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Phenotypic and genotypic characterization of isolated *Flavobacterium psychrophilum* strains in rainbow trout cage farms in Kahramanmaraş city of Türkiye

Caracterizacion fenotípica y genotípica de cepas aisladas de *Flavobacterium psychrophilum* en granjas de jaulas de trucha arcoíris en la ciudad de Kahramanmaraş, Turquía

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ABSTRACT

This research aimed to characterize phenotypically and genotypically characterize *Flavobacterium psychrophilum* strains in juvenile rainbow trout (*Oncorhynchus mykiss*) from 68 cage farms located in Kahramanmaras, Türkiye. The present study was conducted from December 2022 to July 2023, with samples of juvenile fish (5 g or less) being collected alongside larger individuals. The isolation of the bacterium was achieved by employing Tryptone Yeast Salt Agar medium, which was then incubated at 15°C for a period of 5-7 days. The purification of yellow colonies was undertaken, and biochemical testing was conducted using the Biolog GENIII microplate system to evaluate the phenotypic traits. For the purpose of molecular validation, the identity of the strains initially identified by Biolog was confirmed by PCR with specific primers. All isolates underwent sequence analysis, and the resulting data were compared against NCBI reference sequences. Twelve strains of *F. psychrophilum* were conclusively identified through combined phenotypic and genotypic analysis.

Key words: *Oncorhynchus mykiss*; cold–water bacterial disease; bacteriological tests; Biolog GEN III; PCR; Gene sequencing; antibiogram

RESUMEN

Esta investigación tuvo como objetivo caracterizar fenotípica y genotípicamente cepas de *Flavobacterium psychrophilum* en juveniles de trucha arcoíris (*Oncorhynchus mykiss*) de 68 granjas de jaulas ubicadas en Kahramanmaras, Turquía. El presente estudio se realizó entre diciembre de 2022 y julio de 2023, durante el cual se recolectaron especímenes de peces juveniles (con un peso de 5 g o menos) junto con individuos de mayor tamaño. El aislamiento de la bacteria se llevó a cabo mediante la utilización del medio Tryptone Yeast Salt Agar, el cual fue incubado a una temperatura de 15°C por un período comprendido entre 5 y 7 días. El procedimiento llevado a cabo consistió en la purificación de las colonias amarillas, para lo cual se empleó el sistema de microplacas GENIII de Biolog, con el propósito de realizar las pruebas bioquímicas necesarias para la evaluación de los rasgos fenotípicos. En el ámbito de la validación molecular, la identidad de las cepas identificadas inicialmente por Biolog fue confirmada mediante la técnica de Reacción en Cadena de la Polimerasa con cebadores específicos. Todos los aislamientos se sometieron a análisis de secuencia y los datos resultantes se compararon con las secuencias de referencia del NCBI. Se identificaron de manera concluyente doce cepas de *F. psychrophilum* mediante análisis fenotípico y genotípico combinado.

Palabras clave: *Oncorhynchus mykiss*; enfermedad bacteriana del agua fría; pruebas bacteriológicas; Biolog GEN III; PCR; Secuencia de genes; antibiograma

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INTRODUCTION

The aquaculture industry in the region is focused on the cultivation of a single species, *Oncorhynchus mykiss*. In the context of Kahramanmaraş inland waters, two distinct aquaculture methods have been observed. The first method involves the cultivation of trout (Salmonidae) in concrete ponds, constructed on springs or streams. The second method involves the cultivation of trout in cages, installed in dam lakes [1].

The bacterium *Flavobacterium psychrophilum* was first isolated from *Oncorhynchus kisutch* in the USA in 1946 [2, 3]. In Türkiye, *F. psychrophilum* have been isolated from rainbow trout farms [4, 5, 6], and it has been found to cause high mortality (75%) in trout with a weight range of 0.5 – 1.0 grams (g) [7]. Furthermore, the mortality rate was found to be 70% in trout fry weighing between 1 and 5 g, and 10% in trout fry weighing between 10 and 15 g. [8].

F. psychrophilum, the causative agent of bacterial cold water disease, peduncle disease and rainbow trout fry syndrome (psychrophilosis), which has been isolated from cultured salmonid species (*O. mykiss*, *Salmo trutta*, *Salvelinus fontinalis* and *Salmo namaycush*) [2, 3, 7, 9, 10].

In this disease, especially when the hatchery temperature falls below 10°C, various lesions of different sizes are observed on the dorsal and caudal fins, body surface, bleeding on the skin and exophthalmus in juvenile trout. The disease under discussion is an infectious and lethal bacterial infection. The clinical signs include pallor of the gills and liver, abdominal distension, yellow intestinal contents and enlargement of both the spleen and liver [2, 3, 7, 8, 11, 12, 13].

The production of *F. psychrophilum*, the bacterium responsible for significant economic damage to trout farms, is a complex and time–consuming process [14, 15].

The utilisation of microbiological tests is predominantly employed for the purpose of ascertaining the phenotypic characteristics (i.e. the morphological, biochemical and physiological characteristics that are formed by the genotype of the organism in conjunction with the external environment) of *F. psychrophilum* [8].

The Biolog System (the Biolog GENIII microplate) is a tool used in the identification of Gram–positive and Gram–negative bacteria through the execution of 94 biochemical tests. The basis of these tests is the utilization of 71 carbon source assays and 23 chemical sensitivity assays. The Biolog Microbial Identification System (Microstation ID System) has been demonstrated to be a versatile system. Its utilisation is for the identification and characterisation of environmental and pathogenic organisms in a wide range of microbiological fields [16, 17].

Polymerase Chain Reaction (PCR) is a method that facilitates the biochemical amplification of a targeted nucleic acid sequence (DNA) to enable the rapid detection of the genetic material of causative agents [18].

The 16S rRNA gene has been utilised in the domain of human medicine for the classification and genotypic analysis of bacteria. The utilisation of sequence–based molecular identification has become a prevalent method in the identification of bacteria encountered in veterinary clinical practice [19].

An investigation was conducted into the prevalence of *F. psychrophilum*, the causative agent of bacterial cold water disease, peduncle disease and rainbow trout fry syndrome (psychrophilosis), in the rainbow trout population of the Kahramanmaraş province. The investigation used a combination of morphological, phenotypic, biochemical, molecular and genetic characteristics.

MATERIALS AND METHODS

Work area

The study area consisted of 68 aquaculture facilities located in Kahramanmaraş Province, including 48 grow–out farms and 20 integrated farms engaged in both fry and portion–size trout production. The grow–out farms have a total production capacity of 16,279 tons, while the fry farms have a total capacity of 122,592,000 number fry.

Fish material

Between December 2022 and July 2023, a total of 1.088 juvenile rainbow trout (*O. mykiss*) were collected from 68 different aquaculture facilities in Kahramanmaraş Province, with 16 samples taken from each facility.

Water quality parameters

The temperature, pH and oxygen levels of the water in the Fish Farms where samples were taken were measured monthly. The average temperature of the water over 8 months was measured as 9.52 ± 1.85°C, O_2 content as 9.38 ± 2.02 mg·L^{-1}. and pH value as 7.57 ± 0.56.

Feed content

The juvenile fish were fed a commercial granular fry diet (0.6 NM) formulated to meet the specific nutritional requirements of early developmental stages. The feed was administered twice daily at a rate appropriate for the biomass and water temperature, following standard aquaculture practices. The chemical composition of the feed was as follows: 40% crude protein, 11% crude fat, 10.1% crude ash, 1.3% crude fiber, 2.4% calcium, 1.5% phosphorus, and 0.3% sodium. This nutrient profile was designed to promote optimal growth, survival, and physiological development in juvenile fish.

Isolation and identification of *Flavobacterium psychrophilum*

The isolation of *F. psychrophilum* from trout farms was achieved through the preparation of dilutions. To this end, homogenates obtained from samples of fry weighing less than 5 g, taken directly from trout, were treated with 1/10 physiological saline. From these dilutions, 0.5 mL was transferred onto Tryptone Yeast Salt Agar (TYES–A) and spread with a sterile swab. Abdominal incisions were made under aseptic conditions, and the liver, spleen, kidney and intestines of the pups larger than 5 g were inoculated onto TYES–A medium. All inoculated media were then subjected to refrigerated oven incubation at 15°C (Lovibond BOD Incubator, TC 135 S, England) for a period of 5-7 days (d).

Colonies of yellow colour were cultivated on TYES–A medium and subsequently inoculated onto TYES–A medium to obtain subcultures. Gram staining was performed on these colonies in order to ascertain the staining properties of the causative agent and the purity of the culture. The initial testing of rod–shaped and Gram–negative bacteria was conducted for the presence of catalase and flexirubin pigment. Of these tests, catalase is semi–positive and the flexirubin pigment shows an orange hue; The following substances were analysed: cytochrome oxidase, methyl red, voges proskauer, oxidation–fermentation, nitrate, congo red, citrate, indole, ortho–nitrophenyl–beta–D–galactosidase, decarboxylase, urease. A series of analytical procedures were conducted, encompassing the assessment of hydrogen sulphide levels, alongside biochemical investigations. These encompassed the fermentation of carbohydrates, gelatin, hemolysis, starch, esculin, casein, tween 20 and tween 80, hydrolysis, and movement examination.

Flavobacterium psychrophilum identification with biolog system

Flavobacterium psychrophilum isolates were subjected to incubation on TYES–A at a temperature of 15°C for a duration of 48 h. Suspension was prepared in Biolog IF–A buffer solution. In a standard turbidity tube, the bacterial concentration was adjusted to 92–98% by turbidimetry. Densities were adjusted and 100 µl of the bacterial suspensions were placed in each well of the microplate. The microplates were then incubated at 15°C in the Lovibond BOD Incubator (TC 135 S, England) for 4–24 h. Thereafter, a comparison was made between the microplate and the database of the system, which was then read on the reader. This process facilitated the identification of the bacteria.

Flavobacterium psychrophilum Specific PCR and sequence analysis

Tissue and Bacterial DNA purification kit (GeneMATRIX) was used for chromosomal DNA isolation from *F. psychrophilum* strain.

The PCR mixture was prepared in a total volume of 50 µl, comprising the following components; 5 µl of 10× PCR buffer, 5 µl of 25 mM $MgCl_2$, 250 µM each of deoxynucleotides, 1 U of the 25 U Taq DNA Polymerase enzyme (MBI, Fermentas) and two specific primer pairs of the *F. psychrophilum* species. For *F. psychrophilum* strain; 50 pmol of each of the primer pairs GYRA–FP1F (5'–GAAACCGGTGCACAGAAGG-3') and GYRA–FP1R (5'–CCTGTGGCTCCGTTTTATTAA-3') [20] and 5 µl of template DNA were added. The PCR reactions were performed in a PCR–Sprint thermal cycler (Thermo Scientific ARKTIK Thermal Cycler Type 5020 96 Well Lab, USA). The process of PCR amplification incorporated a preliminary denaturation stage at 94°C for 30 s, a PCR cycle denaturation stage at 51°C for 90 s, and a hybridisation stage at 72°C for 2 min. Consequently, the amplification product of 1089 base pairs (bp) was designated as positive [7].

AB3130XL16 capillary device (Hitachi, Japan) was used for DNA gene sequence analysis.

Antibiogram tests

Antibiotic susceptibility tests were performed according to disc diffusion method.

Analysing the data

Following the organisation and sequencing of the sequence results in Clone Manager 9, they were entered into the NCBI GenBank electronic database. The species identification was confirmed by comparing the sequences with those of other *F. psychrophilum* species via BLAST.

RESULTS AND DISCUSSION

Morphological

In the province of Kahramanmaras, the anamnesis of the extracted fish revealed that specimens of particularly diminutive size, with weights below 5 g, exhibited symptoms of inactivity, anorexia, and a lack of responsiveness to nutrition. Furthermore, findings analogous to the symptoms of bacterial cold water disease, which resulted in significant economic losses 20-50 d after the onset of symptoms, were identified. As was established during the course of the examinations that the number of baby trout perishing daily ranged from 100 to 200. Consequently, a 20% mortality of the juvenile fish was observed following the outbreak, which lasted approximately two weeks.

In the ponds containing juvenile fish, all sampled diseased rainbow trout were observed to exhibit clinical signs including loss of appetite, reduced activity, and surface swimming behavior. Some fish demonstrated spiral swimming movements and exhibited balance impairments. Conversely, it was observed that some fish exhibited abdominal distension due to ascites, exophthalmus, darkening of the skin colour and white lesions on the dorsal and caudal fins. In fish with advanced disease, the caudal fin was completely destroyed and radiuses appeared.

The internal organs of infected rainbow trout fry were exposed through an incision made in the abdominal region. The liver and kidneys were observed to exhibit a pallor, while the spleen demonstrated an enlarged appearance.

Phenotypic and biochemical

After the cultures on TYES–A agar were kept in an oven at 15°C for 5–7 d, pure yellow pigmented colonies were observed (FIG. 1).

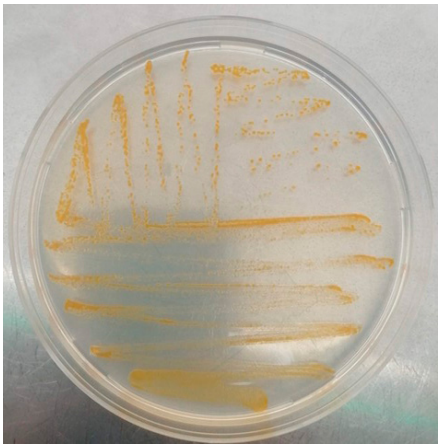


FIGURE 1. Pure colonies grown on TYES–A agar.

The phenotypic and biochemical characteristics of 12 Gram–negative rod–shaped strains isolated from pure cultures were determined (TABLE I). Antibacterial susceptibility testing of 12 *F. psychrophilum* isolates revealed distinct patterns of resistance and sensitivity. All isolates were resistant to amoxicillin, ampicillin, erythromycin, nalidixic acid, and neomycin. Additionally, the majority of isolates (9 out of 12) showed resistance to gentamicin, with the remaining three exhibiting intermediate susceptibility. In contrast, all isolates were sensitive to streptomycin, enrofloxacin, florfenicol, and oxytetracycline. Susceptibility to bacitracin was variable, with 9 isolates classified as sensitive and 3 as intermediate. For trimethoprim–sulfamethoxazole (Batrim), 8 isolates were fully sensitive, while 4 showed intermediate susceptibility. These findings indicate a high level of multidrug resistance among the isolates, particularly against β–lactam and macrolide antibiotics, while demonstrating effective susceptibility to several commonly used antibiotics such as florfenicol and oxytetracycline (TABLE II).

TABLE I
Phenotypic and biochemical characteristics of *Flavobacterium psychrophilum*

Phenotypic and Biochemical Characteristics	12 *Flavobacterium psychrophilum* strains isolated											
	1	2	3	4	5	6	7	8	9	10	11	12
Color of colonies	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Gram Staining	–	–	–	–	–	–	–	–	–	–	–	–
Catalase	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +	Weak +
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–
Flexirubin pigment	+	+	+	+	+	+	+	+	+	+	+	+
Congo red	–	–	–	–	–	–	–	–	–	–	–	–
Movement	–	–	–	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–	–	–	–
Methyl Red	–	–	–	–	–	–	–	–	–	–	–	–
Voges Proskauer	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate test	–	–	–	–	–	–	–	–	–	–	–	–
Oxidation/Fermentation	–/–	–/–	–/+	–/–	–/–	–/+	–/–	–/–	–/+	–/–	–/–	–/–
Ortho–Nitrophenyl–Beta–D–Galactosidase	–	–	–	–	–	–	–	–	–	–	–	–
Ornithinecarboxylase	–	–	–	–	–	–	–	–	–	–	–	–
Gelatine hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Simmons citrate	–	–	–	–	–	–	–	–	–	–	–	–
Urease test	–	–	–	–	–	–	–	–	–	–	–	–
Hydrogen Sulphide	–	–	–	–	–	–	–	–	–	–	–	–
Haemolysis	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–
Esculin hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Tween 20 hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80 hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	–	–	–	–	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–	–	–
Galactose	–	–	–	–	–	–	–	–	–	–	–	–
Arabinose	–	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–
Lizin	–	–	–	–	–	–	–	–	–	–	–	–
Fructose	–	–	–	–	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–
Mannose	–	–	–	–	–	–	–	–	–	–	–	–
Melibose	–	–	–	–	–	–	–	–	–	–	–	–
Amigolalin	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	–	–	–	–	–	–	–	–	–	–	–	–
Reproduction on TYE agar	+	+	+	+	+	+	+	+	+	+	+	+
Reproduction in TYES–A	+	+	+	+	+	+	+	+	+	+	+	+
Reproduction in TSA	–	–	+	–	–	–	+	–	–	–	–	–
Reproduction in TSB	–	–	+	–	–	–	+	–	–	–	–	–

Blood agar	–	–	–	–	+	–	–	–	–	–	–	–
Reproduction in MCA	–	+	–	–	–	–	+	–	–	–	–	–
Reproduction in PCA	+	+	+	+	+	+	+	–	–	–	–	–
Reproduction in MHA	+	+	+	+	+	+	+	+	+	+	+	+
0°C (Reproduction)	–	–	–	–	–	–	–	–	–	–	–	–
5°C	+	+	+	+	+	+	+	+	+	+	+	+
15°C	+	+	+	+	+	+	+	+	+	+	+	+
20°C	+	+	+	+	+	+	+	+	+	+	+	+
25°C	–	–	+	–	–	–	–	–	–	–	–	+
30°C	–	–	–	–	–	–	–	–	–	–	–	–
37°C	–	–	–	–	–	–	–	–	–	–	–	–
0.0% NaCl (Growth)	+	+	+	+	+	+	+	+	+	+	+	+
0.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
1.0% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
2.0% NaCl	–	–	–	–	–	–	–	–	–	–	–	–

+ : Positive reaction, - : Negative reaction, MCA: MacConkey Agar, TSA: Tryptic Soy Agar, TSB: Tryptic Soy Broth, PCA: Plate Count Agar, MHA: Mueller–Hinton Agar

TABLE II
Antibacterial susceptibility status of 12 isolated *Flavobacterium psychrophilum*

Antibacterials	12 *Flavobacterium psychrophilum* strains isolated											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Gentamicin (CN, 10 µg)	R	I	R	R	I	R	R	R	R	I	R	R
Amoxicillin (AX, 25 µg)	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin (AMP, 10 µg)	R	R	R	R	R	R	R	R	R	R	R	R
Erythromycin (E, 15 µg)	R	R	R	R	R	R	R	R	R	R	R	R
Streptomycin (S, 10 µg)	S	S	S	S	S	S	S	S	S	S	S	S
Enrofloxacin (ENR, 5 µg)	S	S	S	S	S	S	S	S	S	S	S	S
Florfenicol (FFC, 30 µg)	S	S	S	S	S	S	S	S	S	S	S	S
Bacitracin (B, 30 µg)	S	I	S	S	I	S	S	I	S	S	S	S
Nalidixic Acid (NA, 30µg)	R	R	R	R	R	R	R	R	R	R	R	R
Oxytetracycline (OT, 30 µg)	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin (N, 30 µg)	R	R	R	R	R	R	R	R	R	R	R	R
Batrim–Trimethoprim + Sulfamethoxazole (BC, 1.25µg+23.7 µg)	S	I	S	S	I	S	S	I	S	I	S	S

S: Sensitive, I: Intermediate, R: Resistant

Biolog (The biolog GENIII micro plate) findings

The Biolog System (The biolog GENIII micro plate) was used to confirm biochemical tests and to determine other phenotypic characteristics (TABLE III).

The investigation revealed that three bacterial species (*Flavobacterium resinovorum*, *Chryseobacterium scophthalmum*, *Flavobacterium tirrenicum* and *Obesum bacteria proteus* biogroup 2) exhibited similarity in different ratios, while one bacterial species (*F. psychrophilum*) demonstrated similarity in percentage terms (FIG. 2).

Polymerase chain reaction

The present study involved the isolation of DNA from 12 strains of *F. psychrophilum*, which were isolated and identified from Kahramanmaraş fisheries. The DNAs were then subjected to a process of amplification by PCR. Subsequently, 12 *F. psychrophilum*, one positive control, one negative control and one 100 bp molecular marker (M) were placed on a 1.5% agarose gel and electrophoresed. The agarose gel was subjected to staining with ethidium bromide and observed under ultraviolet transillumination. The GYRA–FP1F and GYRA–FP1R primer pairs belonging to the *F. psychrophilum* species demonstrated bands of 1089 bp in length (see FIG. 3). It was determined that these bands possessed the same length as the positive control. Consequently, the 12 suspected strains isolated were identified as *F. psychrophilum*.

Sequence analysis

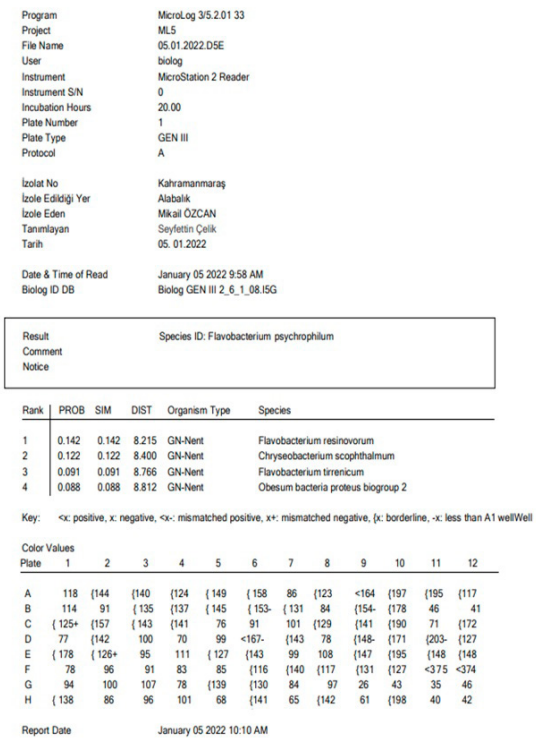
Sequence analysis of *F. psychrophilum* strains was performed by forward reading. Chromatograms of the sequence peaks of *F. psychrophilum* were displayed and individually checked using Bioedit Sequence Alignment Editor program (FIG. 4).

A comparison was then made between the sequence results and reference *F. psychrophilum* strains in NCBI in order to ascertain the degree of similarity (FIG. 5). The closest similarity was determined with *F. psychrophilum* 16S ribosomal RNA (Sequence

TABLE III
Other phenotypic characteristics of *Flavobacterium psychrophilum* isolated from rainbow trout with the biolog GENIII micro plate device

A1 Negative Control	A2 Dextrin	A3 D–Maltose	A4 D–Trehalose	A5 D–Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D–Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
–	+/–	+/–	+/–	+/–	+/–	–	+/–	Weak +	+	+/–	+/–
B1 D–Raffinose	B2 α–D–Lactose	B3 DMelibiose	B4 β–Methyl–D–Glucoside	B5 D–Salicin	B6 N–Acetyl–D–Glucosamine	B7 N–Acetyl–β–D–Mannosamine	B8 N–Acetyl–D–Galactosamine	B9 N–Acetyl Neuraminic acid.	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
–	–	+/–	+/–	+/–	+/–	+/–	–	+/–	+/–	–	–
C1 α–D–Glucose	C2 D–Mannose	C3 D–Fructose	C4 D–Galactose	C5 3-Methyl Glucose	C6 D–Fucose	C7 L–Fucose	C8 L–Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D–Serine
+/–	+/–	+/–	+/–	–	–	–	+/–	+/–	+/–	–	+/–
D1 D–Sorbitol	D2 D–Mannitol	D3 D–Arabitol	D4 Myo Inositol	D5 Glycerol	D6 D–Glucose–6-PO_4	D7 D–Fructose–6-PO_4	D8 D–Aspartic Acid	D9 D–Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
–	+/–	–	–	–	Weak +	+/–	–	+/–	+/–	+/–	+/–
E1 Gelatin	E2 Glycyl–L–Proline	E3 L–Alanine	E4 L–Arginine	E5 L–Aspartic Acid	E6 L–Glutamic Acid	E7 L–Histidine	E8 L–Pyroglutamic Acid	E9 L–Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
+	+/–	–	–	+/–	+/–	–	–	+/–	+/–	+/–	+/–
F1 Pectin	F2 D–Galacturonic Acid	F3 L–Galactonic Acid Lactone	F4 D–Gluconic Acid	F5 D–Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D–Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
–	–	–	–	–	+/–	+/–	+/–	+/–	+/–	+	+
G1 p–Hydroxy–Phenylacetic Acid	G2 Methyl Pyruvate	G3 D–Lactic Acid Methyl Ester	G4 L–Lactic Acid	G5 Citric Acid	G6 α–Keto Glutaric Acid	G7 D–Malic Acid	G8 L–Malic Acid	G9 Bromo–Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
–	–	–	–	+/–	+/–	–	–	–	–	–	–
H1 Tween 40	H2 γ–Amino–Butryric Acid	H3 α–Hydroxy–Butyric Acid	H4 β–Hydroxy–D,L Butyric Acid	H5 α–Keto–Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate
+/–	–	–	–	–	+/–	–	+/–	–	+/–	–	–

+ : Positive reaction, – : Negative reaction



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User	biolog
Instrument	MicroStation 2 Reader
Instrument S/N	0
Incubation Hours	20.00
Plate Number	1
Plate Type	GEN III
Protocol	A
İzolat No	Kahramanmaraş
İzole Edildiği Yer	Alabalık
İzole Eden	Mikail ÖZCAN
Tanımlayan	Seyfettin Çelik
Tarih	05. 01.2022
Date & Time of Read	January 05 2022 9:58 AM
Biolog ID DB	Biolog GEN III 2_6_1_08.I5G

Result	Species ID: Flavobacterium psychrophilum
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.142	0.142	8.215	GN-Nent	Flavobacterium resinovorum
2	0.122	0.122	8.400	GN-Nent	Chryseobacterium scophthalmum
3	0.091	0.091	8.766	GN-Nent	Flavobacterium tirrenicum
4	0.088	0.088	8.812	GN-Nent	Obesum bacteria proteus biogroup 2

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, (x: borderline, -x: less than A1 wellWell

Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	118	(144	(140	(124	(149	(158	86	(123	<164	(197	(195	(117
B	114	91	(135	(137	(145	(153-	(131	84	(154-	(178	46	41
C	(125+	(157	(143	(141	76	91	101	(129	(141	(190	71	(172
D	77	(142	100	70	99	<167-	(143	78	(148-	(171	(203-	(127
E	(178	(126+	95	111	(127	(143	99	108	(147	(195	(148	(148
F	78	96	91	83	85	(116	(140	(117	(131	(127	<375	<374
G	94	100	107	78	(139	(130	84	97	26	43	35	46
H	(138	86	96	101	68	(141	65	(142	61	(198	40	42

Report Date January 05 2022 10:10 AM

FIGURE 2. Output of the biolog diagnostic system of *Flavobacterium psychrophilum* isolated from rainbow trout (*Oncorhynchus mykiss*)

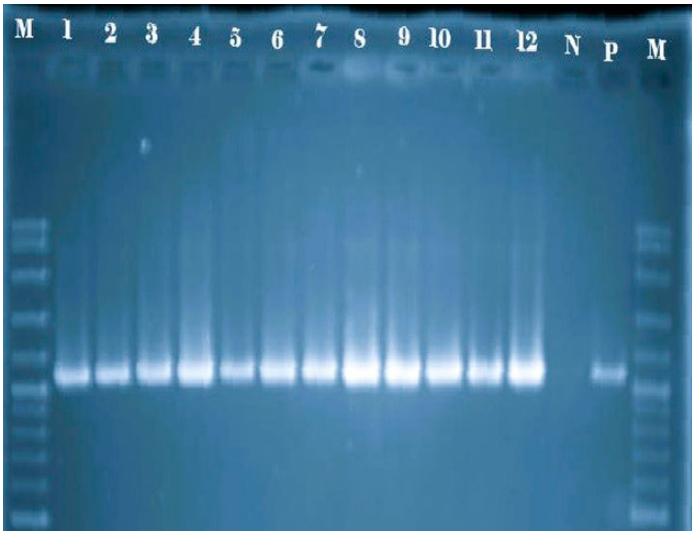

FIGURE 3. Agarose gel showing 1089 bp bands formed by PCR analysis of *Flavobacterium psychrophilum* DNA; 1-12: F. psychrophilum suspect samples; M: 100 bp molecular marker; N: Negative control (*Escherichia coli*) (MBI, Fermantas); P: Positive control reference strain (*F. psychrophilum* NCIMB 1947^T)

ID: KU212250.1). The highest degree of similarity was observed with *F. psychrophilum* 16S ribosomal RNA, with a percentage similarity of 700/700 (100%), an absence of gaps (0/700) and a

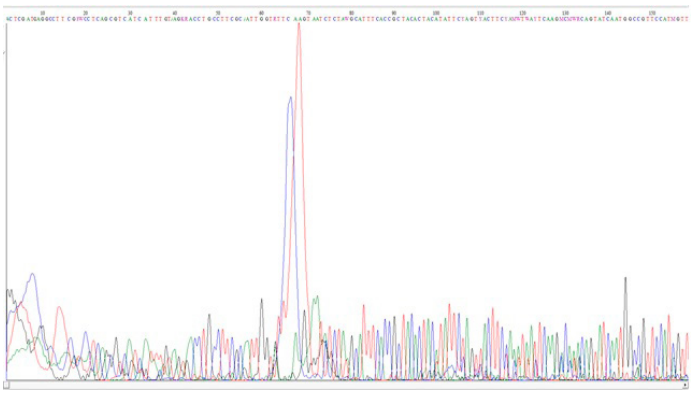

FIGURE 4. *Flavobacterium psychrophilum* sequence chromatogram

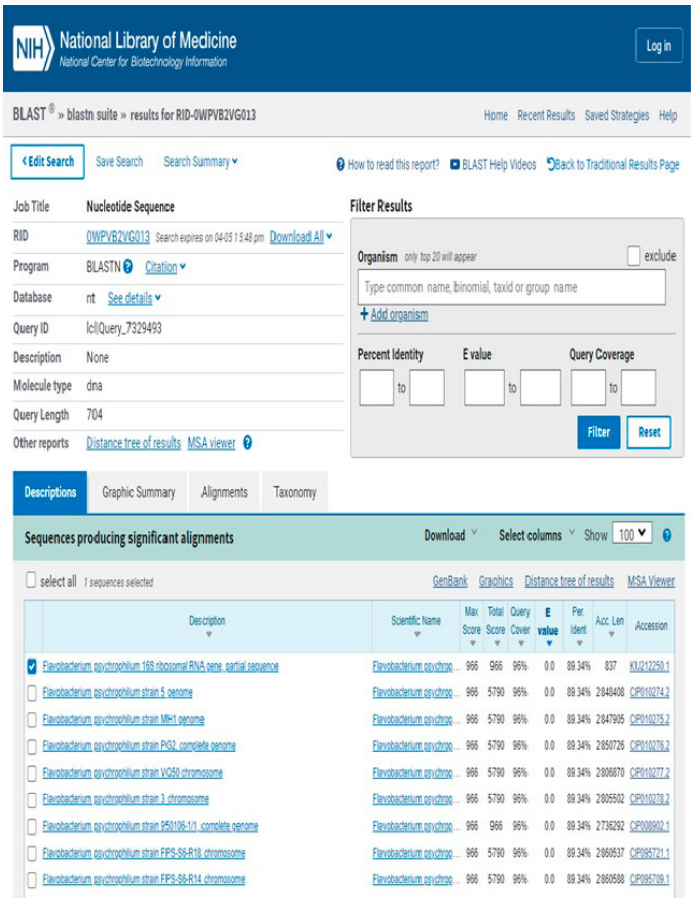

FIGURE 5. Comparison of one *Flavobacterium psychrophilum* sequence results with reference *F. psychrophilum* strains in NCBI for similarity

total score of 1293 bits (700). The following data is provided for reference: similarity values and alignment comparison (FIG. 6).

In this study, *F. psychrophilum* was identified as the causative agent following a comprehensive analysis encompassing morphological, phenotypic, biochemical, molecular and genetic characteristics. This investigation was conducted in response to sudden mortalities and clinical symptoms observed in trout farms

FIGURE 6. Alignment result for the taxon *Flavobacterium psychrophilum* (Query: *F. psychrophilum*, Sbjct: *F. psychrophilum* 16S ribosomal RNA (Sequence ID: KU212250.1)

in Kahramanmaraş province. The anorexia, stagnation, swimming disorders and external surface lesions observed, especially in juvenile trout weighing less than 5 g, coincide with the symptoms of bacterial cold water disease (BCWD). The clinical signs in question have been previously documented by Nematollahi *et al*. [9]

In a separate study [21], fish material was subjected to compression in a porcelain mortar and utilised in bacteriological cultivation. The stomacher homogenizer was selected in this study due to its superior sterility and the rapidity of the results it produces. Consequently, samples of sick fry, with a mass of less than 5 g, were successfully homogenised in the stomacher and utilised in bacteriological cultivation.

Cipriano and Holt [3], Özcan and Sarıeyyüpoğlu [7] and Nematollahi *et al*. [9] incubated Cytophaga Agar, Modified Anacker and Ordal Agar and Tryptone Yeast Extract Salt Agar at 15–20°C for 3–7 d to isolate *F. psychrophilum*. On these media, round, yellow colonies with raised, convex and thinly spreading margins, as well as round, smooth–edged colonies with raised, convex and thinly spreading margins were formed and stained preparations of these colonies showed the presence of Gram–negative rod–shaped and sliding bacteria with a length of 1.20–1.30 μm. By using the media in these studies to determine the phenotypic characteristics, the findings of the researchers on colony structure

and causative properties were once again confirmed. Similar findings were found in 12 *F. psychrophilum* isolated from trout farms in Kahramanmaraş province.

In earlier research [2, 3], it was documented that colonies formed by isolated *F. psychrophilum* exhibited yellow colouration, negative oxidase test results, positive flexirubin–type pigment production test results, positive gelatin and casein hydrolysis test results, and negative Congo red test, H_2S and acid production from carbohydrates test results. In this study, the application of these distinctive biochemical tests was successful, with analogous results obtained in the identification of *F. psychrophilum* isolated from spleen, liver, kidney, intestine and water samples of fry weighing less than 5 g, as well as from healthy, disease–suspected or recently dead trout fry weighing more than 5.

In the course of the study, it was observed that 12 strains isolated from the trout farms in Kahramanmaraş grew at temperatures of 5, 15 and 20°C. This finding was partially consistent with the results reported by Dalsgaard and Madsen [22].

A number of studies have reported negative results of the oxidase test [23], whilst others have reported positive results [24]. All strains isolated in this study were oxidase negative. This finding lends further credence to the hypothesis that phenotypic differences may be observed among *F. psychophilum* strains. In the identification of *F. psychophilum*, Cipriano and Holt [3] and Didinen *et al*. [23] reported that the O/F test yielded a negative result. In contrast, the findings of İspir *et al*. [11] indicated that the oxidation test yielded a negative result, while the fermentation test yielded a positive result. The fermentation test yielded positive results in some strains and negative results in others. The O/F test of the *F. psychrophilum* reference bacterial strain yielded negative results.

As reported by Nematollahi *et al*. [9], the growth of *F. psychrophilum* was observed to occur in 0.8–1% and 2% NaCl. Strains of *F. psychrophilum* isolated from trout production facilities in Kahramanmaraş and the reference strain, *F. psychrophilum* NCIMB 1947T, were found to demonstrate growth in 0%, 0.5% and 1% NaCl, but not in 2% NaCl. Consequently, it was determined that this bacterium does not proliferate in environments with a salinity level of 2% and above.

This is the primary reason why fish become unwell as a result of unfavourable alterations in environmental conditions. The agent has also been detected in the natural flora of the ovarian fluid and digestive tract of water and fish [4, 8, 9]. It has been reported by researchers working on this subject that the agents were isolated from both water and fish samples. Furthermore, the skin colour and white lesions on the dorsal and caudal fin have been noted. In advanced cases of the disease, the caudal fin was completely destroyed, the radii appeared, the liver and kidney were pale and the spleen was enlarged. Analogous findings were ascertained in the trout farm where the research was conducted.

Researchers such as İspir *et al*. [11] and Didinen *et al*. [23] have previously reported that the isolates were susceptible to amoxicillin/clavulanic acid, ampicillin, erythromycin, and gentamicin. However, the present study found *F. psychrophilum* strains to be resistant to these antibiotics. This finding indicates that the susceptibility of *F. psychrophilum* strains to antibacterial agents may be subject to geographical variation, depending on the region within the country where the strains are isolated. Alternatively, the isolates may have developed resistance to these antibacterial agents.

CONCLUSION

In this study, *F. psychrophilum*, one of the causative agents of Bacterial Cold Water Disease, Peduncle Disease, Rainbow Trout Fry Syndrome – RTFS (Psychrophilosis), which leads to significant economic losses in rainbow trout both in our country and worldwide, was isolated and identified using conventional culture methods. In addition, phenotypic analysis was performed using the Biolog System (Biolog GENIII MicroPlate), which consists of 94 tests, including the utilization of 71 different carbon sources and sensitivity to 23 chemical agents (resistance to inhibitory chemicals). Molecular identification of *F. psychrophilum* was carried out using PCR, a rapid diagnostic technique widely used in the identification of bacterial pathogens. Furthermore, 16S rRNA gene sequencing, a critical method employed in clinical microbiology laboratories for the identification of bacterial isolates, was used to characterize *F. psychrophilum*. As a result, rapid diagnosis of *F. psychrophilum* isolated was achieved by PCR, which allows for timely treatment and helps prevent substantial economic losses in aquaculture facilities.

ACKNOWLEDGEMENTS

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Ethical Approval

All animal studies were approved by the Animal Ethics Committee of KSÜZİRHADYEK and Research Institute (Protocol number: 2020/10-4).

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The author have no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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