# Phenotypic and genetic diversity of motile aeromonads isolated from diseased fish and fish farms

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## Summary

Samples from the kidney of 100 diseased fish with signs of haemorrhagic septicemia and 50 samples from outlet water of fish farms were taken aseptically and cultured. In the laboratory, 75 colonies of gram-negative bacteria were biochemically diagnosed as motile aeromonads in our Bacteriology Laboratory Unit using API 20E rapid identification system. The genotype identification using specific primers for 16S rDNA by PCR and direct sequencing of 28 Iranian motile aeromonads isolates were as follow: in diseased fish, *Aeromonas hydrophila* (3 isolates, 15%), *A. veronii* bv. *sobria* (8 isolates, 40%), *A. bestiarum/piscicola* (5 isolates, 25%), *A. media* (3 isolates, 15%), *A. jandaei* (1 isolate, 5%), *A. aquariorum* (0 isolate, 0%) and in water, *Aeromonas hydrophila* (0 isolate, 0%), *A. veronii* bv. *sobria* (6 isolates, 75%), *A. bestiarum/piscicola* (0 isolate, 0%), *A. media* (1 isolate, 12.5%), *A. jandaei* (0 isolate, 0%) and *A. aquariorum* (1 isolate, 12.5%). Results of this study suggest that the incidence of motile aeromonads septicemia due to *A. veronii* bv. *sobria* is the most prevalent motile aeromonads. Nucleotide polymorphisms on the sequencing results of the 16S rDNA were detected as noticeable inter and intra-specific variation within the population of different aeromonads isolates. In total, 10-20 variant nucleotide positions in this region were observed among *Aeromonas* spp.

**Key words:** Phenotype, Genotype, Motile aeromonads, Diseased fish, Fish farms

## Introduction

Species of *Aeromonas* are common inhabitants of aquatic environments and have been described in connection with fish and human diseases (Figueras, 2005). These bacteria have a broad host range, and have often been implicated in the cause of numerous infections, such as humans with diarrhea and fish with hemorrhagic septicemia (Rahman *et al*., 2002). The strains isolated from the environment do not seem to differ from those isolated from cases of infection with respect to the prevalence of virulence factors (Krovacek *et al*., 1994).

Regarding the large numbers of ornamental fish imported from areas of the world where sanitation is often inadequate and where numerous diseases of man are endemic, it is surprising that so little consideration has been given to the role of these aquarium species as vectors of potential pathogens for man. When the occurrence of bacterial pathogens in ornamental fish has been investigated, *Aeromonas* strains were involved in 18 of 23 bacterial disease outbreaks investigated by Hettiarachchi and Cheong (1994).

Among motile aeromonads isolated from diseased fish, pathogenicity of *A. hydrophila* in aquarium fish in Fars province, Iran was investigated by Akhlaghi and Vafaei (2002).

Studies on aeromonads isolates in Iran were based on biochemical techniques and no 16S rDNA sequence data are available for the Iranian *Aeromonas* spp. It has been demonstrated that the 16S rDNA is a useful target for species delineation and for inferring phylogenetic relationships of *Aeromonas* spp. (Nam and Joh, 2007; Burr *et al*., 2012) particularly when the availability of biochemical characters is limited (Orozova, *et al*., 2009). In the current study, biochemical and molecular data were analyzed simultaneously to provide more accurate and reliable information about the nature and extent of variation within *Aeromonas* spp. Accordingly, the 16S rDNA sequence was used as genetic marker to investigate the genetic variation within *Aeromonas* isolates recovered from diseased fish and water of Iranian fish farms and compared with additional closely related bacteria that exist in the GenBank database.

## Materials and Methods

### Fish and water samples

Samples were collected from diseased fish from warmwater aquaculture, ornamental fish and coldwater fish farms, Fars province, Iran. Fish (n=100) with symptoms of weakness were placed into ordinary plastic bags and transported to the laboratory. Water samples (n=50) were taken from the same ponds, aquaria and raceways where the fish were collected (Table 1).

Samples from fish kidneys and the collected water were aseptically cultured on brain hearth infusion agar (Oxoid). After 48 h at 25°C typical colonies were isolated and subcultured again. Following standard procedures, bacterial colonies were subjected to the cytochrome oxidase, catalase activity and nitrate reduction tests for preliminary identification.

*Aeromonas* at the genus level and further key biochemical tests in API 20E (bioMérieux) consisting of 21 tests that allow differentiation among *Aeromonas* species were performed following the manufacturer's instructions.

## PCR and sequence analysis

The nucleotide sequences of the 16S rDNA gene were amplified and sequenced, using the previously described PCR assays for aeromonads (Dorsch *et al*., 1994; Orozova *et al*., 2009). The oligonucleotide primer sets used in this study (Cinnagen Co., Iran) are listed in Table 2. A total of 75 isolates were subjected to genotyping based on the sequencing results of the 16S rDNA region. Nucleic acid extracts were prepared from fresh cultures (Tripticase Soy Broth (Oxoid) at 25°C for 24 h) by suspending the colonies into 300 µl deionized distilled water. Suspensions were heated at 100°C for 7 min and centrifuged at 13,000 × g for 10 min to obtain the nucleic acid-containing supernatant (Akhlaghi and Sharifiyazdi, 2008; Sharifiyazdi *et al*., 2010).

*Reference strains*

DNA of 17 *Aeromonas* strains isolated from diseased fish and water in Turkey provided by Ozer *et al*. (2009) were also used as positive control in this study.

The following PCR conditions were applied to each assay, 50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol of forward and reverse primers, and 2 U *Taq* DNA polymerase (Fermentas) per 50 µl reaction using 4 µl of DNA were extracted as the template. A gradient thermocycler (MG 5331, Eppendorf, Hamburg) was used to determine an optimal annealing temperature for the specific binding of the primer set to the template DNA. The optimal thermal parameters were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min at the end of the amplification cycles was included. Each sample was tested at least in duplicate and sterile water was used as the negative control. The PCR products obtained from 16S rDNA region were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide (1 µg/ml). The amplification products were visualized under a UV transilluminator and photographed (Fig. 1).

The PCR products from each assay were purified using a PCR kit (Bioneer, Korea) and sequenced directly using capillary DNA analyzer (ABI 3730, Applied Biosystems, Foster City, CA, USA) after sequencing reactions with a BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). The forward and reverse nucleic acid sequence data were used to construct a continuous sequence of 16S rDNA. Sequences generated from different isolates were analyzed to assess the diversity of each gene compared to the GenBank database using the BLAST (Basic Local Alignment Search Tool) maintained by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments and construction of a phylogenetic tree were made with the Clustal W and maximum likelihood and neighbor-joining methods based on Kimura two-parameter distances using MEGA 4 software (Tamura *et al*., 2007).

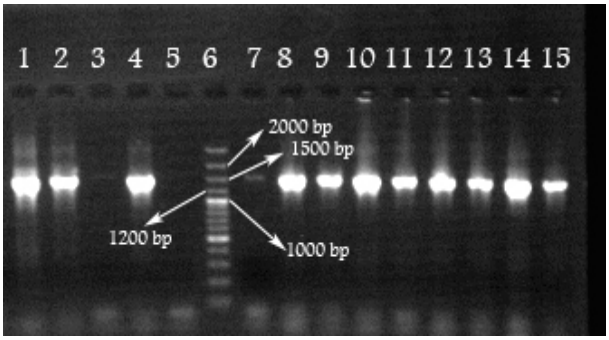


**Fig. 1:** Electerophoretic analysis (1.5% agarose gel) of 16S rDNA amplified fragments from motile aeromonads isolates in this study compared with the marker (Lane 6) and the negative control (Lane 5)

**Table 1:** Fish farms where samples were collected for this study

| No. | Farms (Fars province, Iran) | No. of farms | Sampled diseased fish | Sampled water (Outlets) | Mean temperature (°C) |
|---|---|---|---|---|---|
| 1 | Warmwater fish farm | 12 | 54 | 21 | 26 ± 2 |
| 2 | Aquarium fish farm | 5 | 24 | 15 | 28 ± 1 |
| 3 | Coldwater fish farm | 6 | 22 | 14 | 16 ± 0.5 |
| Total | - | 23 | 100 | 50 | - |

**Table 2:** Oligonucleotide sequences used as primers for PCR detecting 16S rDNA gene

| Gene | Primer sequence | Product Size (bp) | References |
|---|---|---|---|
| 16S rDNA | 5′-agagtttgatcctggctcag -3′<br>5′-acggctaccttgttacgactt-3′ | 1500 | (Orozova *et al*., 2009) |
| 16S rDNA | 5′-gaaaggttgatgcctaatacgta -3′<br>5′-cgtgctggcaacaaaggacag -3′ | 1100 | (Dorsch *et al*., 1994) |

## Results

Cultures of kidney samples of 100 diseased fish with signs of haemorrahagic septicemia and 50 samples from fish farm outlet water on brain heart infusion agar were biochemically diagnosed in our bacteriology laboratory unit using API 20E rapid identification system. Twenty eight isolated motile aeromonads were both biochemically (Table 3) and genotypically identified as motile aeromonads.

Other gram-negative non-aereomonads bacteria such, *Yersinia ruckeri*, *Citrobacter freundi*, *Pseudomonas fluorescens*, *Plesiomonas shigelloides*, and *Shewanella putrefaciens* were also isolated and identified by the methods used in this study. These were phenotypically differentiated from motile aeromonads by using API 20E test strips.

Motile aeromonads were isolated from 16.7% and 4.8% of the diseased cyprinidae fish and their water samples respectively. *Aeromonas veronii* bv. *sobria* isolates were detected in both diseased common carp and grass carp but not silver carp. *Aermonas bestiarum/ piscicola* (deposited in the GenBank as *Aeromonas* spp.) were isolated from diseased common carp and silver carp. *Aeromonas media* isolates were identified from both diseased silver carp and water samples taken from ponds where the diseased fish were sampled (Table 4).

Results of the identification of motile aeromonads from aquarium fish were as follows: motile aeromonads were isolated from 25% and 46.7% of the diseased aquarium fish and their water samples respectively. *Aeromonas veronii* bv. *sobria* isolates were detected in diseased goldfish, Texas cichlid, *Aeromonas hydrophila* isolates were detected in Severum cichlid and parrotfish; *Aeromonas jandaei* and *A. bestiarum/piscicola* in Texas cichlid; *Aeromonas aquariorum* and *Aeromonas v. sobria* both from water samples taken from ponds where diseased fish were sampled (Table 5).

Motile aeromonads were isolated from 21.7% and 0% of the diseased rainbow trout fish and their water samples respectively. *Aeromonas hydrophila*, *A. veronii* bv. *sobria* and *A. media* were detected in 22 diseased rainbow trout. No motile aeromonads were identified from raceways where diseased fish were sampled (Table 6).

The genotype identification using specific primers for 16S rDNA by PCR and direct sequencing of 28 Iranian motile aeromonads isolates were as follows: in diseased fish and water together, *Aeromonas hydrophila* (3 isolates, 10.71%), *A. veronii* bv. *sobria* (14 isolates, 50%), *A. bestiarum/piscicola* (5 isolates, 17.85%), *A. media* (4 isolates, 14.28%), *A. jandaei* (1 isolate, 3.56%) and *A. aquariorum* (1 isolate, 3.56%). The number of isolates and related percent in diseased fish and in fish farm water as well as polymorphism in 16S rDNA gene are shown in Tables 7 and 8. In total, 10-20 variant nucleotide positions in this region were observed among *Aeromonas* spp. The GenBank accession numbers of the nucleotide sequences derived in this study are JF313389 to JF313416. Phylogenic positions of our motile aeromonads were shown in Figs. 2 and 3 using maximum likelihood and neighbor-joining methods.

## Discussion

Motile *Aeromonas* spp. have been recovered from a wide variety of cultured (Nam and Joh, 2007; Orozova *et al*., 2009) and wild freshwater fish species (González *et al*., 2001) which indicates that the microbiota of fish is quite relevant to water quality. This is reflected in the

**Table 3:** Phenotypic characteristics of the isolated *Aeromonas* according to API 20E (after 48 h incubation)

| Motile aeromonads isolates | LDC | CIT | ACE | MAN | SOR | RHA | MEL | AMY | ARA | GLU (g) | ESC (HYD) | VP | CAT | β-HEMO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. hydrophila* | + | $33^{+}/66^{-}$ | + | + | - | $33^{+}/66^{-}$ | - | + | + | + | + | + | - | + |
| *A. veronii* bv. *sobria* | $92^{+}/8^{-}$ | $76^{+}/24^{-}$ | + | $84^{+}/16^{-}$ | - | - | $40^{+}/60^{-}$ | $24^{+}/76^{-}$ | $8^{+}/92^{-}$ | $92^{+}/8^{-}$ | $40^{+}/60^{-}$ | + | $70^{+}/30^{-}$ | + |
| *A. bestiarum/piscicola* | + | - | + | + | $50^{+}/50^{-}$ | $75^{+}/25^{-}$ | - | $50^{+}/50^{-}$ | + | $75^{+}/25^{-}$ | + | + | $50^{+}/50^{-}$ | $75^{+}/25^{-}$ |
| *A. media* | - | - | - | + | - | - | - | + | + | - | + | - | $25^{+}/75^{-}$ | $50^{+}/50^{-}$ |
| *A. jandaei* | + | + | + | + | - | - | - | + | - | + | + | + | - | + |
| *A. aquariorum* | + | + | + | + | - | - | - | + | - | + | + | + | - | + |

ONPG, ADH, IND, GEL, GLU, SAC, OX, MR, MOT, and NIT tests of isolates were all positive. ODC, $H_2S$, URE, TDA, and INO tests of isolates were all negative

**Table 4:** Total fish species/water samples from warmwater fish farms (Fars province, Iran) and results obtained from cultures for aeromonads isolation, genotype identification using specific primers for 16S rDNA by PCR and GenBank data

| Fish species/water sample | Total samples | Negative culture | Positive Culture | | aeromonads isolation code | aeromonads PCR results | aeromonads accession No. |
|---|---|---|---|---|---|---|---|
| | | | Non-aeromonads | Motile aeromonads | | | |
| **Common carp** (*Cyprinus carpio*) | 23 | 12 | 7 | 4 | 13, 39 | *A. v. sobria* | JF313390, JF313395 |
| | | | | | 43, 46 | *A. bestiarum/ piscicola* | JF313408, JF313416 |
| **Grass carp** (*Ctenopharyngodon idella*) | 16 | 9 | 5 | 2 | 8, 41 | *A .v. sobria* | JF313389, JF313392 |
| **Silver carp** (*Hypophthalmichthys molitrix*) | 15 | 8 | 4 | 3 | 11 | *A. media* | JF313404 |
| | | | | | 12, 36 | *A. bestiarum/ piscicola* | JF313409, JF313410 |
| **Water sample** | 21 | - | 20 | 1 | 26 | *A. media* | JF313407 |

**Table 5:** Total fish species/water samples from aquarium fish (Fars province, Iran) and results obtained from cultures for aeromonads isolation, genotype identification using specific primers for 16S rDNA by PCR and GenBank data

| Fish species/water sample | Total samples | Negative culture | Positive Culture | | aeromonads isolation code | aeromonads PCR results | aeromonads accession No. |
|---|---|---|---|---|---|---|---|
| | | | Non-aeromonads | Motile aeromonads | | | |
| **Goldfish** (*Carassius auratus*) | 5 | 3 | 1 | 1 | 42 | *A. v. sobria* | JF313391 |
| **Severum cichlid** (*Heros severus*) | 3 | 1 | 1 | 1 | 15 | *A. hydrophila* | JF313401 |
| **Texas cichlid** (*Herichthys cyanoguttatus*) | 11 | 7 | 1 | 3 | 73<br>80<br>104 | *A. jandaei*<br>*A. v. sobria*<br>*A. bestiarum/ Piscicola* | JF313413<br>JF313414<br>JF313411 |
| **Parrotfish** (*Haplarchus psittacus*) | 5 | 3 | 1 | 1 | 44 | *A. hydrophila* | JF313402 |
| **Water sample** | 15 | 7 | 1 | 7 | 78<br>79,<br>83,<br>84,<br>85,<br>86,<br>91 | *A. aquariorum*<br>*A. v. sobria* | JF313412<br>JF313393,<br>JF313396,<br>JF313397,<br>JF313415,<br>JF313398,<br>JF313399 |

**Table 6:** Total fish species/water samples from coldwater fish farms (Fars province, Iran) and results obtained from cultures for aeromonads isolation genotype identification using specific primers for 16S rDNA by PCR and GenBank data

| Fish species/water sample | Total samples | Negative culture | Positive Culture | | aeromonads isolation code | aeromonads PCR results | aeromonads accession No. |
|---|---|---|---|---|---|---|---|
| | | | Non-aeromonads | Motile aeromonads | | | |
| **Rainbow trout** (*Oncorhynchus mykiss*) | 22 | 13 | 4 | 5 | 47<br>81,<br>103<br>24,<br>25 | *A. hydrophila*<br>*A .v. sobria*<br><br>*A. media* | JF313403<br>JF313394,<br>JF313400<br>JF313405,<br>JF313406 |
| **Water sample** | 14 | - | 14 | - | - | - | - |

**Table 7:** Frequency of aeromonads isolation from diseased fish

| Motile aeromonads isolated in this study | Isolate codes | No. | % | Polymorphism in 16S rDNA gene |
|---|---|---|---|---|
| *Aeromonas hydrophila* | 15, 44, 47 | 3 | 15 | + |
| *Aeromonas veronii* bv. *sobria* | 8, 13, 39, 41, 42, 80, 81, 103 | 8 | 40 | + |
| *Aeromonas bestiarum*/*piscicola* | 12, 36, 43, 46, 104 | 5 | 25 | + |
| *Aeromonas media* | 11, 24, 25 | 3 | 15 | + |
| *Aeromonas jandaei* | 73 | 1 | 5 | + |
| *Aeromonas aquariorum* | - | 0 | 0 | - |

**Table 8:** Frequency of aeromonads isolation from fish farm water

| Motile aeromonads isolated in this study | Isolate codes | No. | % | Polymorphism in 16S rDNA gene |
|---|---|---|---|---|
| *Aeromonas hydrophila* | - | 0 | 0 | - |
| *Aeromonas veronii* bv. *sobria* | 79, 83, 84, 85, 86, 91 | 6 | 75 | + |
| *Aeromonas bestiarum*/*piscicola* | - | 0 | 0 | - |
| *Aeromonas media* | 26 | 1 | 12.5 | + |
| *Aeromonas jandaei* | - | 0 | 0 | - |
| *Aeromonas aquariorum* | 78 | 1 | 12.5 | + |

findings of the present study. In ponds and aquaria whose water is not changed often enough, aeromonads are isolated (4.8% and 46.7%, respectively) (Tables 4 and 5), whereas in raceways where water originates from ground springs and is constantly changed, no aeromonads (0%) were isolated from rainbow trout farms in this study (Table 6). Accurate identification of the aeromonads isolates has important implications for studying population structures, epidemiological model and therefore the effective control program of the disease. Although phenotypic and genetic variation within *Aeromonas* spp. has been reported previously from several countries (Nawaz *et al*., 2006; Nam and Joh, 2007; Burr *et al*., 2012), there is only limited data on phenotypic and genetic diversity of *Aeromonas* spp. circulating in Iran. In this investigation, 50% of the isolates (14/28) were related to *Aeromonas veronii* bv. *sobria*. It was isolated from 8 diseased fish with haemorragic septicemia clinical signs from cyprinidae, cichlidae in warmwater environment and salmonidae in coldwater condition. The bacterium was also isolated from 6 water samples from aquarium water samples.

This shows that this strain in the region constitutes an important causative agent of haemorrhagic septicemia in fish and in the aquatic environment in the geographic area of Iran.

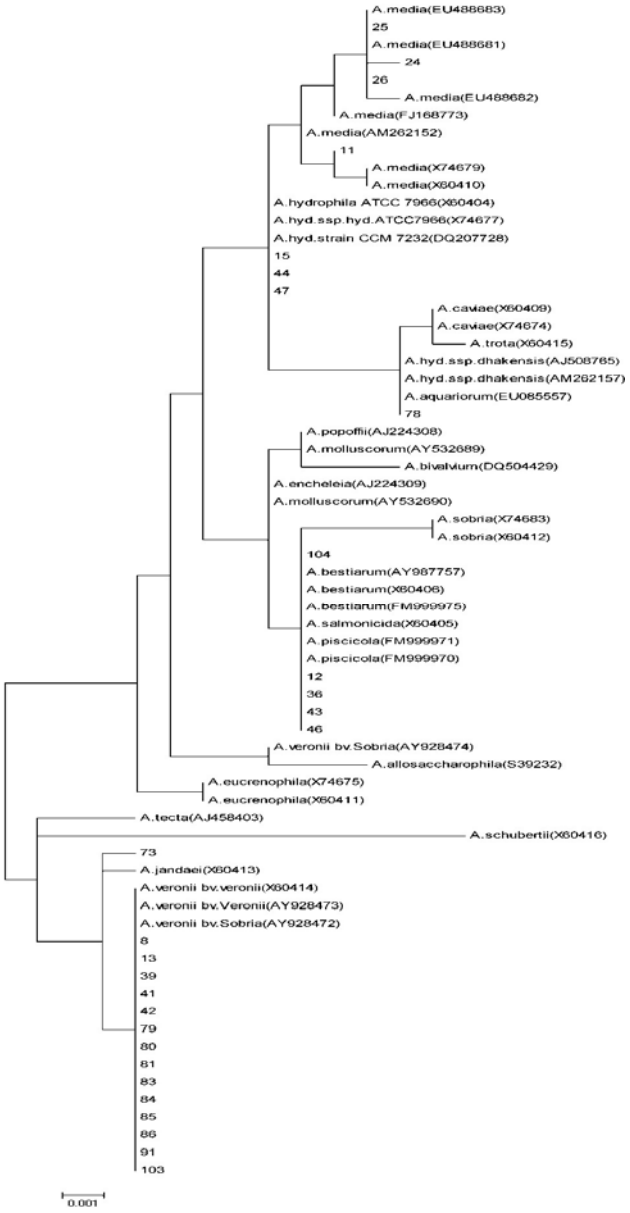


**Fig. 2:** Phylogenic position of the motile aeromonads isolated in this study from fish and water compared with reference strains based on maximum likelihood method

The phylogenetic and taxonomic statuses of *A. veronii* are controversial; other studies have differentiated this species in the two biovars *A. veronii* bv. *veronii* and *A. veronii* bv. *sobria*, but the genotype divergence is very low, even if they represent two heterogeneous phenotypes (Soler *et al*., 2004). In one study Cai *et al*. (2012) confirmed that representative strain PY50, a strain of *A. veronii* bv. *veronii* is highly pathogenic to Chinese longsnout catfish, and this strain is the pathogen that caused outbreaks of ulcerative syndrome in Chinese longsnout catfish.

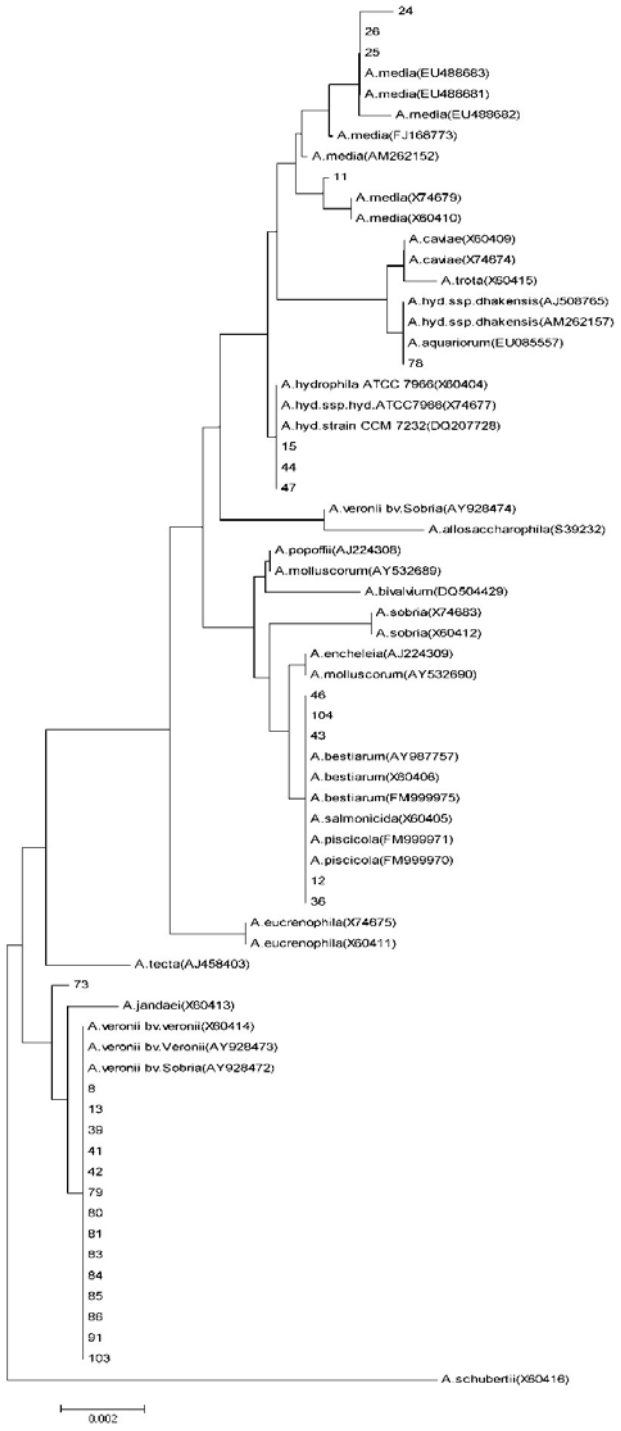


**Fig. 3:** Phylogenic position of the motile aeromonads isolated in this study from fish and water compared with reference strains based on neighbor-joining method

*Aeromonas aquariorum* was isolated from aquarium fish water in this study. Phenotyic characterization of the strain is very close to the *A. aquariorum* isolated from water and skin of imported ornamental fish described by Martínez-Murcia *et al*. (2008). Phylogenetic tree of the *A. aquariorum* isolated in this study from water using maximum likelihood and neighbor-joining methods (Figs. 2 and 3) showed a high sequence similarity with the reference strain of *A. aquariorum* (EU0855557).

In the present study five *A. hydrophila* strains

sampled from water/fish showed genetic similarities with the strain ATCC7966 (X60404). This is reflected in the phylogenic tree of the *A. hydrophila* isolated in this study from diseased fish and the reference strains using maximum likelihood and neighbor-joining methods, which show very close nucleotide sequence. This is the first investigation reporting on commercial cyprinids, aquarium and rainbow trout farms in Iran to identify genetic diversity of motile aeromonads isolated from diseased fish and water samples.

*Aeromonas bestiarum/piscicola* was isolated from diseased cyprinids and Texas cichlids in this study. Phylogenic tree of this isolate shows very close homology to reference strains *A. bestiarum* AY987757 and *A. piscicola* FM999971. *Aeromonas bestiarum/ piscicola* was chosen for our isolate in this study since the 16S rDNA gene is not sufficiently discriminative to clearly differentiate some strains of both species (Beaz-Hidalgo *et al*., 2009).

The phylogenetic likelihood of *A. jandaei* was found very similar to *A. jandaei* X60413; *A. veronii* bv. *veronii* AY60414; *A. veronii* bv. *sobria*, however, it was related as distantly as some other species of the genus, for instance *A. schubertii* X60416 (Fig. 2).

The maximum likelihood and neighbor-joining trees showed that our *A. media* isolates were more closely related to each other and form a monophyltic lineage with other existing reference sequences for *A. media* in GenBank database. However, phylogenetic analysis show higher ancestral 16S rDNA variability for Iranian *A. media* compared to other *Aeromonas* spp. isolates (Figs. 2 and 3). These results provide evidence of a greater diversity of *Aeromonas media* involved in fish pathology and aquatic environment than it was first thought. Further investigations using a considerable number of strains belonging to both species could give more reliable information. The phenotypic traits of *A. media* type strain were in good agreement with previous literature.

In conclusion, genetic diversity of *Aeromonas* spp. of Iranian fish farms was demonstrated in our study. The results could be employed as the preliminary data for studying genetic diversity, molecular epidemiology, and the prevention and control programs of *Aeromonas* spp.

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