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Research Article

# Investigation of infection *Hafnia alvei* in Rainbow trout cage facilities in the Down Fırat Basin

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## Abstract

Some trout cage facilities in the Down Fırat Basin were observed an infection with high mortality in rainbow trout (*Oncorhynchus mykiss*, W., 1792) fry weighing 10-25 g January - March 2019. Ill fish were observed in a yellowish pus-filled exudate and bloody intestines, blackening of the spleen and liver, red spots in the liver, grayish-white abscesses in the kidney, ascites in the abdomen, epizootic hemorrhagic septicemia, darkening of the skin. Eye, liver, spleen, kidney and intestines were made with Tryptic Soy Agar and incubated at 24ºC for 48 hours. 10 suspicious *Hafnia alvei* were detected isolates. These isolates were identified bacteria by applying 94 biochemical tests in the Biolog (The biolog GENIII micro plate) microbial identification system.

**Keywords:** Rainbow trout, *Oncorhynchus mykiss*, *Hafnia alvei*

## INTRODUCTION

The presence of a large number of fish together and in close contact due to aquaculture causes more diseases to occur than free-living fish in nature. The negative changes in the nutritive, physical, chemical, biotic and abiotic optimal living conditions of the fish in which salty, brackish and fresh water is limited, and their persistence and failure in a short time cause many infectious diseases (Arda et al., 2005).

*Hafnia alvei*, which is in the Enterobacteriaceae family, is motile, facultative anaerobic, found in mammals, birds, reptiles, fish, soil, water, sewage and some foods, but also in the external environment. It is an opportunistic rod-shaped bacterium of approximately 1 μm in diameter and 2–5 μm in length, showing Gram-negative, oxidase-negative and catalase-positive properties (Sakazaki 1984; Gelev et al., 1990; Fazal et al., 1997; Rodriguez et al., 1998; Bilgehan, 2000; Ramos & Damaso, 2000; Sakazaki, 2009).

The first occurrence of *Hafnia alvei* as a pathogenic bacterium in fish was reported in 1990. This bacterium has been reported to cause epizootic hemorrhagic septicemia in rainbow trout (Gelev et al., 1990). It has also been reported to cause clinical symptoms such as abnormal swimming, hemorrhagic septicemia, dark body and abdominal swelling in Japanese salmon (*Oncorhynchus masou*) (Teshima et al., 1992). However, Sakazaki & Tamura (1992) reported in their study that this bacterium is not an obligate pathogen and should be considered an opportunistic pathogen. It is also thought to be an extraintestinal pathogen of freshwater fish (Gevlev et al., 1990)

Microbiological tests are generally used to determine the phenotypic characteristics of bacteria (Arda, 2000; Austin & Austin, 1987; Bernardet & Kerouault, 1989; Bernardet et al., 1996).

Biolog System (The biolog GENIII micro plate); It is used to identify Gram-positive and Gram-negative bacteria by applying 94 biochemical tests. These tests are based on 71 carbon source use experiments and 23 chemical susceptibility tests. The biologic Microbial identification system (Micro Station Identification System) is a versatile system. It is used in a wide range of microbiology to identify environmental and pathogenic organisms and to determine their metabolic properties (Singh et al., 2001).

In this study, it is aimed to reveal the factor that causes mass mortality in fry fish in some trout farms in the Lower Euphrates Basin and to suggest the necessary treatment method.

## MATERIAL AND METHODS

In January-March 2019, in 28 different caged rainbow trout farms in the Down Fırat Basin, it was reported that there were mass deaths in juveniles weighing 10-25 g, and the specified facilities were visited. In these facilities, live examinations of fish for diseases were carried out on site. Necessary disease information (anamnesis) was obtained from the business owner.

Kahramanmaras Sutcu Imam University, Faculty of Agriculture, Fisheries Department, Fish Diseases Laboratory brought live to Rainbow Trout (*Oncorhynchus mykiss*, W., 1792) weighing 10-25 g.

The fish brought to the laboratory were subjected to clinical (external) examination. To examine the fish bacteriologically, they were anesthetized with the anesthetic agent 2-phenoxyethanol before autopsy was performed. The body surface of the fish was disinfected with 70% ethyl alcohol. In the laboratory environment, examinations were performed using sterile scissors, forceps, and scalpel in front of a burner flame in a sterile cabinet, according to the autopsy technique (Frerichs & Millar, 1993; Çolak, 1982; Timur & Timur 2003; Arda et al., 2005). The preparations prepared from the internal organs and skin was examined natively under the microscope.

The liver, spleen, kidney and intestines of fish showing disease symptoms were sampled and planted on Tryptic Soy Agar (TSA), Nutrient Broth (NB) and Brain Heart (Infusion) Agar (BHIA). The media were incubated for 48 hours at 24ºC.

Pure colonies were obtained from the colonies on the growth media. Suspension was prepared from these pure colonies with Biologic IF-A buffer solution. Bacteria concentration was adjusted to 92-98% by turbidimeter. Density-adjusted bacterial samples were added to each well of the microplates as 100 µl. These microplates were incubated at 26ºC for 24 hours. Finally, the microplate was read in the reader and compared with the data bank of the system and the diagnosis of the bacteria was carried out.

Antibiogram test was applied to the isolates obtained using Mueller-Hinton agar medium. Zones of Inhibition were measured in millimeters and the results were defined as resistant and susceptible (NCCLS, 2001).

## RESULTS

Since the majority of the enterprises are fattening enterprises, the need for juvenile fish is provided in different provinces of Türkiye.

Wetlands with native birds, which are unique in this area and live in this ecosystem, and foreign bird populations that migrate here at certain times, serve as stations where birds migrate from one place to another.

Samples taken from businesses operating in the Down Fırat Basin are generally samples with suspected disease. With the warming of the weather and the increase in water temperatures, fish samples showing signs of disease were searched in the facilities. In general, fish with darkening color, bleeding and deformity in the mouth parts, operculum, outer surface of the body and fins, swelling in the abdomen, exophthalmos in the eyes, moving irregularly on the edges of the cages,

on the water surface, and standing still, were visually and manually examined and selected as samples.

After the morphological examination of the fish with suspected disease, autopsy was performed and the internal organs were examined. Fluid collection in the abdomen, pallor and enlargement of the liver and spleen, swelling in the kidney, and hemorrhages in the internal organs were observed (Figure 1).

**Figure1.** Discoloration and enlargement of the liver and spleen, swelling in the kidney, hemorrhages in the internal organs

It was diagnosed as *Hafnia alvei* according to the phenotypic and biochemical properties of 12 isolated gram-negative bacillus isolates (Table 1). In addition, the Biolog System (The biolog GENIII micro plate) device was used to confirm the biochemical tests and to determine other phenotypic features (Table 2).

**Table 1.** Morphological and biochemical characteristics of 12 *Hafnia alvei* isolated from Rainbow Trout

| **Biochemical Criterion** | ***Hafnia alvei*** **(n:12)** |
|---|---|
| Colony color | White |
| Gram Staining | - |
| Shape | Stick |
| Oxidase | - |
| Catalase | + |
| Motion | + |
| $H_2S$ | - |
| Metil Red | + |
| Voges Proskauer | - |
| Indole | - |
| Urease Formation | - |
| O/F | F |
| MacConkey Agar | + |
| Mueller-Hinton Agar | + |
| Reproduction at 0°C | - |
| Reproduction at 5°C | + |
| Reproduction at 15°C | + |
| Reproduction at 20°C | + |
| Reproduction at 25°C | + |
| Reproduction at 30°C | + |
| Reproduction at 37°C | + |
| Reproduction at 0.0 %NaCl | + |
| Reproduction at 0.5 % NaCl | + |
| Reproduction at 1.0% NaCl | + |
| Reproduction at 2.0 % NaCl | + |
| Reproduction at 6.5 % NaCl | - |

+: Positive reaction, -: Negative reaction, +/-: Variable, F: Fermentative

**Table 2.** Other biochemical characteristics of 12 Hafnia alvei isolated from Rainbow Trout (These characteristics were determined by Biolog System Device)

| Biochemical Criterion | Isolate reaction | Biochemical Criterion | Isolate reaction |
|---|---|---|---|
| pH 5 | + | %8 NaCl | - |
| pH 6 | + | %4 NaCl | + |
| Positive Control | + | %1 NaCl | + |
| Stachyose | +- | N-Acetyl Neuraminic Acid | + |
| D- Turanose | - | N-Acetyl-D-Galactosamine | + |
| Sucrose | +- | N-Acetyl- β-D-Mannosa-mine | + |
| Gentiobiose | + | N-Acetyl-D-Glucosamine | + |
| D-Cellobiose | +- | D-Salicin | weak + |
| D-Trehalose | + | β- Methyl-D-Glucoside | + |
| D-Maltose | + | D-Melibiose | - |
| Dextrin | +- | α-D-Lactose | - |
| Negative Control | - | D-Raffinose | - |
| D-Serine | +- | Minocycline | +- |
| Fusidic Acid | + | Rifamycin SV | + |
| %1 Sodium Lactate | + | Troleando-mycin | + |
| I Nosine | + | D-Serine | +- |
| L-Rhamnose | + | D-Aspartic Acid | + |
| L-Fucose | + | D-Fructose-6- Phosphate | + |
| D-Fucose | - | D-Glucose-6- Phosphate | + |
| 3-Methyl Glucose | - | Glycerol | + |
| D-Galactose | + | Myo-İnositol | - |
| D-Fructose | + | D-Arabitol | - |
| D-Mannose | + | D-Mannitol | + |
| α-D-Glucose | + | D-Sorbitol | - |
| Niaproof 4 | + | Tetrazolium Blue | + |
| Guanidine HCl | + | Tetrazolium Violet | + |
| Lincomycin | + | Vancomycin | + |
| L-Serine | + | D-Saccharic Acid | - |
| L-Pyroglutamic Acid | - | Quinic Acid | - |
| L-Histidine | + | Mucic Acid | - |
| L-Glutamic Acid | + | Glucoronamide | - |
| L-Aspartic Acid | + | D-Glucoronic Acid | + |
| L-Arginine | - | D-Gluconic Acid | + |
| L-Alanine | + | L-Galactonic Acid Lactone | weak + |
| Glycyl-L-Proline | + | D-Galacturonic Acid | + |
| Gelatin | - | Pectin | - |
| Potassium Tellurite | - | Sodium Bromate | - |
| Lithium Chloride | +- | Sodium Butyrate | + |
| Nalidixic Acid | weak + | Aztreonam | +- |
| Bromo-Succinic Acid | +- | Formic Acid | - |
| L-Malic Acid | + | Acetic Acid | + |
| D-Malic Acid | - | Propionic Acid | - |
| α-Keto-Glutaric Acid | - | Acetoacetic Acid | - |
| Citric Acid | weak + | α-Keto- Butyric Acid | - |
| L-Lactic Acid | + | β- Hydroxy-D, L-Butyric Acid | - |
| D-Lactic Acid Methyl Ester | - | α-Hydroxybutyric Acid | - |
| Methyl Pyruvate | + | γ-Amino-Butryric Acid | - |
| p-Hydroxy-Phenylacetic Acid | + | Tween 40 | - |

As a result of the antibiogram test, it was determined that isolated bacteria were sensitive to Erythromycin, Enrofloxacin and Florfenicol antibiotics and resistant to streptomycin and Sulfafurazole antibiotics (Table 3).

**Table 3.** 12 *Hafnia alvei* isolated from rainbow trout test results the antibiotic

| **Antibiotics** | **Sensitivity** |
|---|---|
| Florfenicol (FFC30) | S (15) |
| Ampicillin (AM10) | R (0) |
| Erythromycin(E15) | S (22) |
| Oxytetracycline(T30) | R (0) |
| Gentamicin (GM10) | S (30) |

S: Sensitive R: Resistant

## DISCUSSION

In this study; In the Karkamış Dam Lake, isolation and identification of *Hafnia alvei*, one of the suspected rainbow trout, was carried out in 28 different trout cage enterprises located in Gaziantep and Şanlıurfa provinces, using the known classical culture method. In addition, biochemical tests were confirmed with the biology System (The biology GENIII micro plate) device and other phenotypic features were determined.

Darkening of the skin, epizootic hemorrhagic septicemia, ascites in the abdomen, grayish-white abscesses in the kidney, red spots in the liver, blackening of the spleen and liver, bloody intestines and a yellowish pus-filled exudate were observed in sick fish. These clinical findings are similar to studies conducted in different countries (Teshima et al., 1992; Gelev et al., 1990).

In this study; Identification test results of bacterial isolates (Table 1) agreed with previous knowledge of *Hafnia alvei* (Rodriguez et al., 1998).

First appearance of *Hafnia alvei* as pathogenic bacteria in fish Gelev et al. (1990). This bacterium is found in the normal microbial flora of the water, and they reported that it is not an obligatory pathogen for fish and should be considered as an opportunistic pathogen (Sakazaki & Tamura 1992). The disease has been seen in farms where environmental conditions have changed, in fish exposed to transport and stress. This confirms that *Hafnia alvei* is an opportunistic pathogen.

The antimicrobial susceptibility test results of *Hafnia alvei* isolated strain are presented in Table (3), showed that this bacterium was sensitive to Florfenicol, Erythromycin and Gentamisin, while it is resistant to Ampicillin and Oxytetracycline. Many studies on *Hafnia alvei* antimicrobial susceptibility showed the same results obtained in this investigation (Janda & Abbott, 2006; CLSI. 2006; Girlich et al., 2000).

## CONCLUSION

In this study, the isolation and identification of *Hafnia alvei*, which causes disease in rainbow trout in our country and in many countries of the world, and causes great economic losses, especially in trout weighing 10-25 g, was carried out by classical culture method. In addition, biochemical tests were confirmed with the biology System (The biology GENIII micro plate) and other phenotypic features were successfully determined.

### Ethical approval

All animal studies were approved by the Animal Ethics Committee of Kahramanmaraş Sütçü Imam University, Faculty of Agriculture (KSÜZİRHADYEK) and Research Institute (Protocol number: 2017/01).

### Authors' Contributions

MÖ: Manuscript design, writing, statistical analyses

OK: Laboratory experiments, reading, draft checking

### Conflict of Interest

The authors declare no conflict of interest for this study.

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