

# Introduce of *Pseudomonas psychrophila*, as a new Pathogen Causing Disease in Cultured Rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

This is the first report on the world that *Pseudomonas psychrophila* is a new etiological agent of disease in fish. A disease case occurred during the fall of 2017 in a rainbow trout farm in the north of Iran (Guilan province). Anorexia, lethargy and mortalities of 10% fish per week were observed in rainbow trout *Oncorhynchus mykiss* weighting 45-107g in three fresh water ponds of a farm on the north of Iran. The fish showed external signs of dark pigmentation, exophthalmia, and hemorrhage at the base of the fins and tissues damage. Internal signs were enlarged spleen, pale Liver and intestine filled with yellowish fluid. Kidney and liver of diseased fish were aseptically streaked on MacConkey and sheep blood agar. The cultured bacterial 16S rRNA gene was sequenced. Causative bacteria were identified as *pseudomonas psychrophila* using morphological and biochemical characteristics and genotypic method based on the 16S rRNA gene.

**Keywords:** *Pseudomonas psychrophila*, *Oncorhynchus mykiss*, New fish disease, Iran

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## Introduction

Bacterial pathogens are naturally present in the fish environment and under some specific stress conditions. They are the etiological agents of the most important disease problems in aquaculture that induces mortalities and severe economic losses to fish farm (Shawna and Brian, 2020). *Pseudomonas psychrophila* is a psychrophilic, Gram-negative bacterium that is responsible for dairy spoilage (Samaržija et al., 2012). However, it has not yet been reported as a pathogen in aquaculture. *Pseudomonas psychrophila* does not produce siderophores, unlike many other members of the *Pseudomonas* genus (Carrion, et al., 2010). The optimal temperature for growth is 30°C, however it can grow between 0°C and 35°C (Hebraud et al., 1994). *Pseudomonas psychrophila* is recognized as pathogenic in rainbow trout fish farm in the north of Iran. Based on 16S rRNA analysis, *Pseudomonas psychrophila* has been placed in the *P. chlororaphis* group (Anzai et al., 2000). Austin and Austin (2007) suggested the reason for the widespread incidence of *Pseudomonas* sp. in the aquatic environment may be due to its spread through the water, which acts as the major reservoir of infection. The genus *Pseudomonas* was identified in different species of fish as etiological agents of *Pseudomonas* septicemia (Altinok et al., 2007; Sakar and Azza, 2008; Eissa et al., 2010; El-Nagar, 2010). The external changes related to infection by *Pseudomonas* sp. bacteria in different fish were fin rot, detached scales,

hemorrhage and darkness of the skin, abdominal ascetic and exophthalmia (Eissa et al., 2010). Various studies have shown that the phenotypic identification methods are solely not enough for the classification of *Pseudomonas* sp. so the genotypic identification systems are required to confirm the traditional identification of particularly potential dangerous *Pseudomonas* (Alatossava and Alatossava, 2008). Molecular techniques using PCR-based methods allow fast, sensitive and exact identification of the bacteria that have been described for the detection of fish diseases. 16S rRNA gene is one of these important methods, especially when used alongside phenotypic characteristics for microbial identification in the diagnostic laboratory (Buller, 2004).

The present study aimed to isolate the causative agent of disease during the sporadic case in rainbow trout and identify the phenotypic and genotypic characterizations identified based on the 16S rRNA gene.

## Materials and methods

### *Isolation of bacteria and characterization of bacterial isolates*

A disease case occurred during the fall of 2017 in a rainbow trout farm in the north of Iran (Guilan Province). Affected fish weights were ranged between 45g and 107g. Microbial examination revealed the presence of bacteria in affected fish at the farm. Fifty fish samples, five fish from each pond, were collected during disease outbreak

and were translated to the Viomed laboratory in Guilan Province. Samples from kidneys and livers of collected fishes were streaked onto McConkey and sheep blood agar plates and incubated at 20°C for 48 h. Some growing colonies were picked up in pure form where the identification of selective colonies was done by morphological and biochemical characters for Gram-negative fish pathogens and they identified to the genus *Pseudomonas*. The identification was confirmed by sequencing the 16S rDNA. Antimicrobial test was applied on Muller Hinton agar.

#### *DNA isolation*

In order to isolate DNA, the pure isolates were incubated in tryptic soy broth (TSB) at 37°C for 24h. Bacterial genomic DNA was extracted using the GeneJET Genomic DNA purification kit based on the manufacturer's instruction.  $2 \times 10^9$  bacterial cells translated in a 2 mL microcentrifuge tube and centrifuged for 10 minutes at 5,000x g. The supernatant was discarded and the pellet was re-suspended in 180 µL of digestion solution. 20 µL of Proteinase K solution added and mixed thoroughly by vortexing to obtain a uniform suspension. Then, the sample was incubated at 56°C while vortexing occasionally until the cells were completely lysed (~30 minutes). 20 µL of RNase A solution added and mixed by vortexing and incubate the mixture for 10 minutes at room temperature. After, 200 µL of lysis solution added to the

sample and mixed thoroughly by vortexing for about 15 seconds until a homogeneous mixture was obtained. 400 µL of 50 % ethanol added and mixed by vortexing. The prepared lysate transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube and centrifuged column for 1 min at 6,000 x g. The collection tube containing the flow-through solution was discarded. The GeneJET Genomic DNA Purification Column (Žilinskien, 2011) placed into a new 2 mL collection tube. 500 µL of wash buffer I added and centrifuged for 1 minute at 8,000 x g. The flow-through discarded and placed the purification column back into the collection tube. 500 µL of wash buffer II added to the GeneJET Genomic DNA Purification Column and centrifuged for 3 minutes at maximum speed. The collection tube containing the flow-through solution discarded and transferred the GeneJET Genomic DNA Purification Column to a sterile 2 mL microcentrifuge tube. The eluted DNA was used as a template for PCR detection of the 16S rRNA gene. A 1500 base pair DNA fragment was amplified from the bacterial DNA extracted from a pure culture *Pseudomonas*. Thus, bacteria isolated from the diseased rainbow trout were confirmed as *P. psychrophila*. (Haghighi *et al.*, 2018).

#### *PCR and 16S rRNA gene sequencing*

Universal bacterial primers specific for detection of 16S rRNA gene of bacteria was used 27F (5'-

AGAGTTTGATCMTGGCTCAG-3') and 1429R (5'-TACGGYTACCTTGTTACGACTT-3'). The location of primers was 1500 bp. These primers were then used to yield a 1500 bp 16S rRNA gene product by PCR. The amplification was done in a thermal cycler with the following parameters: initial denaturation at 95°C for 10 min, followed by 30 cycles of amplification (denaturation) at 95°C for 20sec, annealing at 50°C for 20sec, extension at 72°C for 3 min and a final extension step of 72°C for 10 min (Table 1). After amplification, 10 µL of the PCR sample was loaded on a 1% agarose gel stained with ethidium bromide. PCR product was purified by QIAquick PCR purification kit and directly sequenced with a 3500/3500xl Genetic Analyzer (QIAquick® Spin Handbook, 2020).

**Table 1: PCR program.**

<b>Initial denaturation</b>	95°C	10 min
	95°C	20 sec
<b>30 cycles</b>	505°C	20 sec
	72°C	3 min
<b>Final extension</b>	72°C	10 min

#### *Biochemical characteristics*

Pure colonies of Gram-negative bacteria were isolated on MacConkey agar from the liver and kidney of affected fish. To identify the isolated bacteria, biochemical tests were done. The characteristic of the bacteria was oxidase and catalase-positive, lysine decarboxylase positive, Simmons citrate positive, Urea negative and non-fermentation glucose, β-hemolysis negative, weak motility. It grew well on

MacConkey agar as it formed yellow-pigmented colonies, which indicates lactose non-fermented status.

#### *Antibiotic susceptibility test*

Swab samples were collected from livers and kidneys of infected trout and cultured on 5% Sheep blood agar and MacConkey agar. All the plates were incubated at 20°C for 48 hours. For the determination of susceptibility to antibacterial agents, the disc diffusion method on Muller–Hinton agar according to the procedure of Bauer *et al.* (1966) was used. Some colonies of isolated bacteria from blood agar plate were transferred to a glass tube containing 3 ml saline and the suspension was vortexed and visually matched with 0.5 MacFarland standards for turbidity. The sterile cotton-tipped swab was dipped into the prepared bacterial suspension and then rolled on the upper part of the tube to remove the excess fluid and subcultured onto Muller-Hinton agar (Hudzicki, 2009).

Paper discs with predetermined amounts of antibiotics were used. The following antimicrobial agents based on the OIE List of Antimicrobial Agents of Veterinary Importance 2014, were tested. All antibacterial discs were provided from Padtan Teb Company (Tehran, Iran). The plates were incubated at 20°C for 48 hours. The diameters of the zones of inhibition were measured (millimetres) and were compared to internationally accepted measurements to determine the susceptibility or resistance of the

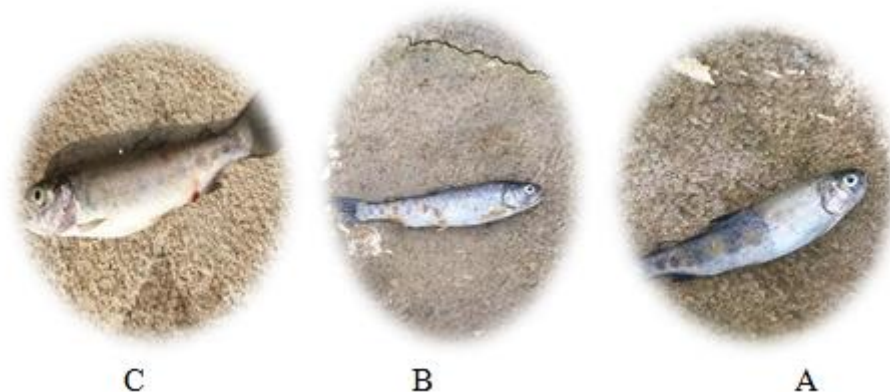
isolates. Results were interpreted to the Clinical Laboratory Standard Institute (CLSI, 2013) guidelines. Drug resistance patterns of the organisms were determined at three levels: Susceptible (S), Intermediate (I) and Resistant (R).

Seventeen antimicrobial drugs were evaluated for efficiency against tested. Some antibiotics include cell wall synthesis (colistin 10 $\mu$ g; lincospectin 15/200 $\mu$ g; enrofloxacin 5 $\mu$ g; difloxacin 5 $\mu$ g), protein synthesis inhibitors (erythromycin 15 $\mu$ g; gentamycin, 10 $\mu$ g; neomycin 30 $\mu$ g; danofloxacin 5 $\mu$ g; streptomycin 10 $\mu$ g; kanamycin 30 $\mu$ g;

florfenicol 30 $\mu$ g; flumequine, 30 $\mu$ g; fosfomycin 200 $\mu$ g; tetracycline 30 $\mu$ g; oxytetracycline 30 $\mu$ g; chlortetracycline 30 $\mu$ g; doxycycline 30 $\mu$ g; and nucleic acid synthesis inhibitors (as listed in Table 1. *Pseudomonas* strain was characterized as sensitive (S), or resistant (R) according to the size of the inhibition zones around the discs (CLSI, 2010).

### Results

Mass mortalities occurred in a rainbow trout farm in the north of Iran (Guilan Province) and cumulative mortality reached 20%. There was dark pigmentation, exophthalmia, hemorrhage at the base of the pectoral, pelvic, and anal fins and surrounding the anus externally (Fig. 1).



**Figure 1: A= Dark pigmentation, B= Hemorrhage at the base of the pectoral, pelvic, and anal fins, C= Hemorrhage surrounding the anus.**

Internal signs were enlarged spleen, pale liver and empty stomach and intestine filled with yellowish fluid. Pure colonies of Gram-negative bacteria were isolated on MacConkey agar (Fig. 2) and sheep blood agar (Fig. 3) from the liver and kidney of affected fish. It grew well on

MacConkey agar as it formed yellow-pigmented colonies, which indicates lactose non-fermented status. Some growing colonies were picked up in pure form where the identification of selective colonies was done by morphological and biochemical

characters for Gram-negative fish pathogens and they identified to the genus *Pseudomonas* (Fig. 4).

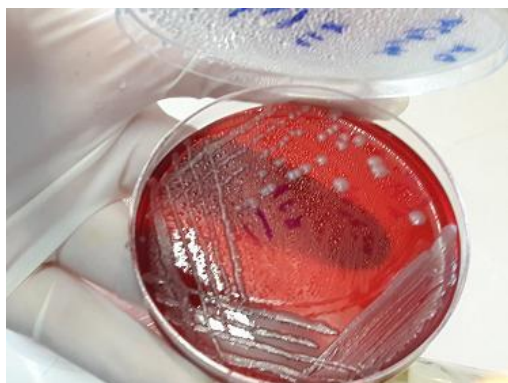


Figure 2: Pure colonies on MacConkey Agar.



Figure 3: Pure colonies on Sheep Blood Agar.

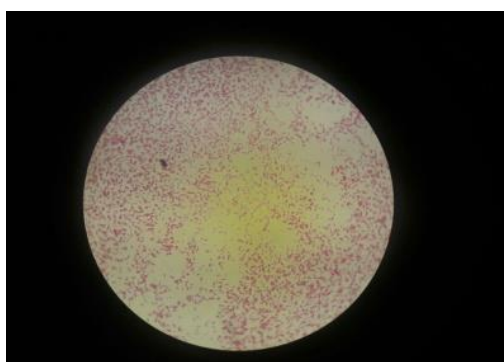


Figure 4: Gram-negative bacteria (genus *Pseudomonas*).

#### Antibiotic sensitivity

Based on antibiotic susceptibility study showed that isolate (*P. psychrophila*) had intrinsically high sensitivity to colistin, fluoroquinolones (enrofloxacin, difloxacin, danofloxacin, flumequine),

aminoglycosides (kanamycin, streptomycin, neomycin, gentamycin), while it had resistance to tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline, erythromycin), fosfomycin which is broad-spectrum antibiotics, and florfenicol (Table 2).

Table 2: Antibacterial activity of different antibiotics against *Pseudomonas psychrophila*

Antibiotic Name	Concentration (µg)	Results
Tetracycline	30	R
Oxytetracycline	30	R
Chlortetracycline	30	R
Doxycycline	30	R
Difloxacin	5	S
Danofloxacin	5	S
Enrofloxacin	5	S
Flumequine	30	S
Erythromycin	15	R
Fosfomycin	200	R
Gentamycin	10	S
Kanamycin	30	S
Streptomycin	10	S
Neomycin	30	S
Colistin	10	S
Florfenicol	30	R
Lincospectin	15/200	S

S= Susceptible; R= Resistant

#### Phenotypic characterization and hemolytic activity

Some growing colonies were picked up in pure form where the identification of selective colonies was done by morphological and biochemical characters for Gram-negative fish pathogens. They were identified morphologically as Gram-negative rod motile bacteria. Biochemically, bacteria was oxidase and catalase-positive, lysine decarboxylase positive, Simmons citrate positive, Urea negative and non-fermentation glucose on MacConkey

agar,  $\beta$ -Hemolysis negative on sheep blood agar, weak motility in SIM medium. Thus, it is characterized as *Pseudomonas* genus. The characteristic of the bacteria has been shown in Table 3.

#### Identification by 16 S rRNA and sequencing

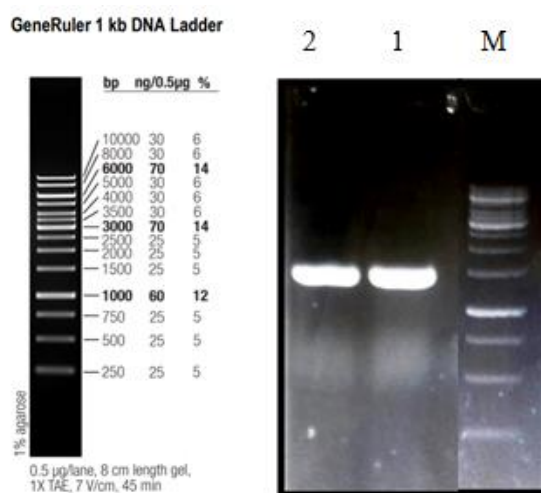
27F and 1429R primers were used to

amplify 16S rRNA of bacterial isolate, with amplicon size 370 bp. The isolates collected and identified by 16S rRNA. The results of the 16S rRNA sequence correlated with the phenotypic classification of The *Pseudomonas* strain. A 1500 base pair DNA fragment was amplified from the bacterial DNA extracted from a pure culture *Pseudomonas* (Fig. 5). Thus, bacteria isolated from the diseased rainbow trout were confirmed as *P. psychrophila* (Fig. 6).

**Table 3: Biochemical characteristics isolated bacteria, *Pseudomonas psychrophila*.**

Characteristics	Results
Glucose	-
Sucrose	-
Lactose	-
Indole	-
H <sub>2</sub> S production	-
Urease	-
Simmon citrate	+
Motility	+
MacConkey agar	+
Sheep Blood agar	+
B-Hemolysis	-
Oxidase	+
Catalase	+

Negative=- Positive=+



**Figure 5: PCR product electrophoresis in 16S rRNA region on 1% agarose gel ethidium bromide staining (by Genfanavaran Co). M: molecular weight marker.**



Figure 6: Identification of 16S rRNA gene sequencing (by MacroGen Co).

## Discussion

A disease case occurred during the fall of 2017 in a rainbow trout farm in the north of Iran (Guilan province) for the first time with cumulative mortality of 20%. Clinical signs were ulceration and hemorrhage. The causative bacteria isolated from this fish farm were identified as *Pseudomonas psychrophila* according to phenotypical characteristics and sequence of 16S rRNA. Despite the fact *P. fluorescens* and *P. aeruginosa* have been considered as opportunistic pathogenic species in aquaculture (Angelini and Seigneur, 1988; Altinok *et al.*, 2006). However, it has been reported that other species of the *Pseudomonas* genus may induce serious infection in various fish for example, *P. anguilliseptica* in eel and *Anguilla japonica* (Wakabayashi and

Egusa, 1972), *P. chlororaphis* in amago trout and *Oncorhynchus rhodurus* (Hatai *et al.*, 1975), *P. plecoglossicida* in ayu (Wakabayashi *et al.*, 1996), *Plecoglossus altivelis* (Kobayashi *et al.*, 2000) and *P. putida* infection in rainbow trout (Altinok *et al.*, 2006). The result of the present study showed that the isolate identified as *Pseudomonas* was characterized by Gram-negative motile rod with oxidase, catalase-positive and lysine decarboxylase and Simmons citrate positive, Urea negative and non-fermentation glucose,  $\beta$ -hemolysis negative. However biochemical identification gave incomplete result at the species level that was identified as *Pseudomonas* sp. PCR was reported to be a sensitive screening technique to detect bacterial communities (Teng *et al.*, 2004). In the present study was used



PCR technique targeting 16S rRNA for detection of bacteria isolate from infected fish through universal primers (27F and 1429R), which was identified as *Pseudomonas psychrophila*. The isolate was well identified at the species level by 16S rRNA sequences that were identified as *Pseudomonas psychrophila*. The antibiotic susceptibility study recorded that *P. psychrophila* exhibited sensitivity to lincospectin, enrofloxacin, difloxacin, danofloxacin, colistin, flumequine, kanamycin, streptomycin, neomycin and gentamycin (Table 1). Eissa *et al.* (2010) reported that strains of *P. putida*, *P. anguilliseptica* and *P. aereginosa* were sensitive to fluoroquinolone antibiotics, erythromycin and gentamicin. Also, Darak and Barde (2015) reported that *P. fluorescens* was very sensitive to kanamycin, nalidixic acid, gentamicin, neomycin, less sensitive to amikacin and tetracycline, and chloramphenicol and the least sensitive to oxytetracycline, erythromycin and penicillin. Also, *P. fluorescens* exhibited susceptibility to oxytetracycline and amikacin more than kanamycin, neomycin and gentamicin (Markovic *et al.*, 1996). Thus, these results showed that *Pseudomonas psychrophila* is sensitive to colistin, fluoroquinolones (enrofloxacin, difloxacin, danofloxacin, flumequin), aminoglycosides (kanamycin, streptomycin, neomycin, gentamycin). However, *Pseudomonas psychrophila* had resistance to tetracyclines (tetracycline, oxytetracycline, chlortetracycline,

doxycycline, erythromycin), fosfomycin which are broad-spectrum antibiotics, and florfenicol (Table 1). Altinok *et al.* (2006) reported that *P. putida* has high resistance to ampicillin, erythromycin, chloramphenicol, tetracycline, naladixic acid, rifampicin and streptomycin. Also, *P. fluorescens* exhibited complete resistance to penicillin and erythromycin (Markovic *et al.*, 1996).

### Conclusion

In conclusion, phenotypic characteristics gave a good identification of *Pseudomonas* isolates. Using 16S rRNA gene sequencing proves that diagnosing fish's bacterial diseases is important for successful epidemiological studies and disease control. The results concluded that pathogenic *Pseudomonas psychrophila* is sensitive to colistin, fluoroquinolone antibiotics (enrofloxacin, difloxacin, danofloxacin), and aminoglycosides (kanamycin, streptomycin, neomycin, gentamycin).

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