Journal of Human Environment and Health Promotion
Print ISSN: 2476-5481 Online ISSN: 2476-549X

Molecular Detection of *Aeromonas hydrophila* in Farmed Rainbow Trout (*Oncorhynchus mykiss*) from Zanjan Province, Iran

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ARTICLE INFO

Article type:
Original article

Article history:
Received: 20 September 2025
Revised: 5 November 2025
Accepted: 22 November 2025
Available online: 2 December 2025

https://doi.org/10.61882/jhehp.738

Keywords:

Aeromonas hydrophila
Rainbow trout
PCR
aerA gene
lip gene

ABSTRACT

Background: Rainbow trout (*Oncorhynchus mykiss*), an economically important fish in Iran, is valued for its nutritional potential. *Aeromonas hydrophila* causes infections that lead to motile septicaemia in fish and humans worldwide. Given the rapid expansion of fish farming in Iran, particularly in Zanjan Province, and the associated losses due to bacterial infections, this study aimed to detect *A. hydrophila* in farmed rainbow trout using molecular methods.

Methods: A total of 103 fish samples were collected from ponds across five cities in Zanjan Province. Cultured specimens were subsequently analyzed, and *A. hydrophila* isolates were confirmed through standard biochemical tests. Genomic DNA was extracted from the isolates, and the *aerA* and *lip* genes were identified as markers for confirmation of *A. hydrophila* using the Polymerase Chain Reaction method.

Results: Overall, 72 (69.90%) samples were positive for A. hydrophila, as determined by both culture and biochemical tests. However, molecular analysis confirmed the aerA gene in 66 (64.07%) of the isolates, while only 3 (2.91%) samples carried both the lip and aerA genes.

Conclusion: The high prevalence of *A. hydrophila* in rainbow trout poses a threat to public health and results in significant economic losses in aquaculture. Collaboration among epidemiologists, microbiologists, veterinarians, and food safety experts is essential to reduce these impacts.

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*), a cold-water species belonging to the family *Salmonidae*, is one of the most economically and ecologically significant fish in Iran and is highly valued for its nutritional potential. This species originates from North America and has now spread throughout the world (Daneshamouz et al., 2020; Patil, 2025).

Bacterial infections cause heavy losses in fish farms, resulting in severe economic damage to the aquaculture industry. Species of the *Aeromonas* genus are among the dominant microbes in freshwater environments and, along with other microorganisms, act as natural and effective biofilters in water self-purification. Although these bacteria are naturally present in the microflora of water and fish bodies, under certain conditions, such as reduced immunity and stress, they can cause substantial losses and economic losses in farmed fish (Saad & Atallah, 2015). Among aquatic bacterial agents, *Aeromonas hydrophila* is of great interest (Sahoo et al., 2011). *A. hydrophila* is a Gram-negative, opportunistic, glucose-fermenting, motile, rod-shaped, facultatively aerobic, oxidase-and catalase-positive bacterium that causes fatal infections leading to motile septicaemia in fish and humans worldwide (Moradi et al., 2025; Perry & Laurent, 1993). It is a common component of the normal intestinal flora of freshwater and marine fish, making infections caused by this bacterium relatively frequent. In nature, it is widely distributed in the digestive tract of farmed fish and in freshwater sediments (Uma et al.,

How to cite: Daneshamooz, Sh., Zeighami, H., & Haghi, F. (XXXX). Molecular Detection of *Aeromonas hydrophila* in Farmed Rainbow Trout (*Oncorhynchus mykiss*) from Zanjan Province, Iran. *J Hum Environ Health Promot*, *XX*(X), XX-XX.

2010). There is some debate regarding whether *A. hydrophila* acts as a primary or secondary pathogen. However, under stressful conditions such as handling, poor water quality, or temperature fluctuations, it becomes a pathogen and causes disease (Adanır & Turutoglu, 2007; Pakravan et al., 2012). This bacterium causes hemorrhagic septicemia in freshwater and, occasionally, in marine fish, leading to high mortality rates in aquaculture and natural ecosystems. It is also associated with red spot disease, fin rot, and epizootic ulcerative syndrome (EUS), which remains a major problem in Southeast Asian countries. Significant losses of warm-water fish have been reported over the past two decades, and although different causes-such as viral infections, environmental, nutritional, and bacterial factors-have been mentioned, several studies have highlighted the prominent role of *Aeromonas* motile septicaemia in these losses (Sahoo et al., 2011; Swaminathan et al., 2004). The causative agent of this disease, *A. hydrophila*, is considered an opportunistic pathogen affecting aquatic and terrestrial organisms, including humans. It can cause various complications such as dysentery, meningitis, and mild to severe septicaemia. This bacterium has been isolated from many fish species, including carp, rainbow trout, catfish, and tilapia (Uma et al., 2010; Wang et al., 2008). *A. hydrophila* causes disease by producing foreign toxins such as enterotoxin, hemolysin (aerolysin), lipase, and protease (Sheikh et al., 2023). Following the suspicious cause of death of a fish, after bacterial culture from its kidney and PCR testing for the extracellular hemolysin (aerolysin) gene, the results indicated that the death was caused by *A. hydrophila* (Choresca Jr et al., 2010). Numerous studies have demonstrated the presence of this bacterium in food products, particularly those of aquatic origin, suggesting that contamination primarily occurs through the consumption of raw or undercooked seafood products (Citterio & Biavasco, 2015; Rasmussen-Ivey et al., 2016). For the identification of *A. hydrophila*, various biochemical, serological, and bacteriological techniques have been used; however, molecular methods such as PCR and DNA fingerprinting are now preferred for their accuracy and speed (Wang et al., 2003). The genes encoding aerolysin (*aerA*) and lipase (*lip*) have each been defined as one of the virulence markers for identifying the pathogenicity of *A. hydrophila* (Christy et al., 2019; Swaminathan et al., 2004). Considering the development of fish breeding in Iran, especially in Zanjan Province, and the frequent occurrence of losses caused by bacterial infections the present research was conducted to investigate the molecular detection of A. hydrophila contamination in farmed rainbow trout.

2. Materials and Methods

2.1 Sampling

To conduct this study on rainbow trout farms in Zanjan Province, the cluster sampling method was used. The province was initially divided into five geographical regions (cities) based on the number of fish farms and their productivity. Subsequently, a total of 17 active farms were selected for sampling. From June to October 2017, all live fish exhibiting significant clinical signs such as corneal opacity, anorexia, hemorrhages around the mouth and urogenital pore, erratic swimming, lethargy, exophthalmia, gill hyperplasia, dorsal rigidity, emaciation, and loss of orientation were considered for laboratory testing. Consequently, a total of 103 fish samples from the five cities were collected. The distribution of collected rainbow trout is presented in Table 1. The weight range of these fish was approximately 200 to 400 g. To euthanize the fish, a spiking method targeting the brain was used. The collected fish samples were carefully packed into clean polyethylene bags, which were labeled accordingly. These bags were transported to the laboratory of food microbiology within 1 h under hygienic conditions, using a polystyrene container containing ice packs to maintain the proper temperature. Four specimens were then taken from the liver, kidney, gills, and eye of each rainbow trout, resulting in a total of 412 specimens being collected (Daneshamouz et al., 2020).

Table 1. Distribution of collected rainbow trout samples by city

City	Number of fish samples	Percentage (%)
Mahneshan	43	41.75
Zanjan	24	23.30
Abhar	20	19.42
Khodabandeh	8	7.76
Qeydar	8	7.76
Total	103	100

2.2 Isolation and Identification of A. hydrophila

All collected fish underwent external and internal examinations. Before accessing the internal organs, the surface of each fish was swabbed with 70% ethanol for disinfection. Liver, kidney, gill, and eye tissues were aseptically placed on tryptic soy broth (TSB) and brain heart infusion broth (BHIB) and incubated at a temperature of 37 °C for 24 h. Then, 100 µL of bacterial suspension was aseptically streaked onto blood agar, tryptic soy agar (TSA), and MacConkey agar (Merck, Darmstadt, Germany) plates. The plates were then incubated at 25 °C for 2-4 days. The identification process for bacterial colonies involved Gram staining as well as biochemical tests, including oxidase and catalase tests (Daneshamouz et al., 2020; Vivas et al., 2005).

2.3 DNA Extraction

A single colony from each bacterial culture sample was selected and inoculated into 5 mL of Luria Bertani broth (LB broth; Merck, Darmstadt, Germany). The culture was then incubated with shaking at a speed of 120 rpm at a temperature of 37 °C until it reached the exponential phase with a turbidity level of 2 McFarland units (6×10^8 CFU/mL). Genomic DNA extraction was performed following the protocol provided by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Briefly, 200 µL of the bacterial suspension was incubated at 56 °C for 10 min after the addition of 10 µL of Proteinase K and 200 µL of lysis buffer. Then, 200 µL of 96% ethanol was added to the lysate, and the mixture was applied to the spin column. The column was washed sequentially

with 500 µL of Buffer AW1 and 500 µL of Buffer AW2, followed by centrifugation to remove residual wash buffer. Finally, nucleic acid was eluted with 100 µL of the elution buffer provided in the kit (Ahmed et al., 2018).

2.4 The molecular validation of bacterial isolates

The PCR assay was conducted to confirm the presence of bacterial isolates. Based on our review of the strengths and weaknesses of different primer sets reported in previous studies, primers targeting the *aerA* and *lip* genes were selected because of their advantages as markers for the identification of *A. hydrophila*, and PCR assays were performed according to previously described protocols (Cascón et al., 1996; Christy et al., 2019). The primers used for amplification are listed in Table 2. For the PCR reaction, DreamTaq PCR Master Mix (Thermo Fisher Scientific, Cleveland, OH, USA) was utilized, which contains dNTPs, Taq polymerase, the appropriate buffer, and $MgCl_2$. Each PCR tube contained a reaction mixture of 25 µL, consisting of 12.5 µL of the master mix, 1 µL each of forward and reverse primer solutions (at a final concentration of 200 nM), 1 µL of DNA with a concentration of 200 ng/µL, and nuclease-free water to complete the final volume. The Gene Atlas 322 system (ASTEC Co., Fukuoka, Japan) was employed for the PCR process. Amplification consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. A final extension step was performed at 72 °C for 5 min. Subsequently, 8 mL of the amplified DNA fragments were separated on a 1% (w/v) agarose gel in 0.5 × TBE buffer and electrophoresed at 100 V for 1 h. The gel was stained with ethidium bromide, and DNA bands were visualized under UV transillumination using a Gel Doc XR+ system (Bio-Rad, Hercules, CA, USA) (Daneshamouz et al., 2020). The reference strain of *A. hydrophila* (PTCC No: 1890) used as a positive control was obtained from the Iranian Research Organization for Science and Technology (IROST) of Iran.

Table 2. Primers used for *A. hydrophila* detection

Bacterial pathogen	Primer pair	Primer sequences (5′–3′)	Amplicon size (bp)	Ref.
Aeromonas hydroghila	*aerA-F*	5-CCTATGGCCTGAGCGAGAAG-3	432	(Christy et al., 2019)
	aerA-R	5-CCAGTTCCAGTCCCACCACT-3		
Aeromonas hydroghila	*Lip-F*	5-AACCTGGTTCCGCTCAAGCCGTTG-3	764	(Cascón et al., 1996)
	Lip-R	5-TTGCTCGCCTCGGCCCAGCAGCT-3		

3. Results and Discussion

Cultivation methods and phenotypic identification using differential biochemical tests are important in the diagnosis of *A. hydrophila* in food and clinical samples. Among these techniques, the PCR method is used as a fast, easier, and more reliable method to identify this bacterium in seafood, including fish, so that today, for the timely control and prevention of diseases, exploration through DNA and PCR tests has been successfully developed to track the presence of pathogens in the environment, body tissues, etc (Janda & Abbott, 2010). For this reason, in our research, previously published studies on specific primers were reviewed to evaluate the strengths and weaknesses of different primer sets. Based on this evaluation, primers targeting the *aerA* and lip genes were selected as markers for the identification of *A. hydrophila* due to their advantages.

In this study, a total of 103 rainbow trout samples were collected from fish farming ponds in five cities of Zanjan Province, and the frequency of the target genes was assessed using culture, biochemical tests, and the PCR method. Overall, 72 (69.90%) samples were positive for *A. hydrophila* based on culture and biochemical tests. However, molecular analysis confirmed the presence of the *aerA* gene in 66 (64.07%) of the isolates, while only 3 (2.91%) samples carried the lip gene in addition to the *aerA* gene. The PCR amplification of the *aerA* and lip genes was confirmed by agarose gel electrophoresis, as shown in Figure 1. According to culture and biochemical tests, 32, 15, 20, 3, and 2 samples from these cities, respectively, were infected with *A. hydrophila*. Molecular analysis showed that 30 (69.76%) of the Mahneshan samples, 14 (58.33%) from Zanjan, 20 (100%) from Abhar, and 2 (25%) from Qeydar carried the *aerA* gene. The frequency of the lip gene was relatively low: only 1 sample (2.32%) from Mahneshan and 2 samples (8.33%) from Zanjan were positive. No lip gene was detected in the Abhar, Khodabandeh, or Qeydar samples. The prevalence of *A. hydrophila* and the distribution of the *aerA* and lip genes among rainbow trout collected from different cities of Zanjan Province are summarized in Table 3. The *aerA* gene is the primary gene that is responsible for producing aerolysin toxin, which is an extracellular protein produced by certain strains of *A. hydrophila* with hemolytic and cytolytic properties. The mechanism of aerolysin involves binding to specific glycoprotein receptors on the surface of eukaryotic cells, followed by insertion into the plasma membrane lipid bilayer and pore formation. This toxin can damage epithelial cells and contribute to gastroenteritis (Christy et al., 2019). The lip gene also encodes a heat-stable extracellular lipase in *A. hydrophila*. Microbial lipases are attracting considerable interest due to their diverse biotechnological uses, including their function as flavor-modifying agents, stereospecific catalysts, and components in detergents. However, their contribution to bacterial metabolism remains unclear. These enzymes can serve as important extracellular factors that support bacterial nutrition and may also act as virulence determinants, influencing immune system responses through the release of free fatty acids during lipolysis (Cascón et al., 1996; Swaminathan et al., 2004).

Comparing the results obtained from this study with the results of the few studies conducted in Iran and some other countries, although they do not show the same pattern, the

pattern of distribution and frequency of contamination of meat and other foods with *A. hydrophila* in all regions is almost the same (Ahangarzadeh et al., 2015; Ahangarzadeh et al., 2022; Fadaeifard, 2014; Khamesipour et al., 2014; Tolouei Gilani et al., 2021; Wang et al., 2003; Zorriehzahra et al., 2020). In a study conducted by Ahangarzadeh et al. (2022) on the frequency of virulence genes in *A. hydrophila* isolates from infected farmed carp in Khuzestan Province, Iran, the results showed that, out of 200 moribund carp with signs of septicaemia, 59 samples (29.5%) were identified as *A. hydrophila* by biochemical methods and 31 isolates (15.5%) by PCR, which indicates a lower prevalence than that observed in the present study. In addition, 16 samples (51.61%) tested positive for the *aerA* gene. In another study conducted in Guilan Province, Iran, to isolate and identify *A. hydrophila* from 100 samples of *Cyprinidae* fish suspected of having hemorrhagic septicaemia in warm-water ponds, 51 samples were identified as positive for *A. hydrophila*. Furthermore, 42 isolates were confirmed by PCR (Tolouei Gilani et al., 2021). In a study conducted by Swaminathan et al. (2004) in India, *A. hydrophila* was identified in 20 water and fish samples using the PCR method, and 9 isolates carrying the lip gene were reported. In contrast, in our study, the lip gene was detected in only 3 out of 103 fish samples. In a study by Vega-Sánchez et al. (2014) in Mexico, the phenotypic, genetic, and antimicrobial susceptibility characteristics of Aeromonas spp. isolated from rainbow trout were investigated. A total of 50 isolates were obtained, among which 20% were identified as *A. hydrophila*, which was less than the results of the present study. Variation in the prevalence of *A. hydrophila* isolates from raw fish samples reported across studies may be attributed to differences in sampling strategies, seasonal factors, and laboratory methodologies (Khamesipour et al., 2014).

Table 3. Frequency of *A. hydrophila* in rainbow trout samples from different cities of Zanjan Province

City	Number of fish samples	Culture	PCR	
			aerA gene positive	*lip* gene positive
Mahneshan	43	32 (74.42 %)	30 (69.76 %)	1 (2.32 %)
Zanjan	24	15 (62.50 %)	14 (58.33 %)	2 (8.33 %)
Abhar	20	20 (100 %)	20 (100 %)	0
Qeydar	8	3 (37.5 %)	2 (25 %)	0
Khodabandeh	8	2 (25 %)	0	0
Total	103	72 (69.90 %)	66 (64.07 %)	3 (2.91 %)

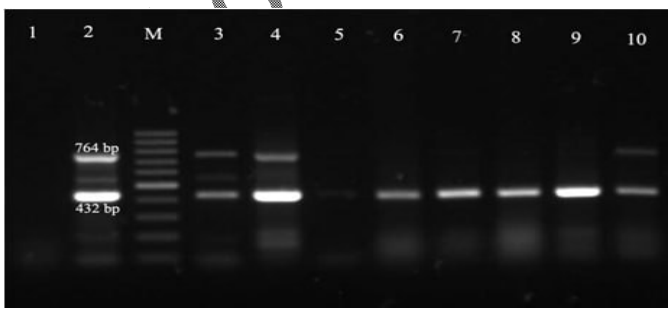

Figure 1. The PCR products of the *aerA* (432 bp) and lip (764 bp) genes were electrophoresed on a 1% agarose gel. Lane M: 100 bp DNA ladder; Lane 1: negative control; Lane 2: positive control containing both genes; Lanes 3-10: test samples

4. Conclusion

In the present study, 103 rainbow trout were obtained from aquaculture ponds across five cities in Zanjan Province, and the occurrence of the target genes was evaluated through culture, biochemical assays, and PCR analysis. Out of these, 72 samples (69.90%) tested positive for *A. hydrophila* using culture and biochemical methods. Molecular testing further verified the *aerA* gene in 66 isolates (64.07%), while only 3 samples (2.91%) carried the *lip* gene alongside *aerA*. The high prevalence of *A. hydrophila* in rainbow trout represents a potential risk to public health and aquaculture sustainability. To minimize this risk, preventive measures such as improving water quality and hygiene, applying probiotics to enhance fish immunity, and considering vaccination strategies against *A. hydrophila* should be encouraged. Close collaboration among epidemiologists, microbiologists, veterinarians, and food safety specialists is crucial to mitigate economic losses and safeguard public health.

Authors' Contributions

Shahrzad Daneshamooz: Conceptualization; Data curation; Investigation; Methodology; Validation; Visualization; Writing-original draft. **Habib Zeighami:** Conceptualization; Data curation; Methodology; Funding acquisition; Project administration; Resources; Supervision; Validation; Visualization; Writing-review & editing. **Fakhri Haghi:** Conceptualization; Data curation; Methodology; Supervision; Validation; Visualization; Writing-review & editing.

Funding

This project was financially supported by Zanjan University of Medical Sciences (project code: A-12-392-25).

Conflicts of Interest

The Authors declare that there is no conflict of interest.

Acknowledgments

This paper is part of a collaborative project between Zanjan University of Medical Sciences and the Veterinary Administration Office of Zanjan. The authors sincerely thank the Veterinary Administration Office of Zanjan for their support in sample collection.

Ethical considerations

This project has been approved by the Ethics Committee of Zanjan University of Medical Sciences with the ethics code ZUMS.REC.1396.17.

Using Artificial Intelligence

This research did not utilize any artificial intelligence techniques.

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