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Isolation, biochemical and molecular detection of *Aeromonas hydrophila* from cultured *Oncorhynchus mykiss*

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Abstract

The isolation of *Aeromonas hydrophila* from *Oncorhynchus mykiss* reared in the farms was the aim of the present study. Ninety samples were collected aseptically from the infected fish with signs of hemorrhagic septicemia in gill and skin, exophthalmia, dropsy and 36 water samples were sub-cultured on Tryptic Soya Agar (TSA), as well as kidney. The genotyping by PCR method was used to amplify the gene of 16 SrDNA using primers 27F and 1492R as primer pairs to achieve an approximate length of 1500 bp. The identified motile *Aeromonas* species were sequenced with electrogram chromas format in chrome version 1/41 software and a phylogenetic tree was drowned by MEGA5.1 software. The results showed that 19% of 24 isolated from gram-negative bacteria were detected as *Aeromonas,* which the sequencing results revealed that all the isolated bacteria had 99% similarities with the standard *A. hydrophila.* The results of environmental factors showed when the levels of dissolved oxygen have decreased and the levels of nitrite and ammonia have increased throughout the year, Aeromonasias is increased. Also, when the water has gone alkaline due to increased ammonia, the disease has increased. It may be concluded that fish farmers should take proper management practices to avoid such disease in cultured fishes especially rainbow trout and to get rid of the infection by such serious bacterial pathogen like *Aeromonas hydrophila* by improving their culture strategy and culture ecosystem.

Keywords: *Aeromonas hydrophila,* Environmental factors, Kerman province, *Oncorhynchus mykiss,* PCR.

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Introduction

Aeromonas spp. have been isolated from the numerous fresh or brackish water fishes worldwide, marine waters, lakes, rivers, swamps, sediments, water distribution systems, drinking water and residual waters (Rashid *et al.,* 2013). There are also in different types of food, such as meat, fish, vegetables and seafood. The genus *Aeromonas* is responsible for a significant number of animal and human infections (Brooks *et al.,* 2007). *Aeromonas hydrophila* is a gram-negative pathogen, rod-shaped, facultative anaerobe, chemoorganotrophic bacteria with an optimal growing temperature of about 22-28°C and causes serious infectious diseases in fish and humans (Abdolnabi *et al.,* 2015; Abeyta *et al.,* 2019). The main pathogenic factors are surface polysaccharides (capsule, lipopolysaccharide, and glucan), S-layers, iron-binding systems, exotoxins and extracellular enzymes, secretion systems, fimbriae and other nonfilamentous adhesions, motility and flagella. *Aeromonas* spp. are divided into two main groups: 1: motile species, such as *A. hydrophila*, *A. caeviae* and *A. sobria.* 2: non motile species including *A. salmonicida* (Tomas, 2012). *Aeromonas* spp., particularly *A.hydrophila* and *A.sobria*, are widely distributed in the environment, in freshwater, brackish water, saline water, lakes and sewage water (Pakravan *et al.,* 2012). They have been also isolated from drinking water and food, such as meat, fish, shellfish, raw milk and vegetable. These bacteria also compose as a part of normal intestinal microflora of healthy fish. Stress can be a contributing factor in outbreaks of diseases caused by these bacteria. Internally, the liver and kidney are target organs of acute septicemia (Jayavignesh *et al.,* 2011). The kidney may become swollen and friable and the liver may become pale or green. These organs are apparently attacked by bacterial toxins and lose their structural integrity (Huizinga *et al.,* 2012). Therefore, the predominant clinical signs include dermal ulceration, with focal hemorrhage and inflammation (Chandra and Mani, 2011; Whitman *et al.,* 2012). The aim of this study was the isolation of bacteria from water, fish farms and some target organs including kidney, skin and liver of rainbow trout (*Oncorhynchus mykiss*) as well as identification of isolated *A. hydrophila* by biochemical and molecular assays Polymerase Chain Reaction (PCR).

Materials and methods

Sampling methods

The study was conducted to measure the important qualitative environmental criteria that could be affected the occurring of Aeromonasiasis such as oxygen, nitrite, nitrate, ammonia and acidity (pH), isolation of diseased fish with clinical signs or typical symptoms from 3 research stations (Bardsir, Kouhpaye and Sirch in Kerman) and suspected fish to Aeromonasiasis and then isolation of *A. hydrophila,* and using PCR and sequencing as a confirmation method.

SPSS statistical software consisted, and non-common letters in each row

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indicate a significant difference among environmental parameters.

Fish sampling method

Samples (n=90) were taken from suspected fish and fish farm's water from 3 locations of Kerman province, Iran in 2018. Also, infected fish were collected with clinical symptoms such as darkening skin, external hemorrhages and internal bleeding in liver, kidney and skin or combination of these symptoms (Fig.1).



Figure 1: **Clinical signs of infected fish.**

Suspensions from kidney were prepared in 100 ml of 0.1% W/V peptone water (pH=7.0) and homogenized at 20 rpm for 30 seconds and were subculture on Tryptic Soy Agar (TSA), Eosin Methylene Blue Agar (EMB) and Thiosulfate Citrate Bile Sucrose (TCBS) and incubated for 24-48 hours at 25±2°C (Ifakat and Evrim., 2014).

Water sampling method

Water samples (n=36) were also taken from the same of coldwater fish farms in 3 locations of Kerman province. Water samples were taken in sterile glass bottles and then transported with ice bags to the microbiology laboratory in Kerman Agricultural and Natural Resources Research and Education Center and diluted with 0.9% saline distilled water for colony counting by pour plate's method (Zaky *et al.,* 2011).

Culture and biochemical methods

Culture characteristics of *A. hydrophila* refer to the growth features and morphology in various kinds of culture media. Pure culture should be taken before morphological observation. The pure culture of *A. hydrophila* can be obtained through the use of spread plates, streak plates, or pour plates on TSA, EMB, and TCBS, and after 24-48 hrs incubation at 25±2°C typical colonies were isolated and pure cultured were obtained (Quinn *et al.*, 2011; Choobkar, 2017). The morphological characteristics of the colonies (fish and water samples) were characterized by Gram staining (Alishahi *et al.,* 2019). Biochemical tests were carried out in all isolates including motility test, Oxidative-Fermentative (O/F) and H_2S production test, SIM (Sulfide, Indole and Motility), esculin and gelatin hydrolysis, lysine and ornithine decarboxylase, sugar

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fermentation, nitrate reduction, and gas production from dextrose, sucrose, maltose, methyl red and Voges-Proskauer (MRVP), triple sugar iron agar (TSI) tests (Table 2) (Markey *et al.*, 2013).

Molecular identification

DNA extraction: A volume of 10 ml of pure culture media from LB broth was collected and centrifuged for 2 minutes at 6000 rpm. The amount of 500 µl of TEB buffer (Tris/Borate/EDTA) plus 15 µl of lysozyme was added to the precipitate and after a vigorous vortex for 2-3 minutes, placed at 55°C for one hour. Then 50 µl of SDS (sodium dodecyl sulfate 10%) and 10 µl of Proteinase K (20 mg ml^{-1}) were added to the precipitate and incubated for 10 minutes at 65° C. The amount of 200 µl of 5 molar NaCl was added to the tube and after the vortex for 30 seconds, 150 µl of CTAB/NaCl buffer was added slowly to the tube and putt at 65°C temperature for 10 minutes. After cooling the solution to 37°C, the volume of the above solution and chloroform/isoamyl alcohol (24/1) was added and shaken for 10-15 minutes, dropped the tube, then shaken for 10 minutes and was centrifuged at 14000 rpm. The supernatant was transferred to a new tube and 0.8 volumes, cold isopropanol (-20°C) were added and then the white layer from DNA was formed by pouring the tubes off and over. The tubes were placed at a temperature of -20°C for 30 minutes. Then, if the DNA sample was thick, the white layer from DNA was transferred to the new tube containing 70% ethanol and centrifuged for 10 minutes at 14000 rpm and then after washing was centrifuged again at 4°C. Otherwise, after leaving the freezer, the samples were centrifuged for 10 minutes at 14000 rpm, and after removing the isopropanol, 200 µl of absolute cold ethanol (-20°C) and 10 µl of sodium acetate (3 mol) were added to each tube. Then samples were placed at -20°C for 2 hrs (Chang *et al*., 2009; Diyana-Nadhirah and Inasalwany, 2016). It was centrifuged for 20 minutes at 14000 rpm. The amount of 100 µl of TEB buffer was added to the DNA precipitate by distillation water twice. Genomic DNA deposition was slowly and completely solved for 30 minutes at 37°C. Finally, the DNA sample was stored at -20°C until use. The purity of the DNA was determined by calculating the absorbance ratio of the sample at 260 and 280 nm wavelengths and the amount of DNA obtained was determined based on the absorbance of the sample at 260 nm (Yogananth *et al*., 2009; Saeed, 2015).

Polymerase Chain Reaction (PCR)

The PCR was used in all isolates. For gene proliferation the primers 27F (forward) and 1492R (Reverse) as primer pairs were used to achieve an approximate length of 1500 bp with the following sequence by PCR:

27F: AGA GTT TGA TCC TGG CTC AG

1492R: CGG TTA CCT TGT TAC GAC TT

The PCR reaction was carried out in a final volume of 25 µl and put in a thermocycler with two replications. The reaction mixture materials are presented in Table 1.

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Table 1: The amount and materials used in PCR

Type of material	used amount
PCR Buffer 10x	2.5µl
$MgCl_2$ 50 mM	1.5µl
dNTP mix 10mM	0.5µl
Fed primer (10 pmol μ^{-1})	1µl
Rev primer (10 pmol μ^{-1})	1µl
cDNA (20 ng μ^{-1})	2µl
Taq DNA polymerase (5u μ^{-1})	0.3µl
Water (ddw)	Up to 25µl

To control the PCR steps, a negative control sample was used containing all the components of PCR except DNA and the reaction was done according to the thermal profile.

Electrophoresis

After completion of the amplification, PCR products were electrophoresed on a 1% agarose gel with a constant voltage of 75 V in a TBE 0.5x buffer for 60 minutes. Gel staining was also used from Gel Red and in order to observe and examine samples, the gel was placed in the UV Gel Document and photographed. For evaluation of PCR products, agarose gel 1% was used and 0.5 g of agarose from Sinagen was added to 50 ml of TBE 0.5x buffer and then placed in medium heat for 1 minute in the macro wave. After removing from the macro wave, 2 µl of Gel Red was added and mixed slowly. Then, the gel fluid was pouring uniformly cassette in a special comb. After the gel was tightened at the laboratory temperature, the comb was removed slowly and the remaining wells were used as the loading site of the specimens. The gel was then transferred to an electrophoresis tank containing a TBE buffer 0.5x (2.7 g Tris-base, 1.37 g boric acid and 1 ml EDTA 0.5 M were dissolved in sterile distilled water and was dispensed to 500 ml). So, the buffer was applied to the surface of the gel and then 5 µl of each PCR specimens was mixed with 1 µl of the loading buffer and loaded in each well. To determine the nucleotide sequence, PCR products that were approved by electrophoresis were used to determine the sequence. The sequencing was performed by South Korea's Macrogen Corporation using Rev and Fwd. primers. The results of sequencing with electrogram chromas format in chromase version 1/41 software were analyzed. Then, the final sequence was compared with the other bacterial sequences in the World Bank Gene (NCBI Gen bank). The MEGA5.1 program was used to plot the phylogenetic tree (James *et al.*, 2010; Ottaviani *et al*., 2011; Tamura *et al*., 2011; Rashid *et al*., 2013).

Results

The bacterial isolates in primary characterization tests were gram-negative, rod-shaped, non-capsulated, non-sporulated and motile. They showed white colonies on TSA and pink colonies on MAC media. *A. hydrophila* strains give violet to metallic green sheen colonies on EMB media due to lactose utilization. They had yellow colonies on TCBS agar due to fermentation of sucrose. All isolates were confirmed at the species level as *A. hydrophila* by differential biochemical tests (Table 2).

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Table 2: Biochemical properties of isolated bacteria.

Characters		Present Isolates
Gram stain		Negative
Shape		Rod
Motility		+
Catalase		+
Oxidase		+
O/F test		Fermentative
Acid and gas production from sugar	Dextrose	+
	Sucrose	+
	Maltose	+
Acid production	Lactose	+
	Mannitol	+
H2S production		+
Voges-Proskauer		+
Esculin hydrolysis		+
Growth in Vibriostatic agent 0/129		+
Methyl red test		-
Growth at	4°C	-
	37°C	+
	40°C	-
Growth in NaCl solution	0%	+
	1%	+
	2%	+
	3.5%	-
	4%	-

About 51 bacterial strains were isolated from 90 fish samples and 36 water samples and strains were encoded as IAUK1001-1126. All isolates were gram-negative, rod-shaped, motile and oxidase positive, encapsulated and non-sporulated similar to the genus of *Aeromonas*. The results of the biochemical tests were compared with the key Bergey's and reference strain *A. hydrophila* (ATCC7965) to determine the species (Whitman *et al.,* 2012). Out of 126 isolates, 24 samples were confirmed at species level as *A. hydrophila* by differential biochemical tests from 90 samples of infected fish, 21.11% and of the 36 water samples, 13.88% *A. hydrophila* were isolated (Tables 3, 4).

Table 3: Results obtained from cultures and genotype identification samples of fish for Aeromonads isolation.

No*	Samples of diseased fish	Non motile Aeromonads	Motile Aeromonad	*A. hydrophila*	Percentage of *A. hydrophila*
1	32	1001-1015	1048-1064	10	31.25
2	34	1016-1033	1065-1080	6	17.64
3	24	1034-1047	1081-1090	3	12.5
Total	90	47	43	19	21.11

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Table 4: Results obtained from cultures and genotype identification samples of water for Aeromonads isolation

No*	Sampled water	Non motile Aeromonads	Motile Aeromonads	*A. hydrophila*	*Percentage of A. hydrophila*
1	14	1091-1101	1119-1121	2	14.28
2	12	1102-1110	1122-1124	2	16.66
3	10	1111-1118	1125-1126	1	10
Total	36	28	8	5	13.88

*Farming ponds in 3 locations of Kerman province: 1. Bardsir, 2. Kouhpaye, 3. Sirch

Meanwhile, the results showed that the amount of oxygen, nitrite and ammonia in the ponds had a significant relationship with the rate of Aeromonasiasis incidence. The percentage of affected fish with symptoms of Aeromonasiasis has increased in the different months when the amount of water dissolved oxygen has decreased as well as the levels of nitrite and ammonia has increased. Also, when the water has gone alkaline due to increased ammonia, the disease has increased (Table 5).

Table 5: Sampling protocol in order to environmental parameters measurements

Environmental Parameter	First (Mean ± SE)	Second (Mean ± SE)	Third (Mean ± SE)	Fourth (Mean ± SE)	Fifth (Mean ± SE)	Sixth (Mean ± SE)
Oxygen	6 ± 0.29^{a}	6.33 ± 0.12^{ab}	7.27 ± 0.15^{ab}	7.17 ± 0.18^{ab}	7.67 ± 0.18^{bc}	7.9 ± 0.06^{c}
Nitrite	0.1 ± 0.1^{a}	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}	0.8 ± 0.1^{d}	0.9 ± 0.1^{d}	0.7 ± 00^{c}
Nitrate	1 ± 0.06^{bc}	1.13 ± 0.09^{cd}	1.23 ± 0.09^{d}	0.93 ± 0.03^{b}	0.85 ± 0.03^{b}	0.8 ± 0.23^{b}
Ammonia	0.37 ± 0.09^{bc}	0.50 ± 0.09^{d}	0.57 ± 0.03^{cd}	0.63 ± 0.06^{cd}	0.60 ± 0.06^{ab}	0.4 ± 0.01^{a}
Acidity (pH)	7.3 ± 0.1^{a}	8.1 ± 0.6^{b}	8.3 ± 0.3^{c}	8 ± 0.1^{b}	7.7 ± 0.2^{ab}	7.4 ± 0.1^{a}
Incidence rate	20%	30%	30%	80%	60%	50%

Non-common letters in each row indicate a significant difference between environmental parameters.

Microorganism detection by sequencing of 16S rDNA

The amplification of the 16S rDNA gene was performed using primers 27F and 1492R, and a piece amplified with a length of 1500 bp. Then the piece was sent for sequencing and purification. The image of the agarose gel obtained from the amplification of the 16S rDNA gene of the target strain. The results of the nucleotide sequence matching using the blast-n algorithm are shown in Fig. 2.

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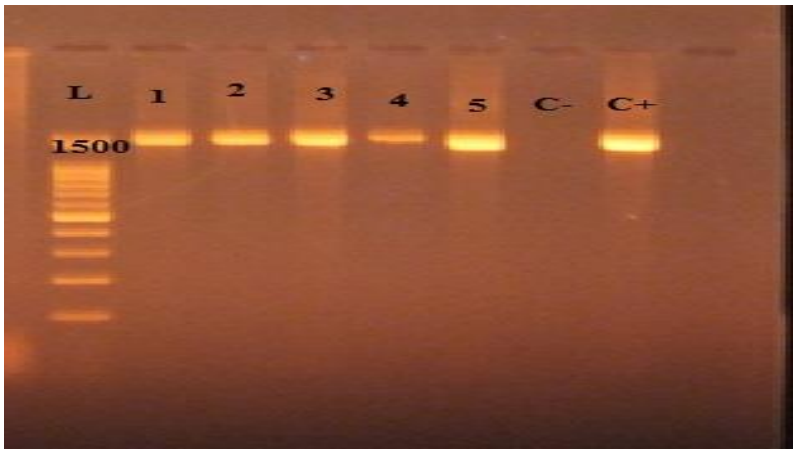


Figure 2: Expansion of 16S rDNA gene fragment from PCR reaction. Well L includes 100 bp ladder, well 1-5 containing PCR product, well 6 was negative control and well 7 was a positive control.

The result of the sequencing

All information about related sequencing was demonstrated as follows and could be compared with the standard phylogenetic analysis of *A. hydrophila*.

CGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGGCGAGCGGCGGACGGGTGAGTAATGCA
TTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGC
CCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTCGGATATGCCCCAGGTGGGA
TTAGCTAGTTGGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGA
TGGATCAGCCACACTGGAACTGAGACACGCCAGACTCCTACGGGAGGCAGCTGAGTGGGGA
ATATTGCACATGGGGGAAACCCTGATGCAGCCAGCTGCCGCGTGTGTGAAGAAGGCCTTCG
GGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATACGTAGTCAACTGTGAC
GTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACTGGAGCGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGAT
GTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCCAGCTAGAGTCTTGTAG
AGGGGGGTAGAATTCCAGGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG

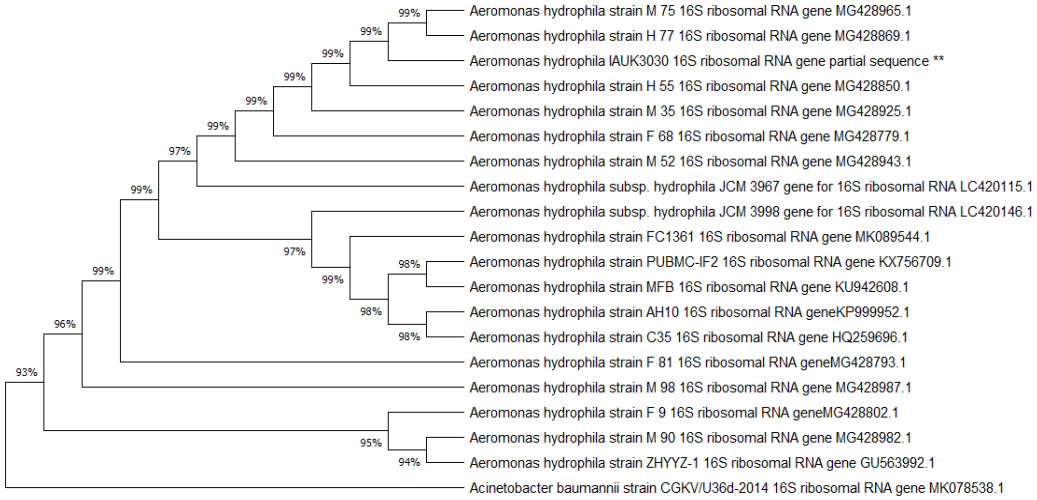


Figure 3: Phylogenetic analysis of *Aeromonas hydrophila* with different 16S rRNA sequences of Aeromonads, collected from NCBI-GenBank.

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The phylogenetic tree of *A. hydrophila* IAUK3030 isolated from infected fish was drawn along with other standard bacteria in the phylogeny of Fig. 3. The isolated bacteria had 99% similarities with the standard *A. hydrophila.* The evolutionary history of *A. hydrophila* was determined by using the maximum likelihood statistical method to compare with 18 other sequences from close relatives in MEGA7 software.

Discussion

A. hydrophila is a widely distributed pathogenic bacterium especially in aquaculture that causes great economic losses to this industry. Isolation, identification and confirmation of fish pathogenic bacteria are important in the accurate diagnosis of suspected disease (Zorrieh, 2008; Yazdanpanah *et al.*, 2020).

One of the types of bacteria that often found in cultivated organisms is *A. hydrophilla* (Rashad *et al.*, 2017). *A. hydrophila* is an opportunistic bacterium that might cause many damages and serious losses to fish breeders (Ottaviani *et al*., 2011; Moori Bakhtiari *et al.*, 2017). It can cause damages to fish farming ponds and in different organs in *O. mykiss*. So, fish farmers should have appropriate health management practices to avoid the disease of fish especially in *O. mykiss*. Diagnosis and phylogenetic relationships within the species can be able to overcome threats and control disease outbreaks of *Aeromonas* species (Uma *et al.*, 2010; Praveen *et al.*, 2016). Alam (2009) isolated *A. hydrophila* from Thai pangus. He found the bacterial load in intestine, liver and kidney. Mamnur Rashid *et al.* (2013) found bacteria in terms of bacterial count in intestine, liver and kidney of naturally infected Thai pangus, respectively. Mostofa et al. (2008) isolated *A. hydrophila* from *Heteropneustes fossilis*. They found the highest bacterial load from the liver and the lowest from the kidney. Mamnur Rashid *et al.* (2013) isolated and identified *Aeromonas* isolates from five apparently healthy indigenous and exotic Carps: Rui (*Labeo rohita*), Catla (*Catla catla*), Mrigal (*Cirrhinas cirrhosus*), and Common carp (*Cyprinus carpio*). Harikrishnan *et al.* (2010) reported a kind of dermal lesions that were associated with *Aeromonas* infection in goldfish (*Carassius auratus*).

In recent study, samples from the kidney of 90 infected fish with Aeromonasis clinical signs and 36 samples of the water of fish farms were taken and purified. In the laboratory, out of 126 samples of fish and water, 51 colonies of gram-negative bacteria were biochemically diagnosed as motile and non-motile Aaeromonads species, using specific morphological, physiological and biochemical characteristics. From 90 sampled infected fish, 21.11% and of 36 water samples, about 13.88% *A. hydrophila* were isolated. Eventually, 24 isolates were *A. hydrophila* that compared with the reference strain *A. hydrophila* (ATCC7965) from Iranian Research Organization for Science and Technology (IROST). *A. hydrophila* was isolated from samples and the results were in line with other studies.

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Similar results with minor variations in biochemical properties of *A. hydrophila* isolates were reported by various authors as negative for lysine decarboxylase, ornithine decarboxylase positive, Voges proskauer positive (Jayavignesh *et al.,* 2011), H_2S positive, negative towards DNase test, nitrate positive, potassium cyanide positive, isolated from rainbow trout in Korea from fish (*Etroplus suratensis*) and the waters of traditional brackish water farms in Cochin, India from *Labeo rohita* and various organs of freshwater fish (Sahu *et al.,* 2013; Sreedharan *et al.,* 2013) from skin ulcerated fish (*Schizothorax prenanti*) in Yaan city, China (Du *et al.,* 2011) isolated from infected fish. In another study, *A. hydrophila* was isolated from infected fish includes rohu, catla, mrigal, catfish, goldfish and *Channa* spp., and compare with reference strain MTCC 646.

Also, it can be concluded that PCR can be a useful tool to identify *Aeromonas* in the species level. Besides that, molecular diagnosis of the fish pathogen can be analyzed using the 16 SrDNA which proved the most reliable method that can be determined and identified rapidly bacterial species in the aquaculture industry. Isolation and identification of *A. hydrophila* from fish ponds and expansion of 16S rDNA gene fragment were done by PCR assay using universal primers. For genotype identification, specific and universal primers for 16 SrRNA by PCR method (Panangala *et al.,* 2007; Chang *et al.,* 2009; Sarkar *et al.,* 2012) can be used.

In the current study, dissolved oxygen, pH, nitrite (NO_2), nitrate (NO_3), ammonium (NH_4) and incidence rate were measured. Pridgeon et al. (2011) observed the species *A. veronii* and *A. hydrophila* that have been associated with diseases in ornamental and farmed fish and temperature, dissolved oxygen, pH, ammonia (NH_3), ammonium (NH_4), nitrite and phosphate (PO_4) of the ponds were measured. Clinically, symptoms such as bilateral exophthalmia, superficial redness of the eye and mouth ulcers were visible. Kidney, spleen, and liver were hemorrhagic, enlarged and the presence of congested blood vessels was noted. The researchers found that water temperature affected the ability to dissolve gases, and in the lower water temperature, more gases can be dissolved, and the activity of aerobic bacteria also changed. As the water temperature is lower, the activity of aerobic bacteria also decreases and their oxygen consumption decreases (Sarkar *et al.,* 2012) which is in agreement with the current study. In our study, relative Aeromonasis and physicochemical factors were measured. In December and January, when the water oxygen level increased and the water temperature decreased, Aeromonasis rate decreased. During the culture period, water quality is controlled by many factors. When oxygen in a fish farm is restricted for a particular species, a new concern regarding water quality including ammonia, nitrite and nitrate and their relationship to aquatic diseases is considered, which has shown in a recent study that there was a significant relationship between the amount of oxygen, nitrite and ammonia

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in rainbow trout ponds. The percentage of fish with symptoms of Aeromonasis increased in different months when the air temperature increased and the water dissolved oxygen content decreased as well as the nitrite and ammonia levels increased. Of course, the results showed that nitrate had little effect on the disease. Therefore, breeders should pay particular attention to nitrite and ammonia levels in fish ponds for disease control. However, according to the findings, nitrate levels did not have much impact on disease. Therefore, water treatment balance and environmental parameters could be recommended as a strategic approach for control and prevention of the mentioned disease in the susceptible fish farms.

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