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Identification of *Vagococcus salmoninarum* from rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) using proteomics-based MALDI-TOF MS

Identificación de *Vagococcus salmoninarum* en trucha arcoíris (*Oncorhynchus mykiss* Walbaum, 1792) mediante espectrometría de masas MALDI-TOF basada en proteómica

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ABSTRACT

Vagococcus salmoninarum is a pathogen causing vagococcosis in rainbow trout (*Oncorhynchus mykiss,* Walbaum 1792) broodstock and large fish. In this study, conventional diagnostic methods, including phenotypic, genomic and high throughput proteomic MALDI-TOF MS were tested together for accurate and rapid identification of *V. salmoninarum.* Twelve isolates from extensively infected internal organs (fins, gills, liver, heart and kidney) of a total of fifty fish were phenotypically confirmed as *V. salmoninarum* by biochemical-enzymatic characteristics using the BBL Crystal™ GP identification system. However, the presence of positive PCR amplification of 16S rDNA gene was detected only in 83% of these isolates and the same samples were identified as *V. salmoninarum* by MALDI-TOF MS method with high mass score value (*m/z*) between 2.00 and 3.00. Based on the comparative data obtained in this study, we conclude that the MALDI-TOF MS method is the most promising and recommended method for the definitive identification of *V. salmoninarum*.

Key words: *Vagococcus salmoninarum*, Vagococcosis, BBL Crystal™ GP, PCR, MALDI-TOF MS

RESUMEN

Vagococcus salmoninarum es un patógeno que causa vagococosis en reproductores de trucha arcoíris (*Oncorhynchus mykiss,* Walbaum 1792) y peces grandes. En este estudio, se probaron juntos los métodos de diagnóstico convencionales, incluidos el fenotípico, el genómico y el proteómico de alto rendimiento MALDI-TOF MS, para lograr una identificación precisa y rápida de *V. salmoninarum*. Doce aislados obtenidos de órganos internos ampliamente infectados de un total de cincuenta peces fueron confirmados fenotípicamente como *V. salmoninarum* mediante características bioquímicas y enzimáticas con el sistema de identificación BBL Crystal™ GP. Sin embargo, la presencia de amplificación de PCR positiva del gen 16S rDNA se detectó solo en el 83 % de estos aislamientos y las mismas muestras se identificaron como *V. salmoninarum* mediante el método MALDI-TOF MS con un valor de puntuación de masa alto (*m/z*) entre 2.00 y 3.00. Con base en los datos comparativos obtenidos en este estudio, concluimos que el método MALDI-TOF MS es el método más prometedor y recomendado para la identificación definitiva de *V. salmoninarum*.

Palabras clave: *Vagococcus salmoninarum*, Vagococcosis, BBL Crystal™ GP, PCR, MALDI-TOF MS

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INTRODUCTION

Vagococcus salmoninarum, is a species of bacteria gram-positive, non-motile, chain-forming pathogen that lives at water temperatures below 10–15°C [1, 2, 3], is the causative agent of "cold water also known streptococcosis" known as "vagococcosis" in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) broodstock and large fish [4, 5, 6]. Cases of *V. salmoninarum* were first reported in rainbow trout [1] and then brook trout (*Salvelinus fontinalis*) [7] in the USA, Australia [8], France [5], Italy [9], Spain [10, 11] and Turkey [12, 13, 14], and in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in Norway [8]. The disease increases fish mortality rates by 30–50% in adult trout during the spawning period [10, 11]. Since diseased fish with vagococcosis show similar symptoms and clinical signs as streptococcosis, i.e. sluggish behaviour, swimming disorder, distortion of the eyeball, boils, external and internal haemorrhages, exophthalmos, ascitices fluid accumulation, body paleness and enlargement of internal organs, it is often difficult to identify the vagococcosis agent with certainty [8, 5]. Phenotypic (traditional microbiological/biochemical tests), histopathologic and molecular based methods [standard polymerase chain reaction (PCR), quantitative PCR (qPCR), random amplified polymorphic DNA (RAPD), ribotyping, enterobacterial repetitive intergenic consensus (ERIC-PCR), repetitive sequence-based PCR (REP-PCR) and genome sequencing analysis of 16S Recombinant DNA (rDNA) are used for the accurate identification of *V. salmoninarum* [1, 8, 10, 15]. However, these techniques take at least one day and usually do not provide a reliable identification. Similarly, standard PCR and simple Sanger sequencing techniques have also been shown to sometimes fail to identify at species level [3, 16]. Though the whole genome sequencing (NGS) technique [17], which is an alternative high throughput analysis, can distinguish close species of *Vagococcus*, it is an application using expensive and labour-intensive platforms.

In recent years, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) based proteomics has emerged as a promising method for diagnosing and differentiating closely related strains that are difficult to distinguish using conventional techniques [18, 19, 20, 21]. The MALDI-TOF MS approach is a rapid, low-cost diagnostic technique that provides results within minutes of culture compared to other conventional or molecular methods [20]. In addition, MALDI-TOF MS provides comparable, sometimes better, results than standard 16S rDNA gene sequencing, allowing taxonomic classification down to the subspecies level [22, 23, 24].

The aim of this study was to compare three diagnostic methods for the identification of *V. salmoninarum* in samples collected during outbreaks of vagococcosis in rainbow trout farms.

MATERIALS AND METHODS

A total of fifty dead rainbow trout (*O. mykiss*) were sampled (average body weight 200–250 g) during a disease outbreak in three different farms in Antalya Province, located in the Mediterranean Region of Türkiye. The fish showing signs of disease were clinically examined and transported to the laboratory within a few hours. Samples of fins, skin, gills, liver, heart, spleen and kidneys from each trout were collected at necropsy and used for phenotypic, genomic and proteomic analyses. Reference strains of *V. salmoninarum* ATCC 5120, *S. warneri* DSMZ 20316 and *S. epidermidis* ATCC 35984 and all clinical samples were cultured on tryptic soy agar (TSA) (Merck, Germany) and blood agar (BA) (Merck, Germany) at 15–20°C for 24–72h. Isolated colonies were subcultured twice and were biochemically characterized using with BBL Crystal™ GP identification system (BD, Becton Dickinson, USA) according to the manufacturer's instructions. Genomic DNA was extracted from bacterial isolates using the DNeasy Blood&Tissue Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated colonies were incubated on tryptic soy broth (TSB) (Merck, Germany) at 20°C for 24 hours for antimicrobial susceptibility testing determined by Kirby-Bauer disk diffusion method. The bacterial suspensions were reduced to 0.5 McFarland turbidity. The bacterial samples were inoculated on a Mueller-Hinton Agar (MHA) (Merck, Germany) plate containing 5% sheep blood. Antibiotic disks were placed on the petri dishes [7, 21]. Nineteen antibiotics were used for susceptibility tests, respectively; sulfamethoxazole/trimethoprim (25 mg), neomycin (10 mg), oxalinic acid (3 mg), enrofloxacin (5 mg), ciprofloxacin (5 mg), flumequine (20 mg), Sulfafurazole (10 mg), tetracycline (30 mg), amoxicillin (25 mg), streptomycin (10 mg), chloramphenicol (30 mg), florfenicol (30 mg), kanamycin (30 mg), erythromycin (15 mg), vancomycin (30 g), ampicillin (10 mg), nitrofurantoin (300 mg) and gentamicin (10 mg) were evaluated according to Clinical and Laboratory Standards Institute (CLSI) guidelines. After incubation of these petri dishes at 20°C for 24–48 hours, the diameters of the growth zone around the antibiotic disks were measured. Isolates and reference strains based on zone diameters measurement references susceptible (S), moderately susceptible (I) and resistant (R).

PCR amplification of the 16S rDNA gene was performed according to the protocol of Ruiz-Zarzuela *et al.* [10]. The identification of reference strains and freshly cultured bacteria by MALDI-TOF MS method was performed with "direct sample spotting" according to the protocol developed by Popovic *et al.* [25]. In this protocol, the targeted extraction method of single colonies grown on TSA and blood media and the Bruker MALDI-TOF Biotyper device (Bruker Daltonics, Germany) were used. A single colony of isolate was transferred to a 96 well plate using a wooden stick. Following this, 1.0 µL of 70% formic acid (Kemika, Croatia) was added to each bacterial colony to lyse bacterial cells proteins. After drying at room temperature, 1.0 µL of CHCA matrix solution (α-Cyano-4-hydroxycinnamic acid) (Bruker Daltonics, Germany) was added to each spot to allow optimal protein crystallisation [26, 27]. A Microflex LT MS with laser operation and MALDI Bruker Biotyper 3.4 software (Bruker Daltonics, Germany) was used for mass spectra acquisition and peak identification of the isolates. Mass spectra in the range 2000 to 21000 Da were obtained for each sample analyzed. Spectra were constructed from 240 individual spectra obtained from each isolate using 60 laser shot stages at random locations. The comparison of each peak with reference mass spectra in the database was recorded on a logarithmic scale ranging from 0 to 3.00. The criterion for successful identification appeared to be reliable at the species level greater than or equal to 2.00 [28, 29, 30].

RESULTS AND DISCUSSION

In the necropsy of 50 diseased fish samples collected, exophthalmos in the ocular area, hemorrhages in the gills and organs, lesions and paleness in the gills, heart and kidney, enlarged spleen and liver, ascites in the abdomen, pericarditis and congestion in the heart and vessels were observed (FIG. 1). The clinical findings of this study are similar to previously reported studies [5, 9, 10, 12, 15, 17].

Samples were taken from the kidney, spleen, liver, gills and heart of each symptomatic fish using swabs sticks. These were inoculated on TSA and BA plates and incubated at 15–20°C for 24–72h. Colonies on TSA plates isolated from heart tissue were generally opaque and

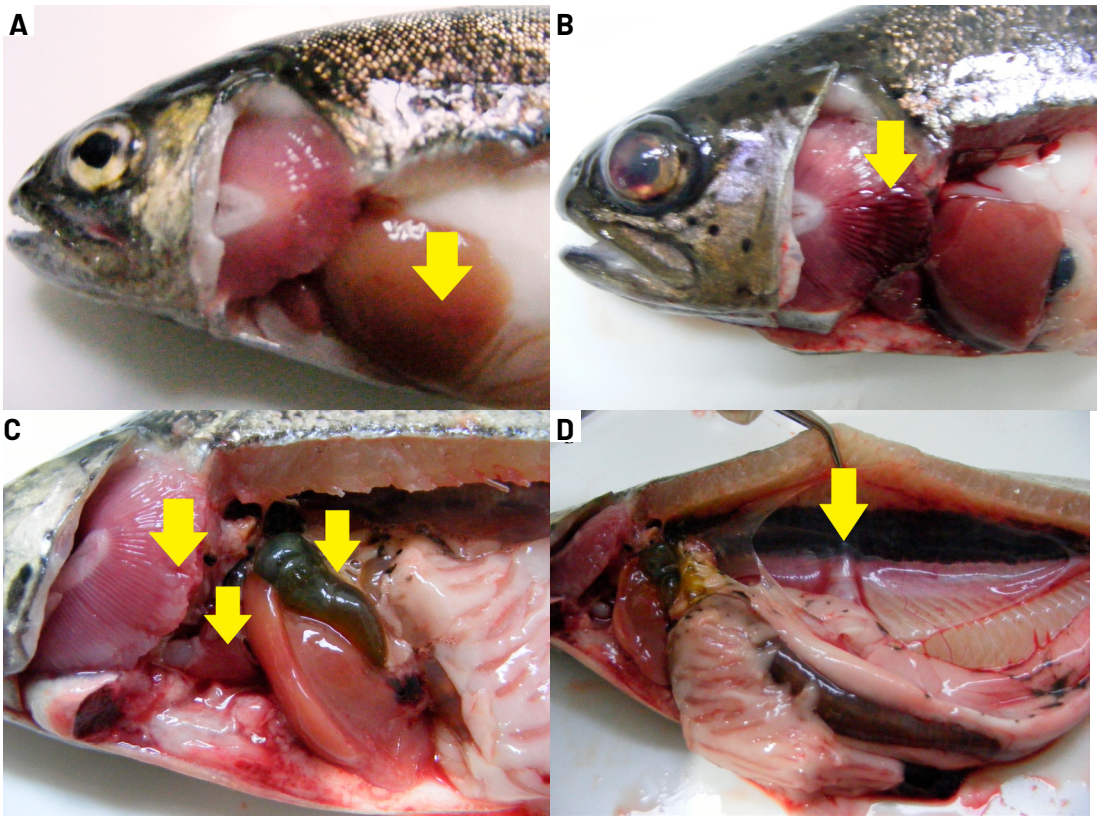

FIGURE 1. Post–mortem examination of fish and arrows show infected areas A) hemorrhage in liver B) hemorrhage in gill C) paleness of heart and gill D) infected kidney

circular with diameters between 0.2 and 0.4 mm (FIG. 2). Our colony findings are consistent with the findings in study of Didinen *et al.* [12] in which white, small and smooth colonies with a diameter of 0.5 mm were found after inoculation in samples taken from the internal organ of fish.

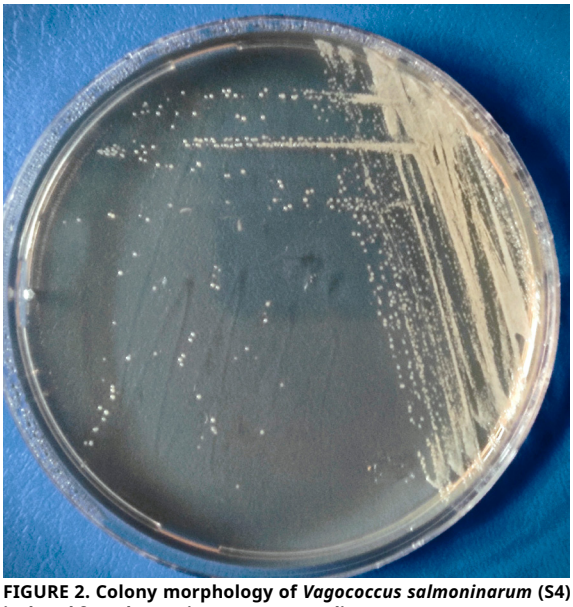

FIGURE 2. Colony morphology of *Vagococcus salmoninarum* (S4) isolated from heart tissue on TSA medium

Twelve isolates (S4 to S15) were identified as *V. salmoninarum* according to biochemical and enzymatic profiles using the commercial BBL Crystal™ GP system for phenotypic characterization and compared with the reference strain (S3) (TABLE I).

TABLE I shows the confirmation of all the characteristics of the twelve isolates and their validation with the reference strain. The biochemical and cultural characteristics of the *V. salmoninarum* isolates obtained from this study were consistent and similar with the BBL Crystal™ GP results used in Cagatay and Gumus [13]. Although Shewmaker *et al.* [31] reported that *V. salmoninarum* was placed in *Streptoccocus* group IV, which is often difficult to identify based on morphological and phenotypic characters, results of morphological identification have been presented in many previous studies such as Michel *et al.* [5], Ruiz–Zarzuela *et al.* [10], Salogni *et al.* [11], Schmidtke and Carson [8] and Tanrıkul *et al.* [14].

Nineteen antibiotics were used for antibiotic susceptibilities of bacterial isolates evaluated by disk diffusion test. The results of our disk diffusion tests show that all isolates were resistant (R) to twelve antibiotics (sulfamethoxazole+trimethoprim, neomycin, oxalinic acid, enrofloxacin, ciprofloxacin flumequine, streptomisin, sulfafurazole, tetracycline, amoxicillin, streptomycin and chloramphenicol), susceptible (S) to five (florfenicol, kanamycin, eritromycin, vancomycin and ampicillin), moderately susceptible (I) to nitrofurantoin and gentamicin was not calculated (NC) for some isolates and susceptible (S) for others (TABLE I). The results that *V. salmoninarum* isolates were resistant to antibiotics such as oxytetracycline, tetracycline, trimethoprim/sulfamethoxazole, erythromycin, chloramphenicol, amoxicillin and florfenicol and sensitive

to gentamicin, vancomycin were found to be consistent with Didinen *et al.* [12] and Tanrıkul *et al.* [14]. Saticioglu *et al.* [17] reported that two of the antimicrobials commonly used in aquaculture are resistant to oxolinic acid and sulfamethoxazole+trimethoprim. These two antibiotics were also found to be resistant in our study. Unfortunately, results suggest that *V. salmoninarum* has developed resistance to many antibiotics currently used in fish farms, but florfenicol and ampicillin which were sensitive [12, 14] could be used for treatment.

TABLE I
Biochemical characterization of isolates and reference strain by BBL Crystal™ GP identification system and antibiotic susceptibility

Characteristics	*Vagococcus salmoninarum* ATCC 51200	Sensitivity of Isolates											
		1	2	3	4	5	6	7	8	9	10	11	12
Growth at													
10°C	+	+	+	+	+	+	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+	+	+	+	+	+	+
20–22°C	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemolysis	α	α	α	α	α	α	α	α	α	α	α	α	α
Growth in													
2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–
Galactose	–	–	–	–	–	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	–	–	–
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+
Urea	–	–	–	–	–	–	–	–	–	–	–	–	–
Glycine	+	+	+	+	+	+	+	+	+	+	+	+	+
Treazolium	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	–	–	–	–	–	–	–	–	–	–	–	–	–
Lysine	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p–nitrophenyl galactosidase	–	–	–	–	–	–	–	–	–	–	–	–	–
p–nitrophenyl α–β–glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Antibiotic susceptibility													
Sulfamethoxazole+trimethoprim	R	R	R	R	R	R	NC	R	R	R	R	R	R
Neomycin	R	R	R	R	R	NC	R	R	R	R	R	R	R
Oxalinic acid	R	R	R	R	R	R	R	R	R	R	R	R	R
Enrofloxacin	R	R	R	R	R	NC	R	R	R	R	R	R	R
Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R
Flumequine	R	R	R	NC	R	R	R	NC	R	R	R	R	NC
Streptomisin	R	R	R	R	R	R	R	R	R	R	R	R	R
Sulfafurazole	R	R	R	R	R	R	R	R	R	R	R	R	R
Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R
Amoxicillin	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptomycin	R	R	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol	R	R	R	R	R	R	R	R	R	R	R	R	R
Florfenicol	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S	S	S	S
Eritromycin	S	S	S	S	S	S	S	S	S	S	S	S	S
Vancomycin	S	S	S	S	S	S	S	S	S	S	S	S	S
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	S
Nitrofurantoin	I	I	I	I	I	I	I	NC	NC	I	I	I	I
Gentamycin	NC	NC	NC	NC	S	S	S	S	S	S	S	S	S

+:positive, – :negative, α:Alpha hemolysis, NA:not available, NC: No calculation, S: Susceptible, I: Moderately susceptible, R: Resistant

The PCR results of the clinical isolates (S4 to S13) and reference strains (S3) using 16S rDNA primers, the gene amplicon of 300 bp in size were positive for eleven isolates and confirmed that the bacteria were *V. salmoninarum*, while the other two isolates (S14 to S15) did not give positive amplicons and thus were not *V. salmoninarum* (TABLE II). The results of the clinical isolates that gave positive PCR results in this study were compared with the previous standard PCR results and it was observed that amplicons of the same size were obtained [1, 10, 12, 14]. However, six of the twelve clinical isolates identified as positive in the BBL Crystal™ GP identification system could not be confirmed as *V. salmoninarum* because they did not produce an amplicon for the 16S rDNA gene. The reason for this is thought to be the inadequacy of the standard PCR amplification the 16S rDNA gene used to distinguish similar *Streptococcus* spp. There are also studies showing that other PCR methods such as ERIC-PCR [14], qPCR targeting the *pheS* gene [7, 32] and partial or whole gene sequencing methods [17] are more accurate for the detection of *V. salmoninarum*.

Mass spectrometry analysis was performed using direct sample spotting technique and the fully automated rapid microbial mass spectrometry detection system (Bruker MALDI-TOF MS Biotyper, Germany). Ten of the twelve isolated bacteria (S4-S13) were identified as *V. salmoninarum* (mass score value between 2.20 to 3.00) and S14 was identified as *V. fluvialis* (mass score value of 2.24) and S15 was *V. lutrae* (mass score value of 2.16) (TABLE II). TABLE II shows the MALDI-TOF MS identification results of isolates and the closet-related bacteria match. The characteristic protein mass peak graph for *V. salmoninarum* (S4) with the highest score value is given in FIG. 3 that represents the peptide mass fingerprint spectrum containing a total of sixteen very consistent mass peaks with values of 2612.161, 3275.430, 3719.485, 4202.990, 4855.407, 5578.718, 5833.827, 6553.199, 7273.375, 7438.837, 8337.143, 9138.511, 9706.730, 10371.131, 12429.907 and 15318.777 Da. These characteristic peaks were found in the spectra of ten *V. salmoninarum* strains analysed. Comparison of these results with the reference spectra in the Bruker database resulted in the 100% correct identification of ten bacteria as *V. salmoninarum*.

TABLE II
Comparison of the results of penotypic, genomic and proteomic methods used in the identification of Vagococcus spp.

Sample No	Isolate/Strain Name	BBL Crystal™ GP Identification	16S rDNA Gene Identification	MALDI-TOF MS Identification	
				Best Match Bacteria	Score Values
S1*	*Staphylococcus epidermidis* ATCC 35984	–	–	*S. epidermidis*	2.80
S2*	*Staphylococcus warneri* DSMZ 20316	–	–	*S. warneri*	2.90
S3*	*Vagococcus salmoninarum* ATCC 5120	+	+	*V. salmoninarum*	3.00
S4	Clinic Isolate 1	+	+	*V. salmoninarum*	3.00
S5	Clinic Isolate 2	+	+	*V. salmoninarum*	2.30
S6	Clinic Isolate 3	+	+	*V. salmoninarum*	2.40
S7	Clinic Isolate 4	+	+	*V. salmoninarum*	2.58
S8	Clinic Isolate 5	+	+	*V. salmoninarum*	2.86
S9	Clinic Isolate 6	+	+	*V. salmoninarum*	2.30
S10	Clinic Isolate 7	+	+	*V. salmoninarum*	2.30
S11	Clinic Isolate 8	+	+	*V. salmoninarum*	2.20
S12	Clinic Isolate 9	+	+	*V. salmoninarum*	2.80
S13	Clinic Isolate 10	+	+	*V. salmoninarum*	2.46
S14	Clinic Isolate 11	+	–	*V. fluvialis*	2.24
S15	Clinic Isolate 12	+	–	*V. lutrae*	2.16

*:Reference strains, *Vagococcus fluvialis: V. fluvialis, Vagococcus lutrae: V. lutrae*

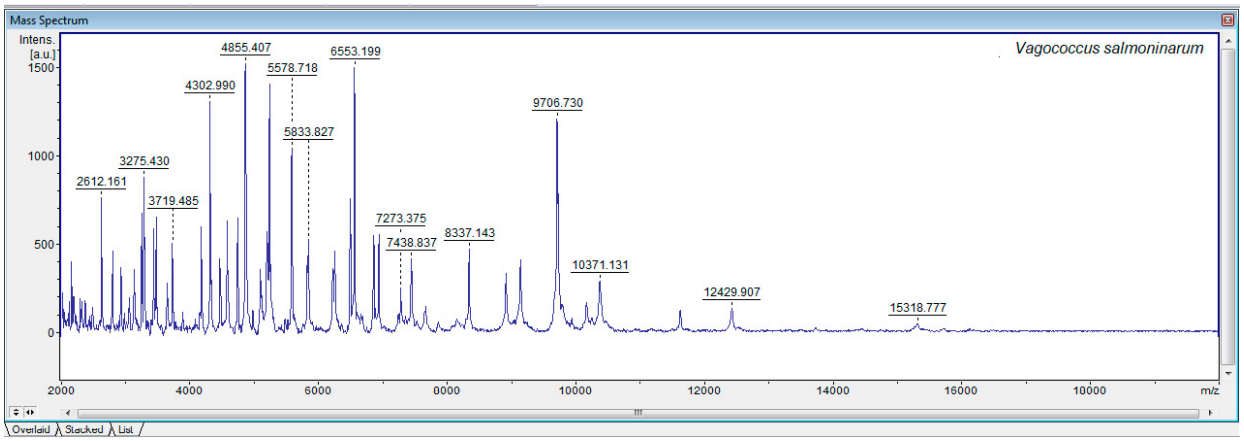

FIGURE 3. Representative MALDI-TOF mass peptide profiles of *Vagococcus salmoninarum* (S4)

Although there are many articles in the literature on the correct identification of most fish pathogenic bacteria by MALDI-TOF MS [33], there are a limited number of published articles on the identification of *V. salmoninarum* by the MALDI-TOF MS method. Torres-Corral and Santos [21] reported the identification of *V. salmoninarum* using this method. The results of our study confirm that the mass score values and protein spectral peaks are similar to the results of this study. However, according to two previous studies conducted by Buller and Hair [34] and Almuzara *et al.* [35] this method may only be reliable at the genus level. The common point in these studies as a reason for the inability to correctly identify *Vagococcus* spp. isolates can be explained as the lack of data for the fish pathogen *V. salmoninarum*, although the databases contain spectrum peak data for *V. fluvialis* and *V. lutrae*. Nevertheless, it is though that these problems will be solve in the new and updated global or in-house databases listed in Çağatay [33].

CONCLUSIONS

This study evaluated the results the higher technological proteomic MALDI-TOF MS method in combination with conventional diagnostic methods for the identification of *V. salmoninarum* isolated from rainbow trout in Türkiye. The results suggest that MALDI-TOF MS analysis is a rapid (2-3 min), inexpensive (~$1.5 USD) and accurate technique for species differentiation. It was concluded that MALDI-TOF MS can be used as an alternative approach for the diagnosis of some fish pathogens and diseases causing rapid mortality in aquaculture.

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Conflict of interest

The author has no declaration of competing interests.

Animal Ethics

Akdeniz University Animal Experiments Local Ethics Committee and their ethical compliance was approved with the protocol number 019/2019.11.02.

Field Study Permissions

The Ministry of Agriculture and Forestry is a government ministry of the Republic of Turkey and approved the study (92190712-288.04-E.2162848).

BIBLIOGRAPHIC REFERENCES

[1] Wallbanks S, Martinez-Murcia AJ, Fryer JL, Phillips BA, Collins MD. 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmoninarum* sp. nov. Int. J. Syst. Bacteriol. [Internet]. 1990; 40(3):224-230. doi: https://doi.org/b6dnph

[2] Mata AI, Gibello A, Casamayor A, Blanco MM, Domínguez L, Fernández-Garayzábal JF. Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in fish. Appl. Environ. Microbiol. [Internet]. 2004; 70(5):3183-3187. doi: https://doi.org/fj6zqn

[3] Austin B, Austin DA. Bacterial Fish Pathogens [Internet]. 6th ed. Chichester (United Kingdom): Springer; 2016. 732 p. doi: https://doi.org/gt7wh4

[4] Toranzo AE, Devesa S, Heinen P, Riaza A, Nuñez S, Barja JL. Streptococcosis in cultured turbot caused by an Enterococcus-like bacterium. Bull. Eur. Ass. Fish Pathol. [Internet]. 1994 [cited 14 Apr. 2024]; 14(1):19-23. Available in: https://goo.su/IzuzUbc

[5] Michel C, Nougayrède P, Eldar A, Sochon E, de Kinkelin P. *Vagococcus salmoninarum*, a bacterium of pathological significance in rainbow trout *Oncorhynchus mykiss* farming. Dis. Aquat. Org. [Internet]. 1997; 30(3):199-208. doi: https://doi.org/cn57cg

[6] Pridgeon JW, Klesius PH. Major bacterial diseases in aquaculture and their vaccine development. CAB Rev. [Internet]. 2012; 7:1-16. doi: https://doi.org/gt7wh5

[7] Standish I, Leis E, Erickson S, McCann R, Puzach C, Katona R, Lark E, Bailey J, Kleman E, Buening J. *Vagococcus salmoninarum* II-qPCR, tropism and egg-associated transmission. J. Fish Dis. [Internet]. 2020; 43(3):317-325. doi: https://doi.org/gt7wh6

[8] Schmidtke LM, Carson J. Characteristics of *Vagococcus salmoninarum* isolated from diseased salmonid fish. J. Appl. Bacteriol. [Internet]. 1994; 77(2):229-236. doi: https://doi.org/bfkxdm

[9] Ghittino C, Agnetti F, Panzieri C, Cabra S, Colussi S, Prearo M. L'infezione da *Vagococcus salmoninarum* della trota iridea in Italia [*Vagococcus salmoninarum* infection in rainbow trout in Italy]. Ittiopatologia [Internet]. 2004 [cited 19 Apr. 2024]; 1:25-33. Italian. Available in: https://goo.su/Az86JfH

[10] Ruiz-Zarzuela I, de Blas I, Girones O, Ghitino C, Mùzquiz JL. Isolation of *Vagococcus salmoninarum* in rainbow trout *Oncorhynchus mykiss* (Walbaum), broodstocks: characterisation of the pathogen. Vet. Res. Commun. [Internet]. 2005; 29(7):553-562. https://doi.org/bt9qmb

[11] Salogni C, Perantoni P, Pitozzi A, Loris G, Alborali GL. *Vagococcus salmoninarum*: descrizione di un focolaio di malattia in riproduttori di trota iridea (*Oncorhynchus mykiss*) [Outbreak of *Vagococcus salmoninarum* in rainbow trout (*Oncorhynchus mykiss*) broodstocks]. Ittiopatologia [Internet]. 2007 [cited 19 Apr. 2024]; 4(1):59-66. Italian. Available in: https://goo.su/bYRplK

[12] Didinen BI, Kubilay A, Diler O, Ekici S, Onuk EE, Findik A. First isolation of *Vagococcus salmoninarum* from cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum) broodstocks in Turkey. Bull. Eur. Ass. Fish Pathol. [Internet]. 2011 [cited 22 Feb. 2024]; 31(6):235-243. Available in: https://goo.su/UXeCK

[13] Cagatay IT, Gumus E. Phenotypic, histopathologic and genomic diagnosis of a novel fish pathogen *Vagococcus salmoninarum* in Turkey. J. Pure. Appl. Microbiol. [Internet]. 2014 [cited 22 Feb. 2024]; 8(Spec. Ed.):119-124. Available in: https://goo.su/HY6EcQh

[14] Tanrıkul T, Avsever ML, Onuk EE, D'Idinen RI. *Vagococcus salmoninarum*, a causative agent of disease in rainbow trout (*Oncorhynchus mykiss*, Walbaum) broodstocks in the aegean region of Turkey. Etlik Vet. Mikrobiyol. Derg. [Internet]. 2014 [cited 22 Feb. 2024]; 25(1):11-16. Available in: https://goo.su/JiDz

[15] Standish I, Erickson S, Leis E, Baumgartner W, Loch T, Knupp C, McCann R, Puzach C, Katona R, Lark E, Bailey J, Buening J, Edwards C, Phillips K. *Vagococcus salmoninarum* I-A chronic

coldwater streptococcosis in broodstock brook trout (*Salvelinus fontinalis*) in Wisconsin, USA. J. Fish Dis. [Internet]. 2019; 43(3):305–316. doi: https://doi.org/gt7wh7

[16] Buller BN. Bacteria and fungi from fish and other aquatic animals: A practical identification manual [Internet]. 2nd ed. Bentley. Wallinford (United Kingdom): CABI Publishing; 2014. Chapter 1, Aquatic animal species and organism relationship; p. 1–424. doi: https://doi.org/ndz3

[17] Saticioglu IB, Yardimci B, Altun S, Duman M. A comprehensive perspective on a *Vagococcus salmoninarum* outbreak in rainbow trout broodstock. Aquaculture [Internet]. 2021; 545:737224. doi: https://doi.org/gt7wh8

[18] Fernandez-Álvarez C, Torres-Corral Y, Santos Y. Use of ribosomal proteins as biomarkers for identification of *Flavobacterium psychrophilum* by MALDI-TOF mass spectrometry. J. Proteomics. [Internet]. 2018; 170:59–69. doi: https://doi.org/gt7wh9

[19] Kim SW, Nho SW, Im SP, Lee JS, Jung JW, Lazarte JMS, Kim J, Lee WJ, Lee JH, Jung TS. Rapid MALDI biotyper -based identification and cluster analysis of *Streptococcus iniae*. J. Microbiol. [Internet]. 2017; 55(4):260–266. doi: https://doi.org/gt7wjb

[20] Popović NT, Kazazić SP, Strunjak-Perović I, Čož-Rakovac R. Differentiation of environmental aquatic bacterial isolates by MALDI–TOF. Environ. Res. [Internet]. 2017; 152:7–16. doi: https://doi.org/gt7wjc

[21] Torres-Corral Y, Santos Y. Identification and typing of *Vagococcus salmoninarum* using genomic and proteomic techniques. J. Fish Dis. [Internet]. 2018; 42(4):597–612. doi: https://doi.org/gt7wjd

[22] Böhme K, Fernández-No IC, Pazos M, Gallardo JM, Barros-Velázquez J, Cañas B, Calo-Mata P. Identification and classification of seafood–borne pathogenic and spoilage bacteria: 16S rRNA sequencing versus MALDI-TOF MS fingerprinting. Electrophoresis [Internet]. 2013; 34(6):877–887. doi: https://doi.org/f2d2kx

[23] Hausdorf L, Mundt K, Winzer M, Cordes C, Fröhling A, Schlüter O, Klocke M. Characterization of the cultivable microbial community in a spinach–processing plant using MALDI–TOF MS. Food Microbiol. [Internet]. 2013; 34(2);406–411. doi: https://doi.org/f4v9qq

[24] Zhu S, Ratering S, Schnell S, Wacker R. Matrix-assisted laser desorption and ionization–time–of–flight mass spectrometry, 16S rRNA gene sequencing, and API 32E for identification of *Cronobacter* spp.: a comparative study. J. Food Prot. [Internet]. 2011; 74(12):2182–2187. doi: https://doi.org/dqmtbq

[25] Popović NT, Kazazić SP, Bojanić K, Strunjak-Perović I, Čož-Rakovac R. Sample preparation and culture condition effects on MALDI-TOF MS identification of bacteria: A review. Mass. Spect. Rev. [Internet]. 2023; 42(5):1589–1603. doi: https://doi.org/gt7wjc

[26] Kazazić Pečur S, Topić Popović N, Strunjak-Perović I, Florio D, Fioravanti M, Babić S, Čož-Rakovac R. Fish photobacteriosis–The importance of rapid and accurate identification of *Photobacterium damselae* subsp. *piscicida*. J. Fish Dis. [Internet]. 2019; 42(8):1201–1209. doi: https://doi.org/gt7wjf

[27] Kazazić SP, Popović NT, Strunjak–Perović I, Babić S, Florio D, Fioravanti M, Bojanić K, Čož–Rakovac R. Matrix–assisted laser desorption/ionization time of flight mass spectrometry identification of *Vibrio (Listonella) anguillarum* isolated from sea bass and sea bream. PLoS One [Internet]. 2019; 14(11):e0225343. doi: https://doi.org/gt7wjg

[28] De Marco ML, Burnham CAD. Diafiltration MALDI–TOF mass spectrometry method for culture–independent detection and identification of pathogens directly from urine specimens. Am. J. Clin. Patho[. [Internet]. 2014; 141(2):204–212. doi: https://doi.org/gpp4vp

[29] Puk K, Banach T, Wawrzyniak A, Adaszek Ł, Ziętek J, Winiarczyk S, Guz L. Detection of *Mycobacterium marinum*, *M. peregrinum*, *M. fortuitum* and *M. abscessus* in aquarium fish. J. Fish Dis. [Internet]. 2018; 41(1):153–156. doi: https://doi.org/gnccr9

[30] Surányi BB, Taczman–Brückner A, Mohácsi–Farkas C, Engelhardt T. Rapid identification of bacteria from agricultural environment using MALDI–TOF MS. Acta Alimentaria [Internet]. 2023; 52(1):113–120. doi: https://doi.org/gt7wjh

[31] Shewmaker PL, Steigerwalt AG, Morey ER, Carvalho MGS, Elliott JA, Joyce K, Barrett TJ, Teixeira LM, Facklam RR. *Vagococcus carniphilus* sp. nov., isolated from ground beef. International J. Syst. Evol. Microbiol. [Internet]. 2004; 54(5):1505–1510. doi: https://doi.org/dgcc4b

[32] Torres-Corral Y, Fernández-Álvarez C, Santos Y. High-throughput identification and quantification of *Vagococcus salmoninarum* by SYBR Green I-based real-time PCR combined with melting curve analysis. J. Fish Dis. [Internet]. 2019; 42(10):1359–1368. doi: https://doi.org/gt7wjj

[33] Çağatay İT. Use of proteomic–based MALDI–TOF mass spectra for identification of bacterial pathogens in aquaculture: a review. Aquacult. Int. [Internet]. 2024; 32:1–37. doi: https://doi.org/gt7wjk

[34] Buller N, Hair S. Identification of bacteria from aquatic animals. Microbiol. Australia. [Internet]. 2016 [cited 22 Feb. 2024]; 37(3):129–131. Available in: https://goo.su/Ezk9hK

[35] Almuzara M, Barberis C, Velázquez VR, Ramirez MS, Famiglietti A, Vay C. Matrix–assisted laser desorption ionization–time–of–flight mass spectrometry (MALDI–TOF MS) as a reliable tool to identify species of Catalase–negative Gram–positive cocci not belonging to the *Streptococcus genus*. Open Microbial. J. [Internet]. 2016; 10:202–208. doi: https://doi.org/gt7wjm