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Detection and distribution of virulence genes in *Aeromonas hydrophila* isolates causing infection in cultured carps

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Abstract

Aeromonas hydrophila is a bacterium associated with many diseases and disorders such as fin rot, skin ulcers and lethal hemorrhagic septicemia in fish. It bears several virulence factors including type III secretion system (T_3SS), *aerolysin*, *cytolytic enterotoxin* and enzymes (*e.g., hemolysins*, lipase) that seem to play an important role in its pathogenesis. Detection of virulence markers by polymerase chain reaction (PCR) is a key procedure in defining the pathogenic ability of pathogenic bacteria and preparing a vaccine for its treatment. In this sense, this study was aimed to determine the frequency of virulence genes in isolates obtained from infected cultured carps in Khuzestan province. Out of 200 moribund carps with septicemic symptoms, 125 isolates were belonged to the motile aeromonads and 59 isolates were identified as *A. hydrophila* by biochemical methods. Finally, using PCR analysis, 31 isolates were identified as *A. hydrophila*. Five virulence genes were detected in these isolates including *hemolysin*, *aerolysin*, *cytolytic enterotoxin* and T_3SS (*aopB and ascV*) by specific primers. Results showed that 23 (74.19%), 18 (58.06%), 16 (51.61%), 13 (41.63%) and 10 (32.25%) isolates possessed *cytolytic enterotoxin*, *hemolysin*, *aerolysin*, and T3SS genes, respectively. The results of the present study showed that among 31 isolates, only five isolates had all of dominant virulence genes. Thirteen other isolates had genotypes including *hlyA*$^+$, *aerA*$^+$, *and act*$^+$. The remaining isolates had at least one virulence gene. This study showed that determination of the virulence genes by PCR can be a reliable method to identify a potential pathogenic *Aeromonad* strain.

Introduction

The pathogenic bacteria are the most important factors that affect general health of farmed fish.[1] *Aeromonas hydrophila* is a straight, coccobacillary Gram-negative bacterium with rounded ends.[2] This motile facultative anaerobe bacterium is an important pathogen causing primary or secondary infectious disease in warm water farmed fish. The stress condition such as temperature fluctuations, handling or poor water quality have critical role in Aeromonad infections in fish.[3] The pathogenesis of *A. hydrophila* infection is multi-factorial and associated with a number of virulence factors including cell structural lipopolysaccharides, outer-membrane proteins, pili and flagella, type III secretion system (T_3SS), and extracellular factors such as enzymes and toxins. The exotoxins such as *aerolysin* (*aerA*), *cytolytic enterotoxin* (*act*), and *hemolysin* (*hlyA*) as well as enzymes such as lipase, proteases, and nuclease seem to play an important role in the pathogenesis.[4-7] The virulence genes of *A. hydrophila* are generally divided into three main groups of *hemolysin*, *aerolysin*, *cytolytic enterotoxins*.[8] The inactivation of either *hemolysin* or *aerolysin* genes reduced the hemolytic and cytotoxic activities of virulent strains of *A. hydrophila,* however, it could not totally inactivate these endotoxins.[9] The type III secretion system (T_3SS) in Gram-negative

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bacteria such as *Aeromonas salmonicida* and different isolates of *A. hydrophila* may result in their virulence.[10-12] *AscV* and *aopB* genes are important components of T_3SS in *A. hydrophila* that encode the T_3SS consolidation proteins associated in internal membrane of bacteria and make holes or channels in the host cell and have been used for the present survey of T_3SS in screening tests of bacterial strains.[13]

The detection method of virulence genes was recently proposed as a reliable approach to identify a potential pathogenic Aeromonas strain. In recent years several molecular methods, particularly polymerase chain-reaction (PCR) based method have been developed for routine identification of Aeromonas species and their putative virulence genes.[4,14,15] The present study was aimed to detect selected virulence genes (*hemolysin*, *aerolysin*, *cytolytic enterotoxin*, and T_3SS) in isolates obtained from diseased cultured carps in Khuzestan province, Iran.

Materials and Methods

Samples collection. A total number of 200 moribund cultured carps with septicemic symptoms including: hemorrhage, ascites, exophthalmos, lethargy, visceral adhesions, and liver discoloration were collected from 30 carp culture farms in Khuzestan province, Iran. Then samples were taken from internal organs including head kidney, liver, spleen, and intestine and also skin lesions. They were streaked on tryptic soy agar (TSA; Merck, Darmstadt, Germany) and incubated at 37.00 ˚C for 24 hr. Following incubation, typical colonies (entire circular, convex, white to greyish, semi-translucent with size of 2.00 to 3.00 mm) were selected from each plate with a pure culture and streaked onto new TSA plates.[16,17]

Phenotypic identification. Gram-negative isolates positive for oxidase and catalase were considered to be suspected for *A. hydrophila*. Then isolates were classified as *A. hydrophila* according to their reactions in the following conventional tests: Motility, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase, indole, Voges-Proskauer, citrate (Simmon's), starch, urea, aesculin, arabinose, cellobiose, sucrose, and salicin. Each substrate was incubated at 37.00 ˚C and reactions were read after 24 and 48 hr.[16-18]

Bacterial DNA extraction for PCR. The genomic DNA was extracted by boiling method. In this regard, each isolate was grown at 37.00 ˚C overnight on tryptic soy broth (TSB) medium. Then they were centrifuged (5,000 *g* for 10 min). The pellets were re-suspended in 1.00 mL phosphate buffer saline (PBS; pH 7.50) in tubes followed by centrifugation (5,000 *g* for 10 min). The pellets were re-suspended in 250 µL of diethyl pyrocarbonate (DEPC) water (Sigma, St. Louis, USA), boiled for 10 min and then centrifuged (5,000 *g* for 10 min). Finally, 200 µL of the cell lysates were stored at –20.00 ˚C until the PCR analysis.[16,19]

The identification of *A. hydrophila* and its virulence genes with PCR. Polymerase chain-reaction was performed with the primers targeted to 16S rRNA and lipase genes in *A. hydrophila*.[20,21] A final volume of 25.00 µL of PCR mixture containing 2.00 µL of template DNA and 12.50 µL of a master mix (containing each deoxy-nucleoside triphosphate, $MgCl_2$ and *Taq* DNA polymerase), 1.00 µL of a 10.00 pM per µL of each primer and 8.50 µL of sterile double distilled water was used for assay with a Thermal Cycler (Corbett Life Science, Sydney, Australia). Each reaction was considered as negative (for negative controls, DNA preparation was replaced by PCR water) and positive controls to confirm the accuracy of the reaction. Then, the PCR products were analyzed by electrophoresis on 1.50% agarose gel with safe stain and visualized by UV trans-illumination. If the 685 bp band was observed for genes 16S rRNA and 763 bp for the lipase gene, the isolate was considered as *A. hydrophila*. In addition, five virulence genes including *hemolysin* (*hlyA*), *aerolysin* (*aerA*), *cytolytic (act) enterotoxin*, *aopB* and *ascV*) in *A. hydrophila* were amplified by PCR with the same procedures with some modifications for providing the optimal condition (Table 1).

Table 1. Cycling conditions in PCR used for each primer of genes.

Genes	Cycles	Temperature-Time	Cycle No.
16S rRNA	Initial denaturation	94.00 ˚C - 5 min	1
	Denaturation	94.00 ˚C - 30 sec	30
	Annealing	55.00 ˚C - 30 sec	
	Extension	72.00 ˚C - 45 sec	
	Final extension	72.00 ˚C - 5 min	1
Lipase	Initial denaturation	94.00 ˚C - 5 min	1
	Denaturation	94.00 ˚C - 30 sec	30
	Annealing	65.00 ˚C - 30 sec	
	Extension	72.00 ˚C - 45 sec	
	Final extension	72.00 ˚C - 5 min	1
Hemolysin (hlyA)	Initial denaturation	94.00 ˚C - 5 min	1
	Denaturation	94.00 ˚C - 30 sec	30
	Annealing	68.00 ˚C - 30 sec	
	Extension	72.00 ˚C - 2 min	
	Final extension	72.00 ˚C - 5 min	1
Aerolysin (aerA)	Initial denaturation	94.00 ˚C - 5 min	1
	Denaturation	94.00 ˚C - 30 sec	30
	Annealing	55.00 ˚C - 30 sec	
	Extension	72.00 ˚C - 30 sec	
	Final extension	72.00 ˚C - 5 min	1
Cytolytic enterotoxin (act)	Initial denaturation	94.00 ˚C - 5 min	1
	Denaturation	94.00 ˚C - 30 sec	30
	Annealing	58.00 ˚C - 30 sec	
	Extension	72.00 ˚C - 30 sec	
	Final extension	72.00 ˚C -5 min	1
AscV - AopB	Initial denaturation	95.00 ˚C - 5 min	1
	Denaturation	95.00 ˚C - 1 min	35
	Annealing	58.50 ˚C - 50 sec	
	Extension	72.00 ˚C - 1 min	
	Final extension	72.00 ˚C - 5 min	1

The primers were designed according to the sequences deposited in GenBank database and references (Table 2).[19,22-24] The size of the expected PCR products for virulence genes in the 1.50% agarose gel were 597, 252, 482, 137 and 129 bp for *hlyA*, *aerA*, *act*, *AscV* and *AopB* genes, respectively.

Results

Isolation and Identification of *A. hydrophila* by biochemical and PCR. Out of 200 samples collected form fish with septicemic signs, 125 isolates were belonged to the motile Aeromonads of which 59 isolates were identified as *A. hydrophila* by biochemical methods (Table 3). Out of 59 isolates, 31 isolates were identified as *A. hydrophila* by PCR analysis (Fig. 1).

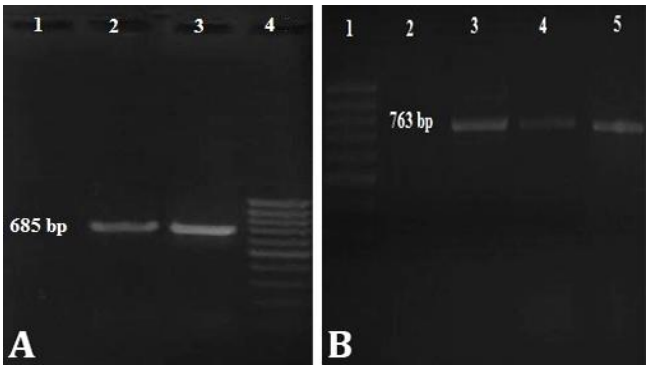

Fig. 1. Detection of 16S rRNA **(A)** and lipase **(B)** genes in on 1.50% agarose gel. **A)** Lanes 1: Control negative, 2: PCR amplification of 16S rRNA gene (685 bp), 3: Control positive; 4: Ladder (100 bp); **B)** Lanes 1: Ladder (100 bp), 2: Control negative, 3 and 4: PCR amplification of lipase gene (763 bp), 5: Control positive.

Table 2. Primers used for the amplification of virulent genes of *Aeromonas hydrophila*.

Virulent genes	Primers	DNA sequences (5'-3')	Product size (bp)	References
Hemolysin(hlyA)	F	GGC CGG TGG CCC GAA GAT GCA GG	597	23
	R	GGC GGC GCC GGA CGA GAC GGG		
Aerolysin (aerA)	F	GCA GAA CCC ATC TAT CCA G	252	20
	R	TTT CTC CGG TAA CAG GAT TG		
Cytolytic enterotoxin (act)	F	ATG ACC CAG TCC TGG CAC GG	482	24
	R	GCC GCT CAG GGC GAA GCC GC		
AscV	F	GCGAGAATGTTGTTGCCGTT	137	25
	R	AACATGCGTGCGATTCTGGA		
AopB	F	TCCAGCAAGTTCGCCTGTTT	129	25
	R	CGCCATGAAAGCCTCAAAT		

Table 3. Biochemical identification of *Aeromonas hydrophila.* Data are presented as percentage.

Biochemical tests	Buller[18]	Characteristics of isolates identified as *A. hydrophila* by both PCR and biochemical tests	
		Positive	Negative
Gram stain	-	-	100
Motility	+	100	-
Oxidase	+	100	-
Catalase	+	100	-
Indole	+	100	-
H_2S	-	-	100
Arginine decarboxylase	+	100	-
Lysine decarboxylase	+	80.64	19.36
Ornithine decarboxylase	-	87.09	12.90
Urea	-	-	100
Simmon's citrate	+(60.00%)	83.87	16.13
Voges-Proskauer	+	64.51	35.49
Nitrate production	+	100	-
Aesculin	+	93.55	6.45
Acid from		-	-
Arabinose	variable	48.39	51.61
Mannitol	+	100	-
Sucrose	+	93.55	6.45
Sorbitol	-	83.87	16.13
Inositol	-	19.36	80.64
Lactose	-	12.90	87.10
NaCl (0-3)	+	100	-

Presence of virulence factors in genomic DNA of *A. hydrophila* isolates. PCR assay showed that 18 (58.60%), 16 (51.61%), 23(74.19%), 13 (41.63%), 10 (32.25%) of the 31 isolated strains were positive for *hlyA*, *aerA*, *act*, *ascV* and *aopB,* respectively (Fig. 2). Based on the detection of virulence genes two dominant genotypes in *A. hydrophila* isolates were found. The *hlyA*$^+$, *aerA*$^+$ *and act*$^+$ genotypes were the most common genotype in 13 (41.93%) isolated strains. In addition, *hlyA*$^+$, *aerA*$^+$, *act*$^+$, *aopB*$^+$ and *ascV*$^+$ genotype was found in 5 (16.12%) of all of isolated strains (Table 4).

Table 4. Virulence properties of *Aeromonas hydrophila* isolates.

Isolates No.	*hlyA*	*aerA*	*act*	*ascV*	*aopB*
1	+	+	+	+	+
2	+	+	+	-	-
3	+	+	+	+	+
4	+	+	+	+	+
5	-	-	+	+	+
6	+	+	+	-	-
7	+	-	+	+	-
8	+	-	-	-	-
9	+	+	+	-	-
10	-	+	+	-	-
11	+	+	+	+	-
12	-	+	-	+	-
13	+	+	+	+	+
14	+	+	+	-	-
15	+	+	+	-	+
16	-	-	+	-	-
17	+	-	+	-	-
18	+	+	+	-	+
19	+	-	+	+	+
20	+	-	-	-	-
21	+	+	+	+	+
22	-	+	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	-	+	-
26	-	-	+	+	+
27	-	-	+	-	-
28	-	-	-	-	-
29	-	-	+	-	-
30	-	-	+	-	-
31	+	+	+	+	-

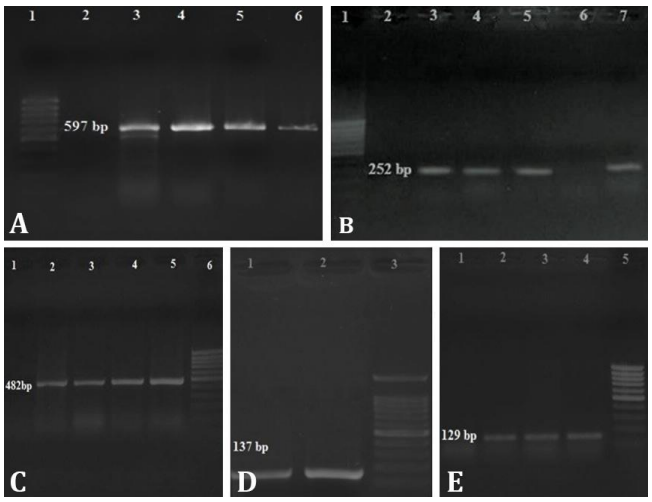

Fig. 2. Detection of **A)** hemolysin, **B)** aerolysin, **C)** act **D)** ascV and **E)** aopB genes in *A. hydrophila* on 1.50% agarose gel. **A)** Lanes 1: Ladder (100 bp), 2: Control negative, 3, 4 and 5: PCR amplifcation of hemolysin gene (597 bp), 6: Control positive; **B)** Lanes 1: Ladder (100 bp), 2: Control negative, 3, 4, 5 and 6: PCR amplifcation of aerolysin gene (252bp), 7: Control positive; **C)** Lanes 1: Control negative, 2, 3 and 4: PCR amplifcation of aerolysin gene (482 bp), 5: Control positive, 6: Ladder (100bp); **D)** Lanes 1: PCR amplifcation of ascV gene (137 bp), 2: Control positive, 3: Ladder (100 bp); **E)** Lanes 1: Control negative, 2 and 3: PCR amplifcation of aopB gene (129 bp), 4: Control positive, 5: Ladder (100 bp).

Discussion

The ability of *A. hydrophila* in causing a wide range of infections suggests a complex pathogenic mechanism that involves proteic toxins such as *hemolysin*, *aerolysin*, *cytotoxin, enterotoxin*, hemagglutinin, surface array proteins and enzymes (*e.g.* protease and elastase).[22,25] There are two hemolytic toxins have been described in *A. hydrophila* including: *hemolysin* (*hlyA)* and *aerolysin* (*aerA*). A virulent strain of *A. hydrophila* produces both hemolysins that their combination results in hemolysis and cytotoxicity.[9]

The activity of these toxins were prohibited when both *hlyA* and *aerA* genes were suppressed.[26] *Aerolysin* is an extracellular, soluble and hydrophilic protein exhibiting both hemolytic and cytolytic properties. *Aerolysin* binds to specific glycoprotein receptors on the surface of eukaryotic cells before inserting into the lipid bilayer and forming holes. Hemolysins are exotoxin proteins produced by bacteria and the lytic activities of hemolysins on red blood cells are reported.[27] Some researchers reported that the screening of virulence determinants was a reliable method to identify a potential pathogenic *Aeromonas* strain by using PCR.[4,8,22,27]

In the present study, the percentage of occurrences of *hlyA* and *aerA* genes were 58.06 and 51.61, respectively using specific primers in *Aeromonas* isolates. Also, in 41.93% of responsible isolates in *Aeromonas* septicemia both genes were observed. In similar studies, different frequency of occurrence from these genes was recorded in *A. hydrophila* isolates.[4,28] The cause of differences could be due to temporal and spatial variations of studied isolates and the used primer.

Yogananth *et al.* showed that 50.00% of the isolated organism had both *hly* and *aer* genes, which to some extent was similar to the present study.[4] Yousr *et al.* have investigated *hly* and *aer* genes in *Aeromonas* isolates and showed 52.63% of isolates were PCR positive for these genes.[28] Ye *et al.* indicated that 85.00% of *A. hydrophila*

isolates from cultured carp with hemorrhagic septicemia were positive for the presence of *hly* and *aer* genes that had higher frequency compared to the findings of our study.[29] Moreover, it has been found that 100% of the isolated *A. hydrophila* from infected tilapia had *hly* and *aer* genes.[30] Uma *et al.* demonstrated the presence of *hly* and *aer* genes in *A. hydrophila* isolates obtained from infected Koi carp and they were presented as virulent strain.[27]

In this study, the frequency of *cytolytic enterotoxin* genes was estimated 74.19%. Twenty-three strains of 31 isolates amplified the sequence with 482 bp, which indicates the presence of *cytolytic enterotoxin* gene. Furthermore, it has been revealed that 35.00% of *A. hydrophila* isolates from cultured carp with hemorrhagic septicemia were *cytolytic enterotoxin* gene positive.[29] The cause of differences could be due to temporal and spatial variations of studied isolates and the used primers.

Type III secretory is an effective system for transportation of toxin into the target cells (effector proteins), and plays an important role in interactions between pathogen and host, and is known as one of the major virulence factors of Gram negative bacteria in plant and animal species.[31] Several studies have shown the importance of this system by inactivation or removing some coding genes in bacterial virulence and pathogenicity. For example, Yu *et al.* showed that inactivation of *aopB* and *aopD* genes of T_3SS in *A. hydrophila* delayed cytotoxicity on carp epithelial cells and reduced its virulence in Gourami fish.[32] Also, the presence of T_3SS in wild species of the *Pseudomonas aeruginosa* compared to a mutant of this bacteria (the secretory system was disabled) was shown to be more virulent and caused higher mortality in infected animals.[33,34] The results of these research demonstrated the importance and role of the T_3SS in the pathogenesis of many Gram-negative bacteria, especially *Aeromonas* sp. Based on the results of the present study five strains were shown to have five dominant genes, $hlyA^+$ $aerA^+$ act^+ $aopB^+$ $ascV^+$ genotypes and 13 isolates had $hlyA^+$ $aerA^+$ act^+ genotypes. The results showed that nine isolates had both genes of T_3SS. Finally, it was shown that the most frequent genotypes were $hlyA^+$ and act^+.

Aslani *et al.* worked on human diarrheal cases and reported that most genotypes among isolates were belonged to $hlyA^+$ $aerA^+$ genotype with an abundance of 78.6 percent.[22] The difference between the results of previous studies with current study might be due to variation in the origin of the isolates of *A. hydrophila.*

Finally, traditional methods for the detection of the virulence properties in *Aeromonas sp.* are based on biological assays *in vitro* and *in vivo*, using cell lines and animal models, respectively.[7] However, these only reveal the phenotypic characteristics of the strains, while the expression of the putative virulence-associated factors in *Aeromonas* appears to be affected by environmental conditions.[35]

For this reason, these methods could in some instances fail to indicate the potential pathogenicity of isolates. Screening of specific cytotoxin and *hemolysin* genes appears to be the most effective way of detecting and characterizing *Aeromonas* virulence factors.[18]

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Conflict of interest

The authors declare there is no conflict of interests.

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