https://doi.org/10.52973/rcfcv-e35574 Revista Científica, FCV-LUZ / Vol. XXXV

Identification of *Staphylococcus warneri* from rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) using proteomics–based MALDI–TOF MS

Identificación de *Staphylococcus warneri* en trucha arcoiris (*Oncorhynchus mykiss* Walbaum, 1792) mediante espectrometría de masas MALDI–TOF basada en proteómica

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ABSTRACT

Staphylococcus warneri, an opportunistic pathogen, is a causative agent of mortal diseases in rainbow trout farming (*Oncorhynchus mykiss*), which are of great economic value for Türkiye. In this study, in addition to traditional phenotypic, biochemical, histopathological and genetic methods, a high throughput proteomics based Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI–TOF MS) method was performed for precise identification of *S. warneri*. Fourteen isolates obtained from skin, gills, liver, spleen and kidney of a total of fifty diseased fish were phenotypically confirmed as *S. warneri* using the BBL Crystal™GP identification system. Only 43% of these isolates showed positive PCR amplification for the 16S rRNA and *sodA* (superoxide dismutase A) gene, while 100% were identified as *S. warneri* by MALDI–TOF MS technique with high mass score value (*m/z*) between 2.35 and 3.05. From the comparative data obtained, it was concluded that MALDI–TOF mass spectrometry analysis can be recommended for the definitive confirmation of *S. warneri*, which showed indistinguishably close similarities with 16S rRNA gene sequences and *sodA* PCR results. To the best of knowledge, this is the first report to validate the results of phenotypic, biochemical, genetic and histological methods by the MALDI–TOF MS and shows that this is a successful identification approach, providing a high mass score (*m/z*) with 100% matching for accurate and faster identification of *S. warneri*. This promising diagnostic technique can identify many different bacterial fish pathogens, although a larger protein mass database for aquatic organisms is needed.

Key words: *Staphylococcus warneri*; MALDI–TOF MS; BBL Crystal™GP; 16S rRNA; *sodA*

RESUMEN

Staphylococcus warneri, un patógeno oportunista, es un agente causante de enfermedades mortales en la cría de la trucha arco iris (*Oncorhynchus mykiss*) de gran valor económico para Türkiye. En este estudio, además de los métodos fenotípicos, bioquímicos, histopatológicos y genéticos tradicionales, se utilizó un método de proteómica de alto rendimiento basado en la espectrometría de masas por ionización/desorción láser asistida por matriz (MALDI–TOF MS) para la identificación precisa de *S. warneri*. Catorce aislados obtenidos de piel, branquias, hígado, bazo y riñón de un total de cincuenta peces enfermos se confirmaron fenotípicamente como S. warneri mediante el sistema de identificación BBL Crystal™GP. Sólo el 43 % de estos aislados mostraron una amplificación positiva por PCR para el gen 16S rRNA y *sodA* (superóxido dismutasa A), mientras que el 100 % fueron identificados como *S. warneri* mediante la técnica MALDI–TOF MS con un alto valor de puntuación de masa (m/z) entre 2.35 y 3.05. A partir de los datos comparativos obtenidos, se concluyó que el análisis de espectrometría de masas MALDI–TOF puede recomendarse para la confirmación definitiva de *S. warneri*, que mostró similitudes indistinguiblemente cercanas con las secuencias del gen 16S rRNA y los resultados de la PCR *sodA*. Hasta donde se sabe, éste es el primer informe que valida los resultados de métodos fenotípicos, bioquímicos, genéticos e histológicos mediante la MALDI–TOF y demuestra que se trata de un método de identificación satisfactorio, que proporciona una puntuación de masa elevada (m/z) con una coincidencia del 100 % para una identificación precisa y más rápida de *S. warneri*. Esta prometedora técnica de diagnóstico puede identificar muchos patógenos bacterianos de peces diferentes, aunque se necesita una base de datos de masas de proteínas más amplia para los organismos acuáticos.

Palabras clave: *Staphylococcus warneri*; MALDI–TOF MS; BBL Crystal™GP; 16S rRNA; *sodA*

Received: 19/11/2024 Accepted: 07/01/2025 Published: 11/03/2025

INTRODUCTION

Staphylococcus warneri, a coagulase–negative and opportunistic pathogen, has been isolated from a variety of sources, including animals, humans and food products. It is frequently associated with the development of spontaneous staphylococcal infections [1, 2, 3]. *S. warneri* has been identifiedas the causative agent in fish disease outbreaks affecting Siberian sturgeon (*Acipenser baerii*) [4], catfish (*Clarias* sp.) [5], bronze gudgeon (*Coreius guichenoti*) [6], seven khramulya (*Capoeta capoeta*) [7] and rainbow trout [8, 9, 10]. Clinical signs of infection caused by *S. warneri* include exophthalmos, abdominal ascites, septicaemia, fin lesions and discoloured kidney and liver [11].

The identifion of *S. warneri* is typically achieved through the isolation of bacterium from diseased fish and subsequent characterisation using biochemical, serological and genetic methods [9, 10, 11]. However, these methods were not always sufficient for distinguish between closely related species of the genus *Staphylococcus* due to the high degree of similarity in the 16S rRNA sequences [12]. Consequently, in order to achieve definitive identification of *S. warneri*, it is necessary to employ a conformative method in addition to the traditional techniques.

MALDI–TOF MS a proteomic based method, has proven to be a powerful diagnostic tool for the determination of microbial diversity in clinical and environmental microbiology during last 20 years [13, 14]. Unlike conventional methods, MALDI–TOF MS provides high throughput, fast, reliable, and easy to use direct strain typing (without subculture) which is relatively inexpensive and does not require specialized laboratory skills [15]. In addition, MALDI–TOF MS provides comparable, sometimes better, results than standard 16S rRNA gene sequencing, allowing taxonomic classification down to the subspecies level [16, 17].

The MALDI–TOF MS technique allows the identification of microorganism through protein/peptide profiling. The technique works by passing a laser through a sample of the bacteria in a specialized matrix solution. The laser energy causes the proteins in the sample to desorb and ionize. Mass signals from the ionized microbial ribosomal peptides, rising into an evacuated detection tube, identify the unique mass fingerprints that each species has based on their distinctive spectrum of mass/charge ratio (*m/z*) peaks [18]. The resulting bacterial peptide mass fingerprints are compared with those in a mass spectral library of pre–existing reference strains in the database [19]. The comparison of these profiles with the database allows for identification of bacterial genus or species based on the peptide composition.

Previous studies have demonstrated that the use of MALDI–TOF MS technique accurately identified bacterial pathogens [20] of significance to fish species such as *Vibrio* [21], *Mycobacterium* [22, 23], *Enterobacterales* [24], *Staphylococcus* [2], *Tenacibaculum* [25], *Photobacterium damselae* [26], *Streptococcus iniae* [27], *Flavobacterium* [28], *Pseudomonas* [29], *Renibacterium salmoninarum* [30], *Vagococcus salmoninarum* [31] and *Yersinia ruckeri*. *S. warneri* has been identified from some aquaculture food products and sea water using MALDI–TOF MS technique, however it has not been used for the identification of *S. warneri* from rainbow trout.

The principal aim of this study was to develop a rapid and accurate proteomic approach utilising MALDI–TOF MS technology for the identification of *S. warneri* in samples obtained during staphylococcosis outbreaks on rainbow trout farms. In addition, the present study sought to evaluate the accuracy of MALDI–TOF MS analysis in comparison with three conventional diagnostic methods. The application of MALDI–TOF MS in the context of aquaculture diseases, bacterial and fungal disease agents is expected to significantly improve the speed and accuracy of diagnoses. It also holds promise in facilitating the assessment of phylogenetic relationships between closely related bacterial species that have been difficult to identify.

MATERIALS AND METHODS

Fish sampling and necropsy

The first sampling of a total of fifty dead rainbow trout with between of 7.5–20 g was carried out in March (2022) when the disease outbreak was reported from two commercial trout farms located in the Aegean (n=10) and Mediterranean (n=15) regions, and the second sampling was carried out in March (2024) from two different commercial farms in the Mediterranean (n=25) region and they were transported to the laboratory under sterile conditions. At the time of sampling, the water temperature in the ponds was between 11 to 15°C, oxygen 10.8–11.0 ppm and pH 7.0 on average. The external surfaces of freshly dead fish showing signs of disease were macroscopically examined and the body surface of the fish was then disinfected with 70–80% ethanol for necropsy in a biological safety cabinet for dissection. During necropsy, aseptic samples were taken from fins, skin, gills, liver, spleen and kidneys for phenotypic, histopathological, genetic and MALDI–TOF MS analyses.

Bacterial strains and growth conditions

Reference strains *S. warneri* ATCC 27836, *S. pasteuri* ATCC 51129 and *S. epidermidis* ATCC 35538 and clinic samples were cultured using tryptic soy agar (TSA) and tryptic soy broth (TSB) (Merck, Germany) at 25°C for 24–48 h [1, 6].

Biochemical identification analysis

Isolated colonies were subcultured and were characterized using the BBL Crystal™GP system (BD, Becton Dickinson, USA) according to the manufacturer's manual.

Antibiotic susceptibility analysis

The isolated colonies were incubated on TSB at 25°C for 24 h for antimicrobial susceptibility testing determined by Kirby–Bauer disk diffusion method [32]. 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. Thirty antibiotics (Merck, Germany) were used for susceptibility tests, respectively; Oxolinic Acid, Tetracycline, Penicillin, Amoxicillin, Nalidixic Acid, Tetracycline, Lincomycin, Nitrofurantoin, Florfenicol, Kanamycin, Gentamycin, Ofloxacin, Enrofloxacin, Cefoperazone, Norfloxacin, Vancomycin, Cefurocime, Flumequine, Sulphamethox, Doxycycline, Apramycin, Cephalothin, Neomycin, Oxacillin,

Colistin, Ciprofloxacin, Oxytetracycline, Tylosin, Spectinomycin and Clindamycin antibiotics were evaluated according to Clinical and Laboratory Standards Institute (CLSI) guidelines The diameters of the growth zones around the antibiotic disks in these incubated petri dishes were measured. Isolates and reference strains based on zone diameters measurement references susceptible (S), intermediate susceptible (I) and resistant (R) [33].

Histopathological analysis

Ten (S1 to S10) dead fish from different farms with positive phenotypic, biochemical and genetic analyses were selected for histopathological analysis. These fish with an average weight of 0.75–20 g were dissected and liver, kidney and spleen tissues were fixed in 10% formaldehyde solution. Tissue samples were subjected to routine tissue tracing and embedded in paraffin. Tissues were sectioned at 5 μm with a microtome (Leica RM2155, Leica Microsystems, Wetzlar, Germany). Tissue sections were stained with hematoxylin and eosin and examined under a light microscope (Olympus CX21, Olympus Corporation, Tokyo, Japan) following staining with hematoxylin and eosin.

Analysis of 16S rRNA and *sodA* genes

The DNeasy blood and tissue kit (Qiagen, USA) was used to extract DNA from seventeen bacterial cultures following the manufacturer's manual. 16S rRNA and *sodA* genes were amplified by polymerase chain reaction (PCR) [12, 34]. PCR protocols and specific primer sets are listed in TABLE I. PCR amplicons of the 16S rRNA gene from RS1, 2, 3 and S1 to S6 were purified and sequenced by Macrogen (Holland) (TABLE I).

BioEdit 7.2 (Ibis Biosciences, USA) was employed to align the DNA sequences. The BLAST program was used to assess the identity of concatenated sequences by comparing them to reference sequences in the GenBank database. The ClustalW algorithm was used in Molecular Evolutionary Genetics Analysis (MegaX) software for phylogenetic tree construction and the jModelTest was used to determine the mutation model of the aligned sequences. MrBayes v3.2 was used for phylogenetic tree construction. Four chains were subjected to Markov chain Monte Carlo (MCMC) analysis, which was carried out for 10 million generations until the split frequency reduced below 0.01. Every 1000 generations, 25% of the trees obtained by sampling were considered "burn–in" and removed. The consensus tree obtained as a result of the analysis was edited in FigTree v1.4.2. The identification was considered reliable if the identification rate was greater than or equal to 100% for the genus level.

MALDI–TOF MS analysis

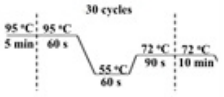
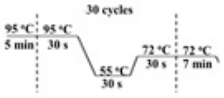
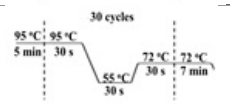
Reference strains (RS1, 2, 3) and freshly cultured isolates (S1-S14) were subjected to analysis using the MALDI–TOF Biotyper (Bruker, Germany). It was performed according to protocol developed by Popovic *et al.* [19] using the on–target extraction method after 24 h of incubation on TSA medium. A wooden stick was used to place an individual colony onto a 96 spot target plate. Following that, each bacterial colony was overlaid by 1.0 μL of 70% formic acid (Kemika, Croatia) to lyse the bacterial cells and release the proteins. After the formic acid had dried, 1.0 μL of α–Cyano-4-hydroxycinnamicacid (CHCA–matrix solution) (Bruker Daltonics, Germany) was applied to each spot and allow to dry at room temperature to allow for optimal protein crystallization [26]. A microflex mass spectrometer equipped with an laser under ion detection mode at a frequency of 60 Hz and MALDI Bruker Biotyper 3.4 software (Bruker Daltonics, Germany) was performed for the collection of MALDI–TOF MS and peak identification of colonies from the bacterial isolates. For each sample examined mass spectra in the range of 2000 to 21000 Da were obtained. The spectra were constructed using 240 single spectra that were acquired from each isolate at random places using 40 laser shot stages. Each peak's comparison to the database's reference mass spectra recorded in ratings from 0 to 3.00 on a logarithmic scale. The criteria for a successful identification appeared reliable at the species level if the number was higher than 2.00 [15, 22].

RESULTS AND DISCUSSION

Phenotypic evaluation

External examination of fifteen infected rainbow trout showed bilateral exophthalmos in the eyes, hemorrhage and dropsy in the lower jaw. In addition, anemia in the kidney and liver, yellow green fluid in the digestive tract, hemorrhage in the adipose tissue and anus, and enlarged spleen tissue were detected in internal examination of this study after necroscopy. In a study reported in the Gil *et al.* [11] reported ulcerated skin lesions on the fins of rainbow trout infected with *S. warneri*, exophthalmos, acidic fluid

TABLE I
Primers and PCR protocols for 16S rRNA and *sodA* gene amplifications

Gene	Primer	Sequence (5'-3')	Size	Conditions	Protocol	References
16S rRNA	*63f* *1387r*	CAGGCCTAACACATGCAAGTC GGGCGGWGTGTACAAGGC	1300 bp	1×PCR buffer, 1.5 mM Mg^{+2}, 1 pmol f/r primers, 0.5 mM dNTP mix, 1 U Taq		34
sodA	*PA237F* *PA237R*	GCTAATTTAGACAGTGTACCTTCTG GCCCGTTATTTACTACTAACCA	237 bp	1×PCR buffer, 1.5 mM Mg^{+2}, 1 pmol f/ r primers, 0.5 mM dNTP mix, 1 U Taq		12
	SW110F *SW110R*	GTAACAAAATTAAATGCAGCTG TCTTACTGCAGTTTGAATATCAGA	110 bp	1×PCR buffer, 1.5 mM Mg^{+2}, 1 pmol f/ r primers, 0.5 mM dNTP mix, 1 U Taq		12

accumulation in the abdomen and anaemia in the liver. Metin *et al.* [9] reported lethargy, anorexia, abrasion on anal fin bases, haemorrhage in eyes, jaw and mouth, darkening of colour, anaemia in internal organs, petechial haemorrhage in liver, splenomegaly and yellow exudate in intestine in the infection caused by *S. warneri* in broodstock rainbow trout weighing 1500–2000 g. In this study, unlike Gil *et al.* [11] and Metin *et al.* [9] and similar to the Diler *et al.* [10], there was no change in the skin in the external examination of infected juvenile rainbow trout, but the symptoms such as bilateral exophthalmos in the eyes, haemorrhage in the lower jaw, dropsy, anaemia in the internal organs, yellow exudate in the digestive tract, haemorrhage in the adipose tissue, haemorrhage in the anus region and enlargement in the spleen tissue were similar (FIG. 1).

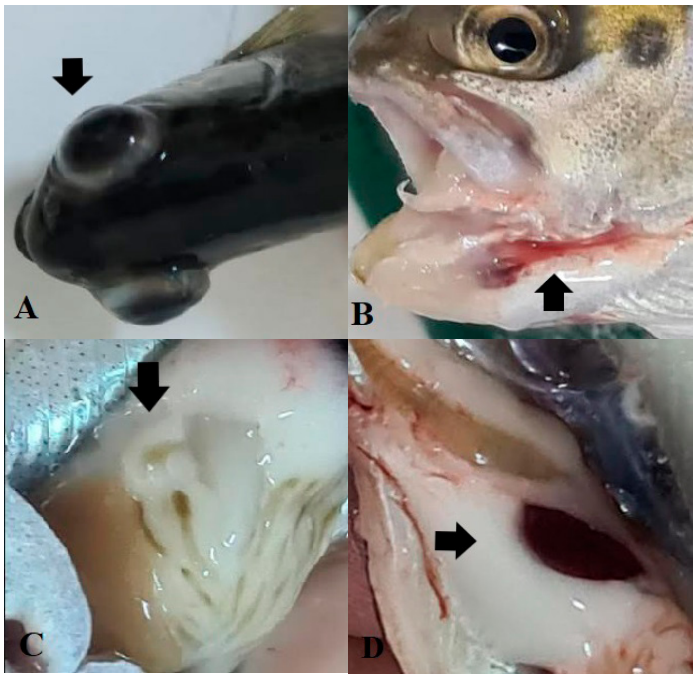


FIGURE 1. A) Exophthalmus in the eyes of juvenile fish, B) Bleeding in the lower jaw, C) Anemic pale liver D) Haemorrhages in adipose tissue, enlarged spleen

Biochemical characterization

Single colonies with a diameter of 1.5-2.0 mm were observed as a result of bacterial cultivation on TSA medium from the anterior kidney, liver and spleen tissues of fish (FIG. 2). Gram staining of the obtained isolates showed gram–positive and staphylococcal morphology.

Fourteen isolates (S1 to S14) were identified as *S. warneri* using the commercial BBL Crystal™GP kit for phenotypic characterisation and identification and compared with the reference strain (*S. warneri* ATCC 27836) (TABLE II). Thirty antibiotics were used for antibiotic susceptibilities of bacterial isolates evaluated by disk diffusion test (TABLE II). As a result, the isolates were found to be susceptible (S) to fourteen antibiotics (Florfenicol, Kanamycin, Gentamycin, Ofloxacin, Enrofloxacin, Cefoperazone, Norfloxacin, Vancomycin, Flumequine, Cefurocime, Sulphamethox, Doxycycline,

FIGURE 2. Morphology of colonies on TSA medium thought to be *Staphylococcus warneri* (S6) isolated from kidney tissue

Apramycin and Cephalothin), resistant (R) to eight (Neomycin, Oxacillin, Colistin, Ciprofloxacin, Oxytetracycline, Tylosin, Spectinomycin and Clindamycin) and intermediate susceptible (I) to Oxolinic acid, Tetracycline, Penicillin, Amoxicillin, Nalidixic acid, Tetracycline, Nitrofurantoin and Lincomycin. In this study, *S. warneri* isolated from rainbow trout were found to be sensitive to Florfenicol, Enrofloxacin, Doxycyclin, Clindamycin, Kanamycin, Gentamicin, Vancomycin antibiotics. The findings were consistent to the results of Metin *et al.* [9] and Diler *et al.* [10]. Xiao *et al.* [6] reported that *S. warneri* species were, however, susceptible to Streptomycin, Doxycycline, Amicacin, Florfenicol antibiotics and the disease was effectively controlled with Doxycycline in infected fish.

Histopathological evaluation

The histopathological examinations in this study, inflammatory infiltration around the vena centralis in liver tissue, hyperaemia in spleen tissue, red pulp, loss of white pulp border, splenitis and necrotic changes in renal tubule epithelium were observed in rainbow trout infected with *S. warneri* (FIG. 3). Although histopathological analyses are an important method that reveal the damage caused by fish pathogens in tissues and can give results in the diagnosis of some diseases, research on the pathological disorders caused by *S. warneri* in fish tissues has remained very limited. The findings of this study were consistent with the findings of Diler *et al.* [10] and Xiao *et al.* [6]. Rusev *et al.* [4] investigated the effects of *Shewanella putrefaciens* and *S. warneri* together on fish tissues in sturgeon (*Acipenser baerii*) and hybrid sturgeon (*Huso huso* × *A.baerii*). Pathological findings showed hyperaemia in the spleen, superficial petechial haemorrhages in the liver and hyperaemia in the mesenteric blood vessels. In another study, histopathological examinations were performed in infections caused by *S. pasteuri* in sturgeon (*A. gueldenstaedtii*) and lymphocyte infiltration around necrotic hepatocyte cells; necrosis in the kidney, hyperaemia in the spleen and intense haemorrhagic areas were determined [35].

TABLE II
Biochemical characterization of isolates and *Staphylococcus warneri* ATCC 27836 by BBL Crystal™ GP identification system and antibiotic susceptibility

Characteristics	*S. warneri* ATCC 27836	Sensitivity of Isolates													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Hemolysis	α	α	α	α	α	α	α	α	α	α	α	α	α	α	α
Growth at															
20-22 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25-27 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30-35 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in															
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p–nitrophenyl galactosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Treazolium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p–nitrophenyl α–β–Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Antibiotics															
Oxolinic acid	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Tetracycline	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Penicillin	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Amoxicillin	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Nalidixic Acid	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Tetracycline	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Lincomycin	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Nitrofurantoin	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Florfenicol	S	S	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	S	S
Gentamycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Enrofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefoperazone	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Norfloxacin	S	S	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	S	S
Flumequine	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cefurocime	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sulphamethox	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Doxycycline	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Apramycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cephalothin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxacillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Colistin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline	R	R	R	NC	R	R	R	NC	R	R	R	R	R	R	R
Tylosin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Spectinomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Clindamycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

(+:positive, – :negative, α:Alpha hemolysis, NC: no calculation, S: susceptible, I: intermadiate susceptible, R: resistant)

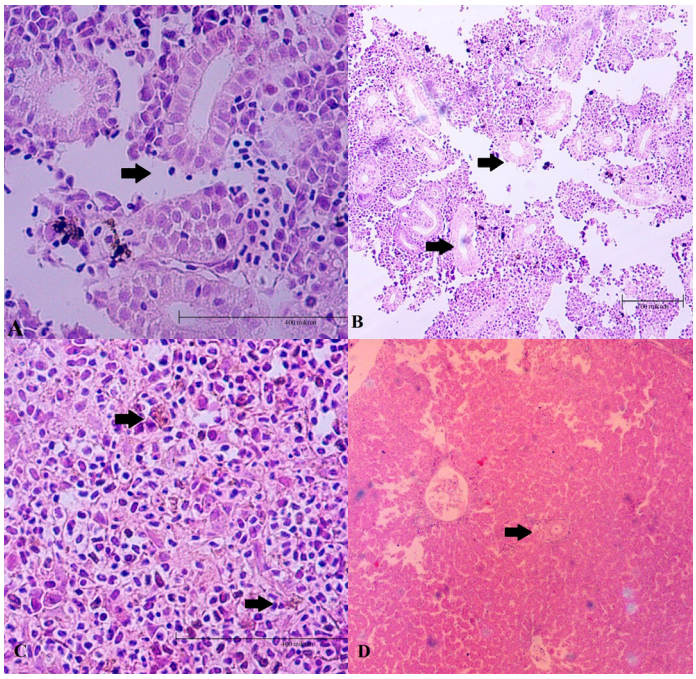


FIGURE 3. A–B: Degenerative and necrotic changes in kidney tubule cells, C: Inflammatory cells in spleen, D: Inflammatory infiltration in the hepatic central vena stage

Genetic characterization

Fourteen phenotypically and histopathologically examined bacterial isolates (S1–S14) were also identified by genetic method. In the conventional PCR conducted using 16S rRNA primers on fourteen clinical isolates, the gene amplicon of 240 bp size was found to be positive in 43% of the cases, confirming it as *S. warneri*. (TABLE III). The positive PCR results of the 16S rRNA gene in this study were compared with previous studies, and it was observed that amplicons of the same size were obtained. [6, 10, 11]. Moreover, the 16S rRNA gene was sequenced for six samples (S1 to S6) randomly selected from fourteen samples and it was observed that the 16S rRNA gene sequence published in GenBank under accession numbers OR144346–1, 2, 3 and OR144347–1, 2, 3 had 100% homology with *S. warneri* (FIG. 4). Kim *et al.* [12] and Iwase *et al.* [36] previously reported that although 16S rRNA analyses are frequently used to identify various bacterial species, they cannot discriminate and misidentify between closely related species such as *Staphylococcus* spp. Therefore, to distinguish between the two closely related species *S. warneri* and *S. pasteuri* and to validate the results, a species–specific *sodA* gene PCR was additionally performed, and it was found that only 50% of the fourteen isolates were positive in the *sodA*–targeted PCR.

As a result of MALDI–TOF analyses, the reference strains and all isolates were identified with *m/z* score values greater than 2.00.

TABLE III
Comparison of the results of phenotypic, genetic methods and MALDI TOF in the identification of *Staphylococcus warneri*

No	Isolate / Strain	BBL Crystal™ GP Identification	PCR Results		16S rRNA Sequencing Result		MALDI–TOF Identification	
			16S rRNA gene	Partial *sodA* gene	*Staphylococcus* sp. AC no	Similarity Scores (%)	Best Match Bacteria	Log Scores
RS 1	*S. warneri* ATCC 27836	+	+	+	*S. warneri* MH426978-1	100	*S. warneri* ATCC 27836	3.00
RS 2	*S. epidermidis* ATCC 35538	-	-	-	*S. epidermidis* L37605-1	100	*S. epidermidis* ATCC 35538	2.80
RS 3	*S. pasteuri* ATCC 51129	-	-	-	*S. pasteuri* KJ623586	100	*S. pasteuri* ATCC 51129	3.00
S 1	CI 1	+	+	+	*S. warneri*_1 OR144346	100	*S. warneri* ATCC 27836	2.50
S 2	CI 2	+	+	+	*S. warneri*_2 OR144346	100	*S. warneri* ATCC 27836	3.00
S 3	CI 3	+	+	+	*S. warneri*_3 OR144346	100	*S. warneri* CICC 23992	2.93
S 4	CI 4	+	+	+	*S. warneri*_1 OR144347	100	*S. warneri* CCUG 7325T	2.40
S 5	CI 5	+	+	+	*S. warneri*_2 OR144347	100	*S. warneri* 5353_2014	2.50
S 6	CI 6	+	+	+	*S. warneri*_3 OR144347	100	*S. warneri* ATCC 27836	3.00
S 7	CI 7	+	-	-	NA	NA	*S. warneri* ATCC 27836	2.86
S 8	CI 8	+	-	-	NA	NA	*S. warneri* Mb18796_1CHB	3.00
S 9	CI 9	+	-	-	NA	NA	*S. warneri* ATCC 27836	3.00
S 10	CI 10	+	-	-	NA	NA	*S. warneri* ATCC 27836	3.00
S 11	CI 11	+	-	-	NA	NA	*S. warneri* DSM 20316	3.05
S 12	CI 12	+	-	-	NA	NA	*S. warneri* DSM 20316	2.93
S 13	CI 13	+	-	-	NA	NA	*S. warneri* 5353_2014	2.80
S 14	CI 14	+	-	-	NA	NA	*S. warneri* CCUG 7325T	2.35

(Reference strain; RS, Sample; S, Clinical Isolate; CI)

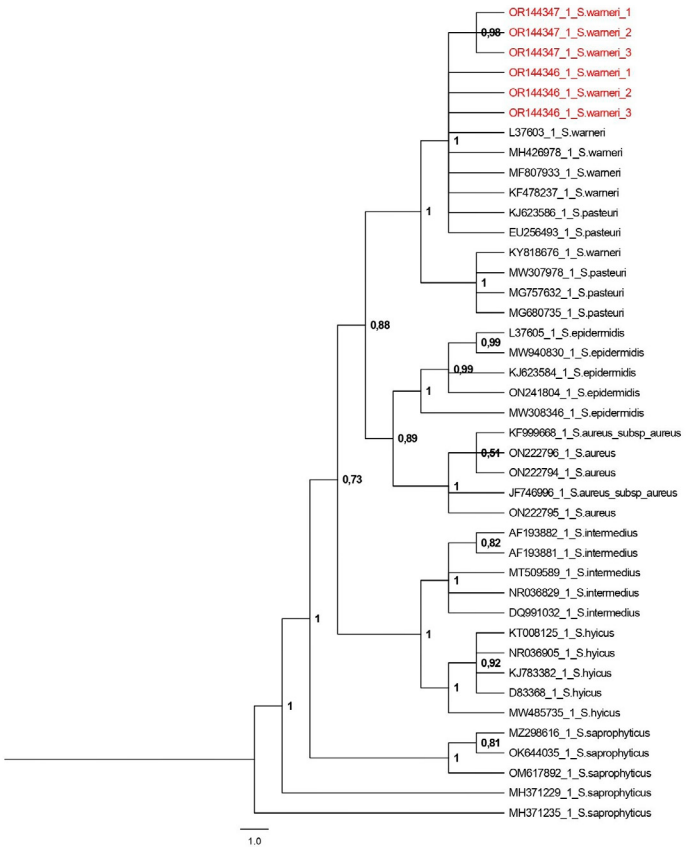


FIGURE 4. Phylogenetic tree based on 16S rRNA gene of six *Staphylococcus warneri* isolates (S1 to S6) that published in GenBank and phylogenetically close members of *Staphylococcus*

The isolates between S1 and S14 were confirmed as *S. warneri* with mass scores ranging from 2.35 to 3.05. TABLE III summarizes the MALDI–TOF MS analysis results obtained for fourteen isolates (S1–14) and associated bacterial identification match for the *Staphylococcus* reference strains.

Besides, FIG. 5 shows the peptide mass fingerprint spectra obtained for *Staphylococcus* reference strains (A) and isolates called *S. warneri* (S6) (B). For *S. warneri*, 2803.295, 4307.366, 5907.798, 8094.116, 9630.956 and 10209.725 Da represent peptide mass fingerprint spectra containing a total of six very consistent mass peaks. The comparison of these characteristic peak results obtained by the MALDI–TOF MS method with reference spectra in the proteomic mass database shows that the bacteria were identified and distinguished as *S. warneri* with 100% accuracy. Numerous studies on the accurate identification of the majority of fish pathogenic bacteria using either MALDI–TOF MS alone or together with other identification techniques have been found in the literature [20]. In some studies, comparative 16S rRNA gene sequencing or PCR analysis has been conducted to show the similarities and phylogenetic relationships among *Staphylococcus* species [1, 2, 5, 6, 17]. Yet, no study has been found that compared MALDI–TOF MS analysis with the traditional techniques for identifying *S. warneri* in rainbow trout.

CONCLUSION

The results presented herein compare conventional and proteomic MALDI–TOF MS analyses to confirm the identification of isolates obtained from diseased rainbow trout in Türkiye as *S. warneri*. This study demonstrated that the MALDI–TOF method is a rapid (2–3 min), cost–effective (~2$ USD) and accurate

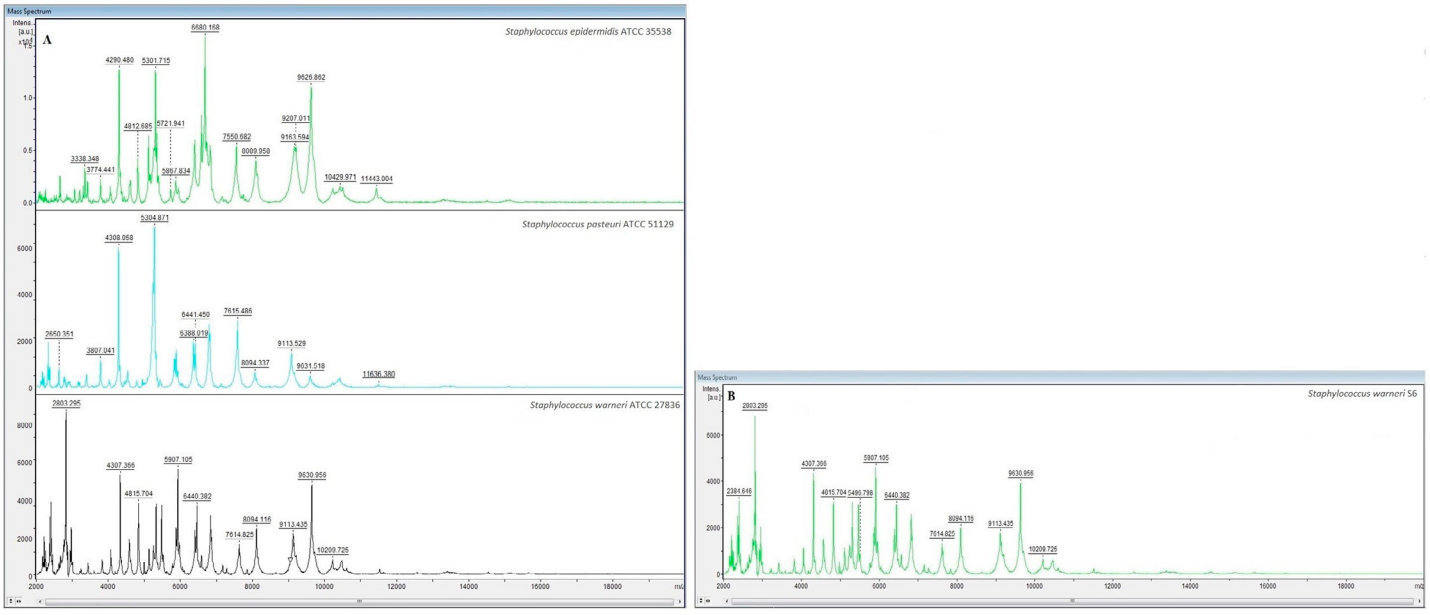


FIGURE 5. MALDI–TOF mass peptide profiles of reference strains (A) and *Staphylococcus warneri* (Sample 6) (B) in the range of 2384.646 to 11636.390 Da

technique that could be used as an alternative to other diagnostic methods used to differentiate genetically similar bacterial species such as *S. warneri* and *S. pasteuri*. Further research into how MALDI–TOF MS can be applied to aquaculture is warranted, such as identification of slow–growing fish bacteria and the direct identification of pathogens from tissues without culturing. In order to make MALDI–TOF MS more accessible and user–friendly for aquaculture practitioners, efforts should be made to improve sample preparation methods and speed up data analysis. This would strongly contribute to future developments in disease diagnosis and the promotion of sustainable aquaculture. However, for precise categorization and identification of fish diseases, more proteomic data need to be submitted to international databases. The widespread application of this technique in the field of aquaculture diseases will contribute to early disease diagnosis, rapid and effective treatment, and will also promote healthier fish populations and sustainability in aquaculture.

ACKNOWLEDGMENTS

The authors would like to thank Akdeniz University, Central Research Laboratory for the use of the MALDI–TOF instrument.

Conflict of interest

The author declare no competing interests.

Animal Ethics

Ethical approval was not required.

Funding

There is no funding.

Field Study Permissions

Field study permissions was not required.

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