicida*	SWASS183	Rainbow trout	2007
Aeromonas salmonicida subsp. *salmonicida*	SW ASS186	Arctic char	2013
Aeromonas salmonicida atypical (vap XVI)	SWASA389	Brown trout	2006
Aeromonas salmonicida atypical (vap XVI)	SWASA410	Brown trout	2012
Aeromonas salmonicida atypical (vap XI)	SWASA415	Arctic char	2013
Aeromonas salmonicida atypical (vap IX)	SWASA416	Brown trout	2013
Flavobacterium columnare	SWFc194	Brown trout	1997
Flavobacterium columnare	SWFc195	Brown trout	1997
Flavobacterium columnare	SWFc196	Brown trout	1997
Flavobacterium columnare	SWFc310	Brown trout	2000
Flavobacterium columnare	SWFc400	Koi carp	2002
Flavobacterium columnare	SWFc463	Brown trout	2009
Flavobacterium columnare	SWFc512	Brown trout	2013
Flavobacterium columnare	SWFc514	Brown trout	2013
Flavobacterium psychrophilum	ATCC49511		
Flavobacterium psychrophilum	NCIMB1947		
Flavobacterium psychrophilum	DK950106-1/1	Rainbow trout	1995
Flavobacterium psychrophilum	DK900406-1/3	Rainbow trout	1990
Flavobacterium psychrophilum	DK99-9A	Rainbow trout	1995
Flavobacterium psychrophilum	THCO90-02	Coho salmon	1990
Flavobacterium psychrophilum	THCO 90-04	Coho salmon	1990
Flavobacterium psychrophilum	CCUG35200		
Flavobacterium psychrophilum	SW6	Rainbow trout	1988
Flavobacterium psychrophilum	SW10	Rainbow trout	1988
Flavobacterium psychrophilum	SW241	Rainbow trout	1988
Flavobacterium psychrophilum	SW497	Rainbow trout	2012
Flavobacterium psychrophilum	SW499	Rainbow trout	2012
Flavobacterium psychrophilum	SW500	Rainbow trout	2012
Flavobacterium psychrophilum	SWFSK1521	Rainbow trout	2013
Flavobacterium psychrophilum	SWFSK1896	Rainbow trout	2013
Renibacterium salmoninarum	SW Rs4.86	Rainbow trout	1985
Renibacterium salmoninarum	SW Rs45 (Iceland)[1]	Atlantic salmon	1988
Renibacterium salmoninarum	SW Rs85	Atlantic salmon	1990
Renibacterium salmoninarum	SW Rs93	Arctic char	1989
Renibacterium salmoninarum	SW Rs 97	Rainbow trout	1991
Renibacterium salmoninarum	SW Rs 102	Rainbow trout	1991
Renibacterium salmoninarum	SW Rs121	Arctic char	1993
Renibacterium salmoninarum	SW Rs 129 (Canada)[2]	Coho salmon	1996
Renibacterium salmoninarum	SW Rs 130 (Canada)[2]	Chinook salmon	1998
Renibacterium salmoninarum	SW Rs 134(USA)[2]	Chinook salmon	1973
Renibacterium salmoninarum	SW Rs 147	Rainbow trout	1999
Vibrio anguillarum	ATCC43314		
Vibrio anguillarum	ATCC43307		
Vibrio anguillarum	ATCC43311		
Vibrio ichthyoenteri	ATCC 700023		
Vibrio splendidus	LGP32 IFREMER[3]		
Vibrio vulnificus	LMG 13545		

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cal isolates of *A. salmonicida* tested. MALDI-TOF typing indicated a unique profile for the A-layer type XI isolate, while only minor differences, inconclusive for a reliable differentiation, were observed in remaining clinical isolates or reference strains (Jansson et al., 2017). Results from an extensive whole genome-based population study of *A. salmonicida* (Gulla et al., unpublished data) verify the existence of multiple lineages within this species that cannot be assigned to any of the validly described subspecies, and MALDI-TOF may possibly prove useful in the future for easy discrimination between some of these. MALDI-TOF typing of international isolates of *V. vulnificus* isolated from eel and humans by Boonstra et al. (2019) gave promising results and these will be published in detail in a separate paper.

Comparison with other methods

Bacterial isolates from routine diagnostic investigations and laboratory archives were tested by MALDI-TOF utilising the Bruker database complemented with the new MSPs produced at DTU, WBVR and SVA. The results were compared with identifications when using standard techniques such as biochemical assays or genotyping. Electronic exchange of MSPs between laboratories allowed efficient comparison of results between laboratories. Introduction of MSPs for *F. psychrophilum, F. columnare* and *R. salmoninarum* were successful and tested isolates all scored above 2.0. As *F. columnare* often adheres to the agar medium it is important to sample this bacterium for MALDI-TOF identification as early as possible, before it attaches too firmly, as this might affect the result, due to agar contamination of the sample. *V. anguillarum* and *Y. ruckeri* are both included in Bruker´s database and all tested reference isolates were successfully identified to species level (Table 2). So far, differentiation of *V. anguillarum* or *Y. ruckeri* to serotype or biotype levels have not been possible. Although in most cases, MALDI-TOF identification of fish pathogenic bacteria was successful, there were some issues. Identification of *A. salmonicida* to species level using MALDI-TOF MS has proven difficult, as false positive identification of motile *Aeromonas* species occurred. In these cases, additional motility testing and other analyses are required. Pending establishment of robust MALDI-TOF spectra allowing reliable identification of *A. salmonicida* to the species level, MALDI-TOF based differentiation of *A. salmonicida* subtypes will likely remain unfeasible.

Basically, it is recommended that samples for analysis should be generated from a culture grown at a similar temperature to that used when generating the reference spectrum. This is one of the challenges working with fish pathogens from coldwater fish, like salmonids, since most of these bacteria grow at temperatures between 15 to 25°C, compared to pathogens of warm-blooded animals with optimum growth of 37°C. Since the Bruker database was primarily developed to identify human pathogens, the majority of strains used as references were cultured at 37°C. In the latest updates of the databases more fish pathogens have been added, including spectra for a large number of bacteria within the genus *Flavobacterium*, including *F. columnare* and *F. psychrophilum*. Also, the number of fish-pathogenic mycobacteria present in the database have been extended. Today, MALDI-TOF MS is implemented in our laboratories for efficient diagnosis of fish bacteria. Proficiency tests for MALDI-TOF MS

Table 2. Comparison of the MALDI-TOF results with the laboratories standard techniques for identification, with use of Bruker database supplemented with the new MSPs, produced in the present project.

Identification with each lab´s standard technique for identification	No of isolates tested	Identification with MALDI-TOF Best match (no. identified)	Score value in MALDI-TOF
Aeromonas salmonicida subsp. *salmonicida**	6	*Aeromonas salmonicida* (4) *Aeromonas bestiarum* (1) *Aeromonas eucrenophila* (1)	2.145 – 2.302 2.214 2.174
Aeromonas salmonicida atypical*	7	*Aeromonas salmonicida* (4) *Aeromonas bestiarum* (3)	2.13-2.171 1.917-2.214
*Flavobacterium columnare**	17	*Flavobacterium columnare*	2,135 – 2,417
*Flavobacterium psychrophilum** NVI 2016-60-459	59 4	*Flavobacterium psychrophilum* *Flavobacterium psychrophilum*	2.204 – 2.544 2.275-2.509
*Renibacterium salmoninarum**	15	*Renibacterium salmoninarum*	2.036 -2.4
Vibrio anguillarum, serotype I and II	7	*Vibrio anguillarum*	2.123 – 2.318
Vibrio vulnificus	3	*Vibrio vulnificus*	2.218 – 2.418
Vibrio splendidus	18	*Vibrio tasmaniensis* *Vibrio gigantis* *Vibrio chagasii*	1.784- 2.035
Yersinia ruckeri	11	*Yersinia ruckeri*	1.997 – 2.239
Pleisiomonas shigelloides	5	*Pleisiomonas shigelloides*	2.311 – 2.447
Pseudomonas anguilliseptica	1	*Pseudomonas anguilliseptica*	2.356
Hafnia alvei	1	*Hafnia alvei*	2.448

*Own MSP produced

have been organised between the labs. This underlines the importance of a long-lasting cooperation to keep active databases up to date, to ensure the quality, and to extend the possibilities for use of MALDI-TOF MS for identification and typing of fish-pathogenic bacteria.

Conclusion

MALDI-TOF MS is suitable for specific identification of pathogenic bacterial species of clinical relevance in veterinary medicine, including many fish pathogens. An advantage is that the companies´ databases can be extended locally through creation of new MSPs based on reference bacterial strains, which may in turn be easily exchanged digitally across labs. Furthermore, typing by MALDI-TOF may allow subspecies classification, as unique peaks may be identified in MALDI-TOF spectra. This re-

quires identification and careful comparison, of unique, stable differences in spectra of bacteria whose proteins are sufficiently different. In sum, MALDI-TOF MS represents a cheap and cost effective diagnostic tool for specific identification and typing of bacteria that enables rapid implementation of targeted ameliorative measures following outbreaks of bacterial fish disease.

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