Identification of bacterial pathogens in cultured fish using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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Abstract

Background Majority of infectious diseases of cultured fish is caused by bacteria. Rapid identification of bacterial pathogen is necessary for immediate management. The present study developed Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for a fast identification of fish bacterial pathogens. *Streptococcus agalactiae*, *S. iniae*, *Aeromonas hydrophila*, *A. veronii*, and *Edwardsiella tarda* obtained from diseased fish were used as representative bacterial pathogens in this study. Bacterial peptides were extracted to create the Main Spectra Profile (MSP), and the MSP of each bacterial species was added into the MALDI Biotyper database. Ten additional field isolates of each bacterial species were tested to validate the technique. **Results** The MSP of all field isolates were clearly distinguishable, and the MSPs of the same species were clustered together. However, the species identification when matching with the public MALDI Biotyper library (Bruker MALDI Biotyper) showed unreliable result at the species level. The accurate identification only obtained when the custom-made database was used, giving a 100% matching result. **Conclusion** This study demonstrates a newly developed technique for the effective identification of fish bacterial pathogens. Further applications require a broad, well-established database to accommodate the prudent identification of many fish bacterial pathogens by MALDI-TOF MS.

Background

Bacterial pathogens are a major etiology of infectious diseases of cultured fish [1]. Among those bacteria, *Streptococcus spp.*, *Aeromonas spp.*, and *Edwardsiella spp.*, are commonly found in several important aquaculture species, such as the Asian catfish *Clarias batrachus* [2], barramundi *Lates calcarifer* [3], and Nile tilapia *Oreochromis niloticus* [4]. In many cases of bacterial infection, clinical signs and lesions are not specific and may mislead the diagnosis. Therefore, identification of bacterial species causing disease is necessary in order to carry out proper disease management.

Conventional microbiology, including morphological, physiological and biochemical tests, and molecular techniques based on 16S rDNA sequencing, are the gold standard for bacterial species identification [5]. However, these techniques require a substantial amount of time and expensive reagents [6]. The recent development of mass spectrometry (MS), the Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been implemented in human and veterinary medicine as an alternative diagnostic tool with increasing popularity due to its quickness, simplicity, cost-effectiveness, and strong discriminating power [7, 8]. The MALDI-TOF MS detects the mass signals from bacterial proteins or peptides and determines their unique mass spectra or peptide mass fingerprints (PMFs). The obtained PMFs are then compared with reference bacterial strains in the public proteomics/genomics databases or in a dedicated mass spectra library (library based approach) [9], which is able to differentiate the bacteria at their genus, species or sub-group levels subject to sufficient reference strains existing in the database [10].

The MALDI-TOF MS approach has been adopted as a routine diagnostic tool for human medicine [11] and also has been widely evaluated for the ability to differentiate bacterial species of veterinary and public health importance. For example, *S. equi* at subspecies level [8], *Streptococcus spp.* isolated from diseased pigs [12], pathogenic Gram-negative bacteria in seafood [13] and *Aeromonad* species found in a drinking water system [14] have been assessed. In fish, MALDI-TOF was evaluated for the fast identification of Gram-positive bacterial pathogens, including *S. agalactiae*, *Lactococcus garvieae*, *S. iniae*, and *S. dysgalactiae* isolated from Nile tilapia [15] and *S. iniae* isolated from the olive flounder *Paralichthys olivaceus* [16]. These studies found that the public database, Bruker MALDI Biotyper library, was insufficient for identifying bacterial species isolated from fish with MALDI-TOF.

Therefore, the present study aims to develop a custom Main Spectra Profile (MSP) database and validate the method using MALDI-TOF MS for a rapid and accurate identification of *S. agalactiae*, *S. iniae*, *A. hydrophila*, *A. veronii* and *E. tarda* isolated from economically important fish species.

Results

MALDI-TOF MS for bacterial species differentiation

The high reliability of the MALDI-TOF MS was indicated by the obtained 100% recognition capabilities and by the cross-validation values of 87.8%, 97.1%, 100%, 100%, and 100% for *S. agalactiae*, *S. iniae*, *A. hydrophila*, *A. veronii*, and *E. tarda*, respectively. The five bacterial species showed distinguishing spectral peaks ranging between 2,000–15,000 Da (Fig. 1). The 3D-PCA scatterplot and the MSP dendrogram presented clearly distinguishable clusters for each bacterial species. For the MSP dendrogram, bacterial isolates within the same species were grouped in the same clade and the same genus were placed closer to each other (Fig. 2A and 2B).

MALDI Biotyper database

The additional field isolates, when blasted against the reference strains available in the Bruker database, gave no-reliable identification for 12 isolates (log score 1.454–1.667), probable genus-level identification for 31 isolates (log score 1.707–1.997), and secure genus-level identification for seven isolates (log score 2.018–2.254). No species-level identification was obtained, especially for *S. iniae* since it is not available in the Bruker database.

A custom database of the five bacterial fish pathogens was then established. Matching results of the field strain were improved, showing nine isolates with a secure genus-level identification to a probable species-level identification (log score 2.115–2.264) and 41 isolates with a highly probable species-level identification (log score 2.301–2.601) (Table 2). Specificity of the method was indicated by the 100% species identification compared with the 16S rDNA sequencing, and no mis-identification at species level was observed.

Discussion

An accurate and repeatable method for the identification of bacterial pathogens of aquaculture species was developed in this study. It can be performed in a relatively short time with a lower cost compared to conventional biochemical methods. However, the correct identification of bacterial species was only obtained when a custom database was constructed because the reference database does not accommodate the tested pathogens. The reference database may only contain bacterial species that are significant to humans but not species of veterinary importance, particularly from aquatic species [21]. The failure in species identification from the Bruker database may also result from inconsistent protein profiles due to the use of different sample preparation protocols. The extraction method usually involves the use of organic acid to extract small-sized protein molecules, such as ribosomal proteins, cold shock proteins, and nucleic-acid binding proteins [22]. The different percentage of acid used in other studies [50% ACN and 2.5% TFA] [16, 23, 24] may alter the pattern of those extracted proteins. Nevertheless, ability to tailor a database expands the application of MALDI Biotyper to be used as an identification tool for bacterial species specific to a host or location, and at below species-level, such as sub-species, strain, or serotype [25, 26].

Besides the identification purpose of MALDI-TOF, the 3D-PCA scatterplot generated from the protein profile can be used for grouping and differentiation of the protein profile of the organism. The protein profile analyzed with MALDI-TOF MS in the present study clearly showed separate clusters of each bacterial species on the 3D-PCA scatterplot and MSP dendrogram, indicating the discriminating power of the method. The MALDI-TOF MS approach has also been used to distinguish antimicrobial resistant Enterobacteriaceae [27], differentiate *Burkholderia pseudomallei* mutants [22], and even in screening for early or late stage cancer [28]. Therefore, MALDI-TOF MS can be a useful tool for investigating aquatic pathogens in many aspects.

In the present study, we provide an example of a MSP dendrogram created by the Biotyper software, by grouping the bacteria based on their phenotypic traits instead of their genetic traits. Interestingly, the ATCC strains from the reference database showed a high distance level to the field strains, and this may explain the failure of species identification described previously. Genotyping is usually based on phylogenetic analysis of a highly conserved region of the ribosomal RNA of the bacteria and this conservative feature may limit classification of the bacteria. Several studies have used MALDI-TOF MS as a discriminatory tool for typing bacterial pathogens [21, 29, 30], and found that genotypic and phenotypic traits do not always concur [31]. However, MALDI-TOF MS can be used as an additional method for bacterial taxonomic classification, which may benefit further research, such as epidemiology, identification of protein biomarkers, and virulence studies.

Conclusions

To our knowledge, the present study is the first to describe a MSP database for both Gram-positive (*Streptococcus*) and Gram-negative (*Aeromonas* and *Edwardsiella*) bacterial pathogens of cultured fish. It

could establish species-level identifications, even when the source of those bacteria was from different geographical locations or host species. However, this specificity can be obtained only when the custom MALDI Biotyper database was constructed with a standard sample preparation protocol. For the most reliable result, we suggested that the database of each user should contain a custom MSP of the active local pathogenic strains.

Methods

Bacterial samples

All bacterial isolates were obtained from clinical cases that were submitted to the Faculty of Veterinary Science, Chulalongkorn University for disease diagnosis. Bacterial isolation was performed on kidney tissues using Columbia blood agar supplemented with 5% sheep blood (Oxoid, Basingstoke, UK) and incubated at 28 °C for 24 h. Bacterial species were identified by conventional microbiology methods, including Gram staining, catalase and oxidase production tests, and API identification (BioMérieux®, France). Bacterial species were confirmed using PCR amplification and sequencing of the 16S rDNA [17]. Pure bacterial isolates were stored in nutrient broth (NB; Oxoid) containing 10% fetal calf serum and 20% glycerol at –80 °C for further analysis.

Sample preparation for MALDI-TOF MS

Each bacterial isolate was revived from the stock onto Columbia blood agar and incubated at 28 °C for 18 h. Extraction of bacterial proteins was performed as previously described by [18]. Briefly, a loopfull of bacterial colonies was suspended in 70% ethanol and the suspension was centrifuged at 11,000 g for 2 min. The supernatant was removed, and the bacterial pellet was resuspended and mixed thoroughly with 100% acetonitrile (ACN) containing 5% (w/v) trifluoroacetic acid (TFA). The suspension was centrifuged and the supernatant was collected for peptide measurement using Lowry's assay at 690 nm absorbance [19]. The concentration of peptide was adjusted to 0.1 µg µL⁻¹ for the MALDI-TOF MS analysis.

MALDI-TOF MS for database generation

Five bacterial isolates of each bacterial species were used as a representative for the MSP database preparation (Table 1). Peptide patterns of all isolates were identified by MALDI-TOF MS to generate a database specific to each bacterial species. The MALDI matrix solution [10 mg mL⁻¹ sinapinic acid in 100% ACN containing 5% (w/v) TFA] was added to each sample (0.1 µg µL⁻¹ peptide) at a 1:1 (v/v) ratio. The mixed samples were spotted and air dried onto a MTP 384 ground steel target plate (Bruker Daltonics, Billerica, MA) as 29 individual replicates. Mass spectra were obtained using an Ultraflex III TOF/TOF (Bruker Daltonik, GmbH) in a linear positive mode with a mass range between 2–20 kDa and a laser frequency of 50 Hz and 500 laser shots.

Fingerprint spectra were calibrated and analyzed by the flexAnalysis software version 3.4. The reliability of the results was analyzed by t-test/ANOVA and a construction of three-dimensional principal component analysis (3D-PCA) scatterplot was performed using ClinProTools software version 3.0. Database construction was performed according to Bruker's recommendation, where a total of 20 apparent peptide spectra of each isolate were selected to be uploaded into MALDI Biotyper (version 4.0). The MSP dendrogram of the MALDI-TOF MS derived data was then created from the constructed database in order to evaluate the relatedness of each bacterial species based on their peptide spectra.

Method validation and bacterial species identification

The same bacterial isolate was processed twice using a similar protocol and analyzed by MAILDI-TOF MS to evaluate the repeatability of the method. To assess the specificity of the method, additional field isolates of the five pathogens (10 additional isolates/pathogen) were used. All isolates were identified using 16S rDNA PCR and sequencing, as previously described [17], and were then identified by the MALDI-TOF MS method. The samples were spotted as eight individual replicates on the MALDI plate, blasted with both the Bruker database and then the created database. The reliability of the method was determined based on log score values; < 1.700 = no reliable identification, ≥ 1.700 – 1.999 = probable genus-level identification, 2.000 – 2.229 = a secure genus-level identification and a probable species-level identification, and 2.300 – 3.000 = a highly probable species-level identification [20].

Declarations

Acknowledgments

Not applicable.

Authors' contributions

PP and JW participated to the study design and planning. PP, SR and JW organized the project and agreed the objectives of the study. PP and TQH executed the sample collection. PP and JJ performed the sample treatment, method validation, and instrumental analysis. PP and JW prepared initial draft of the manuscript. SR and JW made revision and refinement of the manuscript. SN and JW applied for project's funding. All Authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the study are available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Bacterial pathogens used for the development the MSP database. The isolates were obtained from cultured Nile tilapia *Oreochromis niloticus*, red tilapia *Oreochromis spp.*, barramundi *Lates calcarifer*, hybrid catfish *Clarias macrocephalus* × *C. gariepinus*, and Snakehead fish *Channa striata*.

Bacterial species	Isolate number	Year	Source	Region
<i>S. agalactiae</i>	S147-J	2013	Nile tilapia	Western Thailand
	S183-J	2015	Red tilapia	Central Thailand
	S187-J	2017	Nile tilapia	Eastern Thailand
	S190-J	2018	Red tilapia	Southern Thailand
	SV1/1-J	2018	Nile tilapia	Northern Vietnam
<i>S. iniae</i>	NS12-J	2007	Red tilapia	Northeastern Thailand
	NS70-J	2012	Nile tilapia	Northeastern Thailand
	NS74-J	2014	Barramundi	Eastern Thailand
	NS76-J	2014	Barramundi	Eastern Thailand
	NS185-J	2018	Barramundi	Eastern Thailand
<i>A. hydrophila</i>	A28-J	2011	Nile tilapia	Eastern Thailand
	A29-J	2011	Hybrid catfish	Eastern Thailand
	A49-J	2013	Red tilapia	Central Thailand
	A50-J	2015	Nile tilapia	Eastern Thailand
	A84-J	2017	Snakehead fish	Central Thailand
<i>A. veronii</i>	SB1-J	2017	Nile tilapia	Eastern Thailand
	SB2-J	2017	Nile tilapia	Eastern Thailand
	SB3-J	2018	Barramundi	Eastern Thailand
	SB4-J	2019	Barramundi	Eastern Thailand
	SB7-J	2019	Red tilapia	Eastern Thailand
<i>E. tarda</i>	Ed10-J	2012	Hybrid catfish	Central Thailand
	Ed12-J	2013	Hybrid catfish	Central Thailand
	Ed14-J	2015	Nile tilapia	Eastern Thailand
	Ed16-J	2016	Nile tilapia	Eastern Thailand
	Ed18-J	2017	Nile tilapia	Central Thailand

Table 2 Method validation of the MALDI Biotyper using field strains of five bacterial pathogens showing the matching results with the custom MSP and the Bruker MSP database.

Isolate number	Source	Custom MSP database		Bruker MSP database	
		Organism best match	Log score	Organism best match	Log score
<i>Streptococcus spp.</i>					
S71	Red tilapia	<i>S. agalactiae</i>	2.244	<i>S. urinalis</i>	1.666
S96	Red tilapia	<i>S. agalactiae</i>	2.377	<i>S. agalactiae</i>	1.756
S100	Nile tilapia	<i>S. agalactiae</i>	2.442	<i>S. agalactiae</i>	1.582
S101	Nile tilapia	<i>S. agalactiae</i>	2.375	<i>S. parauberis</i>	1.829
S102	Nile tilapia	<i>S. agalactiae</i>	2.544	<i>S. parauberis</i>	1.876
S183	Red tilapia	<i>S. agalactiae</i>	2.388	<i>S. agalactiae</i>	1.716
S184	Red tilapia	<i>S. agalactiae</i>	2.591	<i>S. agalactiae</i>	1.749
S191	Red tilapia	<i>S. agalactiae</i>	2.347	<i>S. urinalis</i>	1.564
S195	Nile tilapia	<i>S. agalactiae</i>	2.455	<i>S. agalactiae</i>	1.632
S198	Nile tilapia	<i>S. agalactiae</i>	2.544	<i>S. agalactiae</i>	1.908
NS11	Nile tilapia	<i>S. iniae</i>	2.376	<i>S. agalactiae</i>	1.997
NS18	Nile tilapia	<i>S. iniae</i>	2.419	<i>S. agalactiae</i>	1.707
NS26	Red tilapia	<i>S. iniae</i>	2.534	<i>S. pyogenes</i>	1.728
NS34	Barramundi	<i>S. iniae</i>	2.544	<i>S. agalactiae</i>	1.886
NS50	Barramundi	<i>S. iniae</i>	2.308	<i>S. pyogenes</i>	1.679
NS84	Barramundi	<i>S. iniae</i>	2.118	<i>S. pyogenes</i>	1.679
NS85	Barramundi	<i>S. iniae</i>	2.445	<i>S. agalactiae</i>	1.864
NS89	Red tilapia	<i>S. iniae</i>	2.342	<i>S. agalactiae</i>	1.975
NS90	Red tilapia	<i>S. iniae</i>	2.221	<i>S. agalactiae</i>	1.873
NS91	Nile tilapia	<i>S. iniae</i>	2.464	<i>S. parauberis</i>	1.593
<i>Aeromonas spp.</i>					
A90	Nile tilapia	<i>A. hydrophila</i>	2.312	<i>A. veronii</i>	2.018
A108	Hybrid catfish	<i>A. hydrophila</i>	2.464	<i>A. hydrophila</i>	1.956
A109	Red tilapia	<i>A. hydrophila</i>	2.115	<i>A. hydrophila</i>	1.848
A110	Snakehead fish	<i>A. hydrophila</i>	2.394	<i>A. hydrophila</i>	2.181
A112	Snakehead fish	<i>A. hydrophila</i>	2.601	<i>A. hydrophila</i>	2.011
A114	Nile tilapia	<i>A. hydrophila</i>	2.451	<i>A. veronii</i>	1.995
A115	Nile tilapia	<i>A. hydrophila</i>	2.551	<i>A. ichthiosmia</i>	2.045
A120	Hybrid catfish	<i>A. hydrophila</i>	2.009	<i>A. veronii</i>	1.454
A126	Snakehead fish	<i>A. hydrophila</i>	2.567	<i>A. veronii</i>	1.777
A127	Snakehead fish	<i>A. hydrophila</i>	2.301	<i>A. shigelloides</i>	1.985
SB5	Nile tilapia	<i>A. veronii</i>	2.327	<i>A. ichthiosmia</i>	2.027
SB6	Nile tilapia	<i>A. veronii</i>	2.454	<i>A. hydrophila</i>	1.787
SB8	Barramundi	<i>A. veronii</i>	2.382	<i>A. hydrophila</i>	1.844
SB9	Barramundi	<i>A. veronii</i>	2.511	<i>A. veronii</i>	1.906
SB10	Barramundi	<i>A. veronii</i>	2.377	<i>A. veronii</i>	1.733
SB12	Barramundi	<i>A. veronii</i>	2.401	<i>A. veronii</i>	1.667
SB13	Barramundi	<i>A. veronii</i>	2.359	<i>A. hydrophila</i>	1.872

Isolate number	Source	Custom MSP database		Bruker MSP database	
		Organism best match	Log score	Organism best match	Log score
SB14	Barramundi	<i>A. veronii</i>	2.228	<i>A. ichthiosmia</i>	1.560
SB15	Barramundi	<i>A. veronii</i>	2.553	<i>A. veronii</i>	1.924
SB17	Barramundi	<i>A. veronii</i>	2.198	<i>A. veronii</i>	1.855
<i>Edwardsiella tarda</i>					
Ed8	Hybrid catfish	<i>E. tarda</i>	2.382	<i>E. hoshinae</i>	1.584
Ed9	Hybrid catfish	<i>E. tarda</i>	2.367	<i>E. hoshinae</i>	1.624
Ed11	Hybrid catfish	<i>E. tarda</i>	2.203	<i>E. hoshinae</i>	1.782
Ed17	Nile tilapia	<i>E. tarda</i>	2.471	<i>E. tarda</i>	1.977
Ed20	Nile tilapia	<i>E. tarda</i>	2.457	<i>E. tarda</i>	1.945
Ed23	Hybrid catfish	<i>E. tarda</i>	2.457	<i>E. tarda</i>	2.254
Ed25	Hybrid catfish	<i>E. tarda</i>	2.342	<i>E. tarda</i>	1.963
Ed27	Nile tilapia	<i>E. tarda</i>	2.264	<i>E. tarda</i>	2.014
Ed30	Nile tilapia	<i>E. tarda</i>	2.445	<i>E. tarda</i>	2.002
Ed31	Nile tilapia	<i>E. tarda</i>	2.116	<i>E. tarda</i>	1.956

Figures

Figure 1

Mass peptide fingerprints of five bacterial pathogens (*S. agalactiae*, *S. iniae*, *A. hydrophila*, *A. veronii* and *E. tarda*) isolated from cultured fish.

Figure 2

Cluster analysis of the five bacterial pathogens (*S. agalactiae*, *S. iniae*, *A. hydrophila*, *A. veronii* and *E. tarda*) isolated from cultured fish. (A) The 3D-PCA scatterplot representing clusters of each species (dashed circles) and the (B) MSP dendrogram of the representative bacterial isolates analyzed in the present study with the reference ATCC strains.