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ORIGINAL ARTICLE

Isolation and Identification of *Aeromonas hydrophila* from *Cyprinidae* Suspected with Hemorrhagic Septicemia in Pools of Warm Water Fishes in Gilan Province

Javad Tolouei Gilani[1], Ali Goudarztalejerdi[1*], Morteza Yavari[2], Mahdi Nouri Kalourazi[1]

1. Department of Pathobiology, School of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran
2. Department of Clinical Sciences, School of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran

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**Corresponding author:*
Ali Goudarztalejerdi, PhD;
Department of Pathobiology,
School of Veterinary Medicine,
Bu-Ali Sina University, Felestin Sq.,
Postal code: 6517658978,
Hamedan, Iran.
Tel: +98-8134227475.
Email: a.goudarz@basu.ac.ir
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ABSTRACT

Background: Bacterial diseases in aquaculture fishes are one of the most important risk factors in fish industry. *Aeromonas hydrophila* is the main causative agent of hemorrhagic septicemia in warm-water fishes, especially *Cyprinidae* that plays an important role in public health through consumption of contaminated fish meat that can cause gastroenteritis, traveler's diarrhea, wound infection, pneumonia, and meningitis. Considering the importance of culturing *Cyprinidae* in Gilan province and the need to identify and detect the presence of *A. hydrophila* in *Cyprinidae* fishes suspected with hemorrhagic septicemia, this study aimed to isolate and identify *A. hydrophila* in *Cyprinidae* fishes suspected with hemorrhagic septicemia in Gilan province.

Methods: In this Experimental study, during summer and autumn of 2020 by referring to 71 different warm fish farms of Gilan province, 100 specimens of *Cyprinidae* fishes were collected. *A. hydrophila* strains were isolated from the surface wounds (n=15) and kidney (n=85) of fish suspected to hemorrhagic septicemia by inoculation of samples on blood and MacConkey agar media.

Results: Among samples from fish kidneys and skin wounds, 51 samples were positive for *A. hydrophila*. Forty two isolates were confirmed as *A. hydrophila* by PCR technique using specific primers which targeted 16S rDNA gene fragment.

Conclusion: Due to the significant role of *A. hydrophila* in *Cyprinidae* mortality in Gilan province, preventive and diagnostic strategies are necessary to control the occurrence of hemorrhagic septicemia by undertaking identification tests of *A. hydrophila* in fish pools that can reduce costs in fish industry management.

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Introduction

Following the rapid growth and expansion of aquaculture in the country, the occurrence of some problems has led to damages to this industry. Annually, 25% of the world's aquatic production is lost due to various factors, including mismanagement and occurrence of various diseases. It is estimated that annually, 10% of aquaculture production in the country is directly destroyed due to different diseases and indirectly reduce

production, lead to weight loss, and decrease food efficiency (1). Among the major aquatic diseases, bacterial infections were shown to have heavy mortality in fish farms, resulting in severe economic losses in fish aquaculture industry. Among the fish aquatic bacterial pathogens, Aeromonas, especially *Aeromonas hydrophila* is the most important bacterial agent, which can cause extensive losses in fish farms (2-4).

A. hydrophila is the main causative agent of hemorrhagic septicemia in freshwater fishes such as *Cyprinidae*, *Anguilloidei*, *Scomberomorus commerson*, *Ictalurus punctatus,* Tilapia and Ayu. It also causes red-sore disease, columnaris, and epizootic ulcerative syndrome, which are major problems in Southeast Asian countries (5-9). Although this bacterium is usually a secondary pathogen, it is sometimes the primary cause of death in fish farms and is one of the most important pathogens in warm-water fishes (9). In general, symptoms of hemorrhagic septicemia, due to *A. hydrophila* are tissues swelling, ascites, redness, necrosis, septicemia, ulceration, and bleeding. Sometimes the disease is acute and symptoms of sepsis include bleeding in the lower abdomen, in the fins, around the mouth and anus, as well as, sores on the skin of caudal fin. If generalized, death occurs within 24 to 48 hours (10, 11). Infection with *A. hydrophila* in humans through the consumption of contaminated fish meat can cause gastroenteritis, traveler's diarrhea, wound infection, pneumonia, and meningitis; therefore, this bacterium plays an important role in terms of public health (12-14).

A. hydrophila is a heterotrophic, Gram-negative, rod-shaped, and motile bacterium that moves by polar flagella, and is found mainly in warm climates. This bacterium can live in fresh and brackish waters as well as aerobic and anaerobic environments (5, 15-18). In our country, the mortality in aquaculture fish has been considered as one of the important health problems of this industry in the last two decades. Fish diseases are also the biggest risk factor in aquaculture. Therefore, understanding the ecology and epidemiology of harmful infectious agents in the aquaculture system is important. Considering the importance of *Cyprinidae* culturing in Gilan province, and the need to identify the presence of *A. hydrophila* in *Cyprinidae* suspected of hemorrhagic septicemia; the present study was performed to isolate and identify *A. hydrophila* by phenotypic and genotypic methods in fishes suspected to hemorrhagic septicemia.

Materials and Methods

In this Experimental study, sampling was performed in the summer and autumn of 2020 by referring to 71 different warm fish farms from ponds with a history of mortality and clinical signs. Samples were taken from 100 diseased fishes with clinical sings such as hemorrhagic septicemia, columnaris, wound, or wart in the body. These fishes were collected in a sterile container, and then the details of the sampling site, the last used drug, and the history of used antibiotics were recorded on the containers. Fishes with clinical signs were kept next to the ice and were immediately transferred to the laboratory. From each pool, only one fish was sampled, and a questionnaire enrolled the information and medications obtained from fish farmers in details. Samples were taken from pools where symptoms of the disease were observed. Table 1 provides details of the samples taken, based on the type of fish.

Table 1: Details of the collected samples.

Fish type	No. of samples
C. carpio	35
H. molitrix	35
C. idella	15
H. nobilis	15
Total	100

A. hydrophila strains were isolated from the surface wounds (n=15) and kidney (n=85) of fish suspected to hemorrhagic septicemia by inoculation of samples on blood agar and MacConkey agar (Merck, Germany) aerobically for 24-48 h at 22.5ºC. Single colonies were achieved by the repeated streaking method on MacConkey agar. Suspected bacterial colonies were identified via subculture on thiosulfate citrate bile salt agar (TCBSA) medium (Merck, Germany), and additional identification was made using biochemical tests. Gram staining was performed and Gram-positive bacteria were removed and due to the gram-negative nature of *A. hydrophila*, they were further identified according to the biochemical tests mentioned in Bergey's manual of systematic bacteriology (19). Most important of these tests which were used to identify *A. hydrophila* were oxidase, catalase, motility, methyl red, Voges Proskauer, citrate, urea, and gelatinase tests.

Molecular techniques, including PCR were used as a sensitive and specific method to confirm and identity the isolated *A. hydrophila*. For DNA extraction, the boiling method was used. For this purpose, a complete loop of colonies grown on the nutrient broth medium was transferred into a 1.5 mL microtube and was thoroughly mixed with 200 µL of sterile distilled water, and then was heated at 100°C in a thermoblock for 10 minutes. Then the microtubes were centrifuged at 2400 g for 8 minutes.

Table 2: Details of primers used in this study.

Primer	Oligonucleutide sequence (5′→′3)	Target gene	Amplicon size (bp)	Annealing temperature (ºC)	Reference
A16S-F	GGGAGTGCCTTCGGGAATCAGA	16S rDNA	356	55	(21)
A16S-R	TCACCGCAACATTCTGATTTG				

Table 3: Results of isolation of *A. hydrophila* from *Cyprinidae* suspected to hemorrhagic septicemia using conventional microbiological and molecular methods.

Sample	No. of collected sample	No. of *A. hydrophila* isolates based on biochemical tests	% of *A. hydrophila* isolates based on biochemical tests	No. of *A. hydrophila* isolates confirmed by PCR	% of *A. hydrophila* isolates confirmed by PCR
C. carpio	35	23	65.71	19	54.28
H. molitrix	35	20	57.14	18	51.42
C. idella	15	3	20	1	6.66
H. nobilis	15	5	33.33	4	26.66
Total	100	51	51	42	-

Afterward, 50 µL of the supernatant was removed as total DNA and was transferred to 0.2 mL microtubes, and stored at -20°C for further tests (20).

Isolates suspected to *A. hydrophila* were confirmed by simple polymerase chain reaction (PCR) using primers A16S-F and A16S- R targeting the 16S rDNA gene fragment. The primer details and expected amplicon size were presented in Table 2. The PCR reaction was performed at a final volume of 20 µL containing 7 µL of distilled deionized water, 10 µL of Taq DNA polymerase 2X Mastermix (Ampliqon, Denmark), 1 µL of the template DNA, and 1 µL of each primer (10 pmol– DynaBioTM TakapouZist Co., Iran). PCR amplification was performed in Applied Biosystem thermal cycler (Applied Biosystem, USA) in the following PCR conditions: 95ºC for 1 min (Initial denaturation), 50 cycles of 95ºC for 1 min (denaturation), 55ºC for 45s (annealing), 72ºC for 1 min (extension) and 72ºC for 10 min (final extension). In this reaction, distilled water was used as negative control and, *A. hydrophila* ATCC7966 was used as a positive control to optimize PCR conditions. Amplified products were analyzed in 1.0% (w/v) agarose gel (SinaClon, Iran) stained with ethidium bromide (0.5 µg/mL-, SinaClon, Iran) and electrophoresed at 110 V for one hour. Gels were viewed under UV light and photographed using UV Imager (Transluminator, France). A 100 bp molecular weight marker (100 bp; SinaClon, Iran) was used as the size standard.

Results

Totally, 100 samples of *Cyprinidae*, including *C. carpio*, *H. molitrix*, *C. idella* and *H. nobilis* were examined for the presence of *A. hydrophila* contamination from fish culturing pools in Gilan province. After culturing, obtaining a pure colony, Gram staining, and performing biochemical tests, 51 isolates suspected to *A. hydrophila* were identified. More details of the obtained results, using conventional microbiological methods were presented in Table 3.

PCR technique was used for identification and confirmation of 51 isolates suspected to *A. hydrophila* isolated from *Cyprinidae* suspected to hemorrhagic septicemia by conventional microbiological methods, as a molecular method with high sensitivity and specificity. The PCR results showed that out of 51 isolates suspected to *A. hydrophila*, and 42 samples (80.76%) were confirmed as *A. hydrophila*. These samples showed a 356 bp band in agarose gel electrophoresis (Figure 1). Table 3 shows the results of the identification of isolates suspected to *A. hydrophila* in different species of *Cyprinidae* using PCR (Table 3).

Discussion

In this study, 100 samples were taken from warm-water fish farms in Gilan province that had symptoms of hemorrhagic septicemia, and after transferring the samples to the laboratory, biochemical tests were performed. Fifty-one percent of the total samples were positive for *A. hydrophila*. Afterward, 42 percent were confirmed as *A. hydrophila* using the PCR technique. According to the results of molecular technique and biochemical tests, the presence of *A. hydrophila* in warm-water fish pools of Gilan province was verified. Bacterial infections caused heavy mortality in fish farms and aquaculture industry. Among bacterial

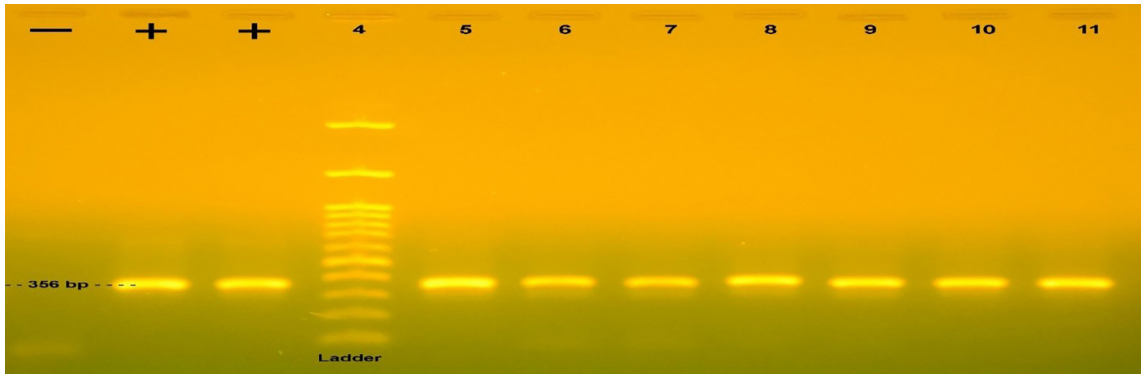

Figure 1: Agarose gel electrophoresis results for confirmation of isolates suspected to *A. hydrophila* isolated from *Cyprinidae* suspected to hemorrhagic septicemia. Lane 1: Negative control (distilled water), lane 2: Positive control (*A. hydrophila* ATCC7699), lane 3: Positive control (*A. hydrophila* ATCC7699), lane 4: 100 bp DNA ladder, lane 5-11: Suspected strains of *A. hydrophila* isolated from *Cyprinidae* suspected to hemorrhagic septicemia.

agents, especially in freshwater fish, *A. hydrophila* was highly visible (6). This bacterium causes hemorrhagic septicemia in freshwater fishes and sometimes in marine fishes. The prevalence of *A. hydrophila*'s contamination in summer is higher due to stresses; such as parasitic infections, high temperatures, and low oxygen levels in the water (7). Furthermore, in a study conducted in 1994, it was shown that the prevalence of septicemia caused by motile Aeromonas among cultured and wild Tilapias was 10% and 2.5%, respectively, and it was 18.75% and 6.25% in cultured and wild Karmout catfish, respectively (22).

Aeromonas species are naturally present in aquatic environments and the gastrointestinal tract and are also considered as opportunistic pathogens in animals and humans (17), especially *A. hydrophila* as a zoonotic agent causing wound infections and gastroenteritis in humans (14). *A. hydrophila* is widely found in all environments and causes dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease, and scale protrusion disease (23). The pathogenicity of these bacteria in humans has been attributed to the release of virulence factors from bacteria and endotoxins (17). The most important problem that all these zoonotic pathogens cause in humans is digestive disorders and food poisoning, which are mostly due to contaminated food consumption (14, 22). Although the number of species belonging to motile Aeromonas is very diverse, zoonotic species seem to be more important than other species. Regardless of their geographical distribution, *A. hydrophila* is the most important species of motile Aeromonads, which has been extensively investigated. As a result, the *A. hydrophila* is the most important fish pathogen among other motile Aeromonads (17, 23-26).

In investigating the cause of deaths in Amur fish, Alishahi *et al.* (2009) concluded that 11% of deaths were due to *A. hydrophila* and 17.6% were due to other Aeromonads (27). In the present study, the PCR technique was used to target 166 rDNA gene fragments with a size of 356 bp to identify *A. hydrophila* isolates, which has been used in many studies (14, 28, 29). In 2004, Aslani and Seyed Hamzeh isolated strains of *A. hydrophila* from those with diarrhea and seemingly healthy individuals in Ilam. They reported that from the 50 isolated strains of *A. hydrophila*, 28 (56%) were isolated from diarrhetic samples and 22 (44%) from healthy and asymptomatic cases (30).

In a study conducted in Iraq from 2011 to 2012 on hospital specimens, including saliva, urine, feces, blood, and burn wounds to detect *A. hydrophila* by various culture and PCR methods, 28 strains of *A. hydrophila* were identified by 16S rDNA specific primer using PCR (31). In a study on the deaths of warm-water fishes and crabs, Nielsen *et al.* (2001) reported that Aeromonas were present in 72.6% and *A. hydrophila* in 30.5% of samples (7). Although the above study differs from the present study in terms of breeding conditions and geographical location, it is almost consistent in estimating the role of *A. hydrophila* in fishes with symptoms. Yi *et al.* (2013) isolated 20 isolates of *A. hydrophila* from 60 diseased *Cyprinidae* with symptoms of hemorrhagic septicemia and concluded that 33.3% of septicemia was due to *A. hydrophila* (32).

Ahangarzadeh *et al.* (2015) investigated the strains of *A. hydrophila* as a cause of hemorrhagic septicemia in *Cyprinidae* farms in Khuzestan province. For this purpose, after sampling of *Cyprinidae*, including phytophagous, *C. carpio*, and Amur; biochemical and molecular studies were performed. The results showed that 31 strains of *A. hydrophila* were identified in these fields and the role of this bacterium in the incidence of septicemia was determined to be 62.5%. Moreover, the molecular method was evaluated as a faster and more accurate

method for identifying strains of *A. hydrophila* compared to the biochemical method (33).

In order to determine the presence of *A. hydrophila* in diseased fishes in China, Nielsen *et al.* (2001) also reported that after PCR, out of 35 suspected isolates, 6 isolates were not *A. hydrophila*. The reason for this discrepancy may be due to the fact that biochemical diagnoses were mostly based on the analysis of human isolates, and isolates belonging to fishes may differ in several biochemical characteristics (7). In the investigation by Castro-Scarpoli *et al.* (2003), out of 82 isolates belonging to the genus *Aeromonas*, 17 isolates with biochemical tests were confirmed as *A. hydrophila*. After molecular testing, only 2 isolates, equivalent to 2.5% have been confirmed as *A. hydrophila* (34).

Borrell *et al.* (1997) conducted a study on identification of Aeromonas isolates from clinical specimens obtained by PCR to investigate 16S rDNA genes. Thus, after culturing and isolating bacterial samples, biochemical tests such as cytochrome oxidase, glucose, sorbitol and salicin fermentation and nitrate were performed. Then, bioinformatics analysis of 16S rDNA gene sequences and molecular studies were performed. The results showed that although in most cases, the identification of isolates using both biochemical and molecular methods had similar results, in some cases, discrepancies were visible. For example, a species that was identified as *A. veronii* by biochemical methods of hydrolysis of esculin and salicin fermentation was identified as A. *hydrophila* by PCR (35).

Besides, the three species, which were identified as *A. hydrophila* by the method of hydrolysis of of esculin and salicin, by molecular method, were *A. veroni*. Based on these results, it seems that the two biochemical tests mentioned in the detection of A. *hydrophila* and *A. veronium* were not reliable, and serotyping, whole-cell protein electrophoresis, and phage typing tests were more sensitive to species identification (35). Various studies have shown that the rate of *H. molitrix* mortality in Iran is directly related to increasing temperatures and the highest deaths were reported in August. The most common infectious agent of *H. molitrix* in Iran was *A. hydrophila*, which appeared with symptoms of lethargy, increased secretions and mucous membranes of the gills, general and spotty bleeding on the surface of the body, inflammation of internal organs, ascites, and death. To a much lesser extent, the genus *Pseudomonas* was identified. In other countries, these symptoms have been introduced as clinical signs of *H. molitrix* in aquaculture with bacterial septicemia caused by *A. hydrophila* and *Pseudomonas* (36-38).

In different farms, the rate of mortality has varied according to the general conditions, and management of each farm, which can be due to lack of proper preparation of the pond before the start of the culturing season, poor health management conditions, and poor water and nutrition status can lead to mortality. Due to the rapid growth of aquaculture in the country, especially the warm water culture system and also the spread of infectious diseases, such as *Aeromonas* sepsis, for the diagnosis of aquatic infectious, it is recommended to use rapid molecular methods such as PCR. In general, due to the significant role of *A. hydrophila* in *Cyprinidae* mortality in Gilan province, preventive strategies, monitoring of health and quarantine conditions are recommended.

Conclusion

The results of this study revealed that 42% of fish samples taken from farm fishes in Guilan province were positive for the presence of *A. hydrophila*. Since the present study showed that *A. hydrophila* contamination was present in fish samples, it is necessary to make available good hygiene condition and management to prevent the occurrence of hemorrhagic septicemia caused by Aeromonas and especially by A. *hydrophila* to reduce the costs. Besides, the results of this study showed that PCR is a fast, sensitive, and accurate molecular method for identification of *A. hydrophila*. Although traditional culture-based methods are still used, they generally do not have all the characteristics of a desirable method for identifying microorganisms. Moreover, in cases where antibiotic treatment is given before sampling, a negative culture result is reported. Therefore, in order to determine the definitive identity of *A. hydrophila*, in addition to culture, the simultaneous use of other methods such as PCR can be very helpful.

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Conflict of Interest

None declared.

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