Isolation, phenotypic and molecular characterization of motile *Aeromonas* species, the cause of bacterial hemorrhagic septicemia in affected farmed carp in Iran

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Key words:
Farmed carp, Motile *Aeromonas* septicemia, 16S rRNA gene

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Received: 27 January 2016
Accepted: 11 May 2016

Abstract:

BACKGROUND: Motile *Aeromonas* species cause heavy mortalities in carp farms during spring and summer in Iran. **OBJECTIVES:** The aim of this study was to detect phenotypic and genotypic characterization of motile *Aeromonas* species isolated from diseased carps in some northern and southern provinces of Iran. **METHODS:** A number of 40 samples from 22 fish farms were collected. The identified motile *Aeromonas* species were sequenced and phylogenetic tree was drawn by MEGA6 using UPGMA analysis. **RESULTS:** A number of 19 bacterial isolates were identified as motile *Aeromonas* sp. by biochemical tests, and the DNA segments of 16S rRNA gene of all these strains gave 1200 bp after running on 1% agarose electrophorus gel. Also, the sequencing results showed that the bacterial samples were determined as *A. hydrophila* and *A. veronii* biovar veronii. **CONCLUSIONS:** Phylogenetic analysis revealed that motile *Aeromonas* strains in this study were separated in two clusters and four genogroups with high similarities.

Introduction

Aeromonas hydrophila and other motile *Aeromonas* species which cause motile *Aeromonas* septicemia are known as the most common bacterial infections in freshwater aquaculture worldwide (Borrell et al., 1997; Leblanc, et al., 1981; Woo et al., 2011; Henebry et al., 1988). The species of *A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii* are the cause of carp mortalities (Popoff and VéEron, 1976; Garrity et al., 2004). Motile *Aeromonas* species cause some pathologic conditions including acute, chronic and latent infections with different clinical signs (Soltani, 2002). The disease occurrence is common in spring and summer in farmed carp that is related to high temperature, fish metabolism and organic loading rate (Soltani, 2002). According to the Iranian veterinary organization, the most epizootics occur in the north and south regions of Iran where the carp aquaculture is located (Iranian Veterinary Organization, unpublished data, 2011). So far, some epizootics of the disease have occurred in various species of carp in the north and south provinces (Peyghan and Esmaili, 1997; Soltani et al., 1998; Alishahi et al., 2009). Although the morphological and biochemical approaches have been applied to identify the genus *Aeromonas* in Iran, there is no adequate data on the phylogenetic position of the *Aeromonas* species involved in morbid-

ity and mortality of carps. Therefore, the aim of this study was to isolate and characterize the motile *Aeromonas* species involved in carp mortality in the north and south regions of the country. Such data are useful for prevention and control criteria of disease and outbreaks.

Materials and Methods

Collection of bacterial isolates and phenotypic identification: After collecting 40 samples from different carp ponds, a total of 19 *Aeromonas* strains were isolated from common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*) with clinical symptoms of bacterial infection such as hemorrhages in skin and gill, exophthalmia and dropsy. The samples were collected from three different geographical locations in Iran during a two-year period (2014 - 2015): 10 samples from Mazandaran province, 14 samples from Gilan province and 16 samples from Khuzestan province. The samples were isolated from kidney, cultivated on blood agar and incubated at 30°C for 72 h. First, the morphological characteristics of the colonies were identified by Gram staining which confirmed the bacterial colonies were Gram negative. Biochemical tests were carried out in all strains including SIM (Sulfide, Indole, and Motility), gelatin and esculin hydrolysis, lysine and ornothine decarboxylase, nitrate reduction, fermentation and gas production of glucose, maltose and inositol, triple sugar iron agar (TSI), methyl red and voges-proskauer (MRVP) tests.

DNA extraction: The DNA Biospin Bacteria Genomic Extraction Kit, Bioflux Co. (Gapan) was used to extract the genomic DNA. Briefly, a volume of 10 ml of culture was collected by centrifugation for 1min at 14000 g and resuspended in 100 µl of EL buffer incubated at 37°C for 40 min. Then RS buffer and PK solution were added, respectively and incubated at 56°C for 15 min. 200 µl of GA buffer was then added and centrifuged at 12,000 x g for 1 min and the supernatant was transferred to a new 1.5ml tube. An aliquot of 400 µl of BA buffer was then added and centrifuged at 10,000 x g for 1 min. A volume of, 500 µl of G binding buffer was then added into the spin column and centrifuged at 10,000 x g for 1 min. A volume of 500 µl of washing buffer was added to the spin column and centrifuged at 10,000 x g for 1 min two times. After transferring the spine column to a sterile 1.5ml micro centrifuge tube, 100 µl of elution buffer was added and the mixture was incubated at room temperature for 1 min, centrifuged at 12,000 x g for 1 min. The spin column was removed with the buffer in the micro centrifuge tube containing the DNA. The analysis of DNA concentration and quality were based on the 260 /280 nm and absorbance ratio using the spectrophotometer (Biophotometer Eppendorf) according to manufacturer's instructions. The solution was kept at -20°C when stored.

Polymerase chain reaction: The polymerase chain reaction was used to detect the presence of the 16S rRNA gene in all isolates. Briefly, the 25 µl PCR mix consisted of 2.5µl 10x reaction buffer, Fermentas Co. (Lithuania), 0.2U Taq polymerase, 1µl of the two primer solutions (forward: 5′-AGA GTT TGA TCA TGG CTC AG -3′; reverse: 5′-GGT TAC CTT GTT ACG ACT T-3′), 4µl mix dNTP (2.5mM each) and 1µl of DNA sample. PCR was carried out on the thermocycler Bio-Rad Co. (USA) performed the following cycles of denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1/5 min and an extension cycle at 72°C was allowed for 10 min. Negative controls with no template DNA were included. Also, PCR products were electrophoresed in 1% agarose gel in TBE buffer. The gel was stained with GenDireX (Biohelix-USA) and photographed on a UV transilluminator XR-plus, Bio-Rad Co. (USA). The identified *Aeromonas* strains by 16S rRNA PCR analysis on 1% agarose gel electrophoresis were then sequenced directly by

Table 1. Biochemical characteristics of *A. hydrophila* isolates in this study.

Tests	M1	M2	M3	M4	G1	G2	G3	G4	G5	K1	K3	K4	K5	K7	K9
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H2S	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TSI	-/+	-/-	-/-	-/-	-/-	-/+	+/+ Gas	+/+ Gas	+/+ Gas	+/+	+/+ Gas	+/+ Gas	+/+	+/+	+/+ Gas
Lysine decarboxilase	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Esculin hydrolysis	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Inositol fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose fermentation	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas production of glucose	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-

Table 2. Biochemical characteristics of *A. veronii* isolates in this study.

Tests	K2	K6	K8	K10
Indole	+	+	+	+
Motility	+	+	+	+
H2S	+	+	+	+
Gelatin hydrolysis	-	+	+	+
Ornithine decarboxylase	-	-	-	-
Nitrate reduction	+	+	+	+
MR	+	+	+	+
VP	+	+	+	+
TSI	+/+	+/-	+/+	+/+
Lysine decarboxilase	+	+	+	+
Esculin hydrolysis	-	-	-	-
Inositol fermentation	-	-	-	-
Maltose fermentation	-	-	-	-
Glucose fermentation	-	+	+	+
Gas production of glucose	-	-	-	-

Takapouzist Co. (Tehran, Iran). The results were aligned by the Finch TV software and then compared with available sequences in GeneBank using BLAST service. The phylogenetic tree was drawn by MEGA6 using UPGMA analysis.

Results

Clinical signs and gross pathology: Fish samples collected from 22 farms showed distinct clinical signs of lethargy, anorexia, loss of balance, exophthalmia, ulcers, hemorrhages in skin and gill and abdominal distention (Figs. 1 and 2). Internally, the spleen was enlarged and hemorrhagic. Petechial hemorrhages were observed on the surface of kidney, intestines and many tissues. Also, accumulation of ascetic fluid was observed in almost all the diseased fish.

Biochemical tests: In primary phenotypic tests, grown colonies were gram negative, cocobacilli shaped, motile and oxidase positive which are supposed to be in genus *Aeromonas*. The results of the biochemical tests were compared with the key table in Bergey's manual systematic bacteriology to determine the species. The results are shown in Tables 1and 2.

Analysis of 16S rRNA gene PCR: The results of PCR amplification of DNA from bacterial strains showed the expected amplicons of 1200 bp (Fig. 3).

Sequencing and phylogenetic analysis: The sequencing of the PCR products resulted in detection of KT378601, KT378602, KT378603, KT378604, KU201534,

Table 3. The Aeromonas strains isolated from carp ponds (sampling region, number of fish samples, number of isolated strains of motile Aeromonas, laboratory code and accession number).

Region	Number of fish samples	Number of isolates identified as Aeromonas sp.	Laboratory Code	Accession Number
Khuzestan State	16	10	K1, K2, K3,K4, K5,K6, K7, K8, K9, K10	KU201536, KU201537, KU201538, KU201539, KU201540, KU216160, KU216161, KU216162, KU216163, KU216164
Gilan State	14	5	G1, G2, G3 G4, G5	KT378601, KT378602, KU201534, KU201535, KU257639
Mazandaran State	10	4	M1, M2, M3, M4	KT378603, KT378604, KU257637, KU257638

KU201535, KU201536, KU201537, KU201538, KU216160, KU216161, KU216164, KU257637, KU257638 and KU257639 as *A. hydrophila* and KU201539, KU201540, KU216162 and KU216163 as *A. veronii* biovar veronii (Table 3). The MEGA 6 software has been applied to show the phylogenetic relationship between all isolates in this study (Fig. 4). According to the phylogenetic tree, high similarities were observed among motile *Aeromonas* species isolated from carps in Iran. The strains were classified in two clusters, A and B with 1.6% variations. There were two genogroups in cluster A, genogroup A1 and A1. In genogroup A1, the similarity between the strain, KU216163 and the other strains including KU216162, KU257637 and KU201154 was 99.4%. Also, the cluster A was divided to two genogroups, B1 and B2 with 99.3% similarity. In genogroup B1, the similarity between the two strains, KU216160 and KU216164 and the strains, KU216161, KU257637, KU 257638 and KU257639 was 99.5%. Also, the strains in genogroup B2 showed high similarities so that the similarity between the strain, KU201537 and the others strains was 99.5%.

Discussion

Recently, there have been many reports of heavy mortalities in carps during spring and summer in Iran. Although motile *Aeromonas* species are usually known as secondary opportunistic pathogens, they can be as primary pathogens in some conditions. So, there are no effective approaches for control and treatment of disease in carp farms since Aeromons infection is related to the environmental conditions and physicochemical parameters of water. Therefore, isolation and identification of the bacteria would be useful for control and prevention of disease. Today, molecular techniques are used widely since they provide a relatively rapid and highly sensitive method for the detection of bacterial pathogens (Chu and Lu, 2005). In this regard the most commonly used gene for taxonomic and phylogenetic purposes in bacteria is the 16S rRNA which is the most conserved gene in bacteria (Clarridge, 2004; Roy et al., 2013). In the present study, various biochemical tests have been applied to identify 40 clinical isolates of motile *Aeromonas* sp. Also, 19 isolates have been characterized as motile *Aeromonas* sp. by PCR analysis of 16S rRNA gene. Most results from both approaches have shown similarities, however, some discrepancies have been observed. Four strains, KT378602, KU257637, KU257638 and KT378603 that were characterized as *A. veronii* biochemically (esculin hydrolysis negative), were identified by PCR and DNA sequencing as *A. hydrophila*. Also, the gelatin hydrolysis test was negative in five

Figure 1. Skin hemorrhage and exophthalmia of silver carp (H. molitrix).

Figure 2. Hemorrhage in gill filaments of silver carp (H. molitrix).

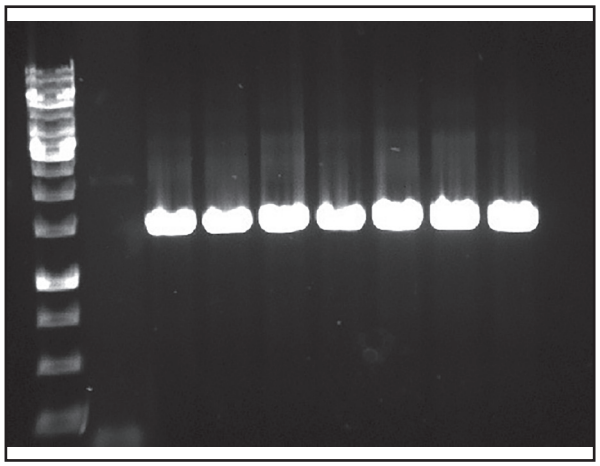

Figure 3. PCR detection of 16S rRNA genes in several Aeromonas recovered from diseased carp in Iran. Lane M= 1 kbp DNA ladder, Lane 1= negative control, Lane 2= positive control (*Aeromonas hydrophila* - IRTCC1032), Lane 3 to 8= several test samples.

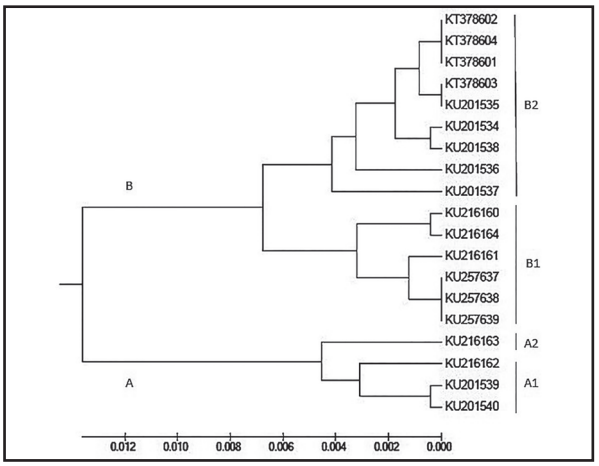

Figure 4. Phylogenetic analysis of Aeromonas strains based on the 16S rRNA sequences using UPGMA method.

strains, KT378601, KT378602, KU216160 and KU201537 and KU201439. But they identified phylogenetically as *A. hydrophila* and *A. veronii*. These results were confirmed by repeating the tests. Such discrepancies may result from the fact that biochemical tests are not reliable for identification of *Aeromonas* sp.. Other strains showed the same results in both biochemical and molecular tests. The phylogenetic tree of 19 bacterial isolates of this study showed 97.2-100% similarity based on the analysis of 16S rRNA gene. Such similarities among the isolates of the country can be applied to promote some prevention methods like vaccination.

Alzahrani (2015) used the 16S rRNA gene to identify and characterize *Aeromonas* species in drinking water since this gene is known as a specific molecular marker for bacterial identification and is useful for the bacterial molecular taxonomy. The results showed that all bacterial samples were *A. veronii* or a new species. Moreover, classical tests as well as molecular studies on the 16S rRNA gene have been applied by Sarkar et al. (2012) for classification of the phylogenetic relationships among *Aeromonas* species isolated from various sources. The biochemical and molecular results were similar to those of the present study and the isolates registered in GeneBank were used for comparison with the isolates of this study by using MEGA6 software. The

results showed similarities in 16S rRNA sequences of the strains even though the samples have been collected from different sources. Arora et al. (2006) applied several methods to identify *Aeromonas* species and concluded that both indirect ELISA and duplex-PCR are reliable methods to detect *Aeromonas* species in foods of animal origin due to their specificity and duplex-PCR is the best method among others used in the study on account of its rapidity, sensitivity and specificity. Borrel et al. (1997) used the same gene to identify *Aeromonas* species from different clinical samples such as blood, soft tissue infection and urine. Most results from the biochemical tests and RFLP method were similar but some discrepancies were seen. For instance, some species detected biochemically as *A. veronii* were characterized as *A. hydrophila* and *A. caviae* by RFLP analysis. In conclusion, the RFLP method is proposed as a rapid and reliable method without the need for sequencing to identify *Aeromonas* species. In addition to the 16S rRNA gene, many virulence genes have been detected to identify *Aeromonas* species (Sechi et al., 2002; Yogananth et al., 2009; Uma et al., 2010; Cagatay and Şen, 2014; Hussain et al., 2014). Among the virulence factors of motile *Aeromonas* species, antigen-O, the presence of capsule, S layer, exotoxins such as hemolysins and enterotoxins, exoenzymes such as lipase, amylase and protease and the type III secretion system are well known (Oliveira et al., 2012). Hussain et al. (2014) studied the ahh1, asa1 and 16S rRNA genes by using multiplex PCR to detect the hemolytic strains of *Aeromonas* isolated from fish and fishery products. The results proved the presence of 16S rRNA and asa1 genes in *A. sobria*, 16S rRNA and ahh1 genes in *A. hydrophila* and 16S rRNA gene in *A. liquifaciencs* and provided reliable data to detect the hemolytic strains of *Aeromonas*. In the study of Cagatay and Şen (2014) on the virulence genes of *A. hydrophila* regarding the cause of bacterial hemorrhagic septicemia in rainbow trout, the AHCYTOEN, Hly and OmpTS genes have been detected. All these genes were detected in the *Aeromonas* strains and are known as specific virulence determinants and genetic markers to identify the bacteria before the disease spread. Biochemical tests as well as PCR for detecting the aerolysin and hemolysin genes of *Aeromonas* strains have been carried out by Yoganath et al. (2009) in which seven strains of 15 strains have been identified as *A. hydrophila*. The PCR assay applied in this study was known as a useful tool to detect the aerolysin and hemolysin genes which can be the genetic virulence markers. A similar study has been done by Uma et al. (2010) that indicated the aerolysin and hemolysin genes are a better indicator of the potential health risk since the pathogenicity of *Aeromonas* strains is associated with such virulence factors. Besides the molecular and biochemical tests, the LD50 determination can be used to identify the virulent *Aeromonas* strains, as Alishahi et al. (2009) applied this method to detect the pathogenic motile *Aeromonas* species. The results showed the presence of *A. hydrophila* (%11), *A. veronii* (%4) and *A. sobria* (%2.7) from a total of 300 samples. Also, *Aeromonas* species were known as the secondary pathogens since they have not presented enough virulence needs for mortalities.

Furthermore, motile *Aeromonas* infection is related to the physicochemical parameters of water quality which can exacerbate the disease outbreaks. In the present study, water temperature was higher than the standard levels and the acidity of water was in the maximum range. Other parameters including dissolved oxygen, carbon dioxide, total hardness as calcium carbonate, nitrite, nitrate and total dissolved solids were in the normal ranges. According to previous studies on detecting *Aeromonas* sp. in carp ponds, this bacteria was known as a secondary pathogen and the disease can occur at stress conditions (Alishahi et al., 2009). Recently, the motile *Aeromonas* species have

been isolated from warm water fish in Iran and numerous factors can be associated with the incidence of the disease. Some of these factors were mentioned in the following: The temperature in summer is more than 40 °C as a result of climate change and the ammonia level and the acidity of water occasionally increase in warm weather. Also, the atmospheric pressure decreases in summer and as a result the water capacity to keep oxygen reduces. Another reason of reducing the dissolved oxygen level is toxins produced by algae which increase the organic loading range in water. Further, unsuitable structure of fish ponds leads to an inadequate water exchange and prevent refilling water. Waste water is periodically used for filling the ponds. The organic loading rate increases by the use of animal-based fertilizer in feeding fish. Besides, the owners do not pay attention to the rules and regulations defined by the responsible organizations about the control and prevention of the disease in summer. In this regard, several methods are suggested to prevent the disease such as systematically preparing the ponds before new larva and fish are put into the ponds, water filtration, quarantine process and collecting the dead fish on a daily basis and reporting the mortality rate to the veterinary organization.

In addition, the carp species are sensitive to some viral diseases such as Spring Viremia of Carp (SVC) and Koi Herpes Virus (KHV). Recently, several fresh water ornamental fishes were imported to the country for research aims and experimental studies. SVCV and KHV were detected in the imported ornamental fishes (Soltani, unpublished data, 2012; Zamani et al., 2014). So occurrence of the viral diseases in carp pond is possible and needs further studies.

Acknowledgments

This work was financially supported by Iranian Veterinary Organization, Center of Excellence of Aquatic Animal Health and Research Council of University of Tehran.

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مجله طب دامی ایران، ۱۳۹۵، دوره ۱۰، شماره ۳، ۲۱۶-۲۰۹

جداسازی و مطالعه ویژگی‌های فنوتیپی و مولکولی گونه‌های آئروموناس‌های متحرک عامل سپتی‌سمی هموراژیک باکتریایی در برخی مزارع مبتلای کپورماهیان پرورشی کشور

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(دریافت مقاله: ۷ بهمن ماه ۱۳۹۴، پذیرش نهایی: ۲۲ اردیبهشت ماه ۱۳۹۵)

چکیده

زمینه مطالعه: آئروموناس‌های متحرک عامل تلفات سنگین در مزارع پرورش کپورماهیان ایران در فصول بهار و تابستان می‌باشند. **هدف:** هدف از انجام مطالعه حاضر جداسازی و شناسایی ویژگی‌های فنوتیپی و مولکولی گونه‌های آئروموناس‌های متحرک در برخی از مزارع پرورشی کپورماهیان کشور بوده است. **روش کار:** به این منظور نمونه‌های بافت کلیه ۴۰ نمونه ماهی دارای علائم بالینی از ۲۲ مزرعه مورد بررسی قرار گرفت. پس از کشت از بافت کلیه ماهیان بیمار روی محیط ژلوز خوندار، آزمایش‌های بیوشیمیایی جهت شناسایی جنس‌های باکتریایی انجام شد. به منظور تأیید تشخیص پس از استخراج DNA نمونه‌ها و ارزیابی کیفیت DNAهای استخراج‌شده، آزمایش PCR صورت گرفت. در این آزمایش از یک جفت آغازگر برای شناسایی ژن ۱۶S rRNA باکتری استفاده شد. نمونه‌های تأییدشده تعیین توالی گردید و مطالعات فیلوژنتیک بر روی جدایه‌ها با استفاده از نرم‌افزار MEGA نسخه ۶ انجام شد. **نتایج:** از کل نمونه‌های مورد مطالعه ۱۹ نمونه به عنوان گونه‌های آئروموناس‌های متحرک با استفاده از آزمایش‌های بیوشیمیایی و نیز تولید باند ۱۲۰۰ بازی از ژن موردنظر روی ژل الکتروفور ۱٪ شناسایی شدند. پس از تعیین توالی، این گونه‌ها به عنوان *Aeromonas hydrophila* و *A. veronii biovar veronii* تشخیص داده شد. **نتیجه‌گیری نهایی:** مطالعات فیلوژنتیک نشان داد که سویه‌های آئروموناس‌های متحرک مورد مطالعه در دو خوشه و چهار گروه ژنتیکی قرار دارند.

واژه‌های کلیدی: کپور پرورشی، سپتی‌سمی آئروموناس‌های متحرک، ژن کدکننده RNA ریبوزومی

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