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Original Article

Virulence Factors and Molecular cloning of Outer Membrane Protein (OMP) gene from virulent *Aeromonas hyrophila* isolated from infected gold fish *Carassius auratus*

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Aeromonas hyrophila **strains AHV1, AHV2 and AH3 were isolated and identified from Muscle tissue, intestine, body fluid and gills of infected gold fish** ***Carassius auratus.*** **In order to study their virulence, LD_{50} tests against normal gold fish, proteolytic, haemolytic and challenge studies were performed. The virulence studies revealed that, both AHV1 and AHV2 strains are highly positive for proteolytic and haemolytic properties. The LD_{50} data showed that, the fish** ***C. auratus*** **are highly susceptible to** ***A. hyrophila*** **strains AHV1 and AHV2 at cent percent lethal rate. The survival of** ***C. auratus*** **significantly ($P<0.05$) decreased when challenged with virulent strains of** ***A. hydrophila*** **AHV1 and AHV2. The outer membrane protein (OMP-TS) gene was successfully amplified generating an the amplicon size of 1008 bp. The amplified product from the genomic DNA of AHV1 strain was cloned in to pTZ57R/T vector, transformed into $DH_5\alpha$ cells and sequenced. The sequenced clone is resembling to various** ***A. hydrophila*** **isolates and submitted to NCBI GenBank database (accession no: HQ331525).**

Keywords: ***Aeromonas hyrophila,*** **Outer Membrane Protein,** ***Carassius auratus,*** **Cloning**

Introduction

Globally the ornamental fish culture is a powerful income and employment generating industry. In the aquaculture sector, ornamental fish breeding, culture and trade provide excellent opportunities as a non–food fishery activity for employment and income generation. It is environment friendly, socially acceptable and involves low investment for adopting as a small scale enterprise with high return. The attractive coloration and quiet disposition of ornamental fish provide a source of joy and peace for people irrespective of age group[1].

Goldfish are one of the most popular pets in the world. They are, undoubtedly, characterized by brilliant metallic gold and reddish coloration. The Goldfish, *C. auratus* contain the golden colour pigment due to the presence of erythrophore. It is highly adaptable species gaining important commercial value in the export trade. This fish has high susceptibility to aeromonads and are commonly valuable for experimental animals[2].

In Asian countries fish culture continues to be destroyed by bacterial diseases such as Motile Aeromonads Septicaemia (MAS), furunculosis and edwardsiellosis. Among these, MAS caused by *A. hydrophila* is most widespread in freshwater fishes[3]. *A. hydrophila* is a ubiquitous, opportunistic free-living Gram negative bacterium prevalent in crowded aquatic habitats[4]. MAS infect a number of species producing stress related diseases with the common symptoms of ulcerations, exophthalmia and abdominal distension[5,6]. Recently, aquaculture is facing lots of problems due to aeromonads outbreaks. Potential virulence factors of *A. hydrophila*, which contribute to their pathogenicity, include the production of endotoxins, extra cellular enterotoxins, hemolysin, cytotoxins and protease, the ability to adhere the cells, and the possession of certain surface proteins[7].

Outer membrane proteins (OMPs) from *Aeromonas* spp. have been identified as suitable targets for vaccine development in fish[8,9]. Bacterial OMPs play a significant role in virulence as they comprise the outermost surface in contact with host cells and immune defense factors. Recombinant OMPs have been tested as possible vaccine antigens for *A. hydrophila*. Fang *et al.*[10] showed significant protection against two isolates of *A. hydrophila* in blue gourami, *Trichogaster richopterus* immunized with a recombinant 43 kDa OMP. More recently, a recombinant OMP (37 kDa) of *A. hydrophila* was produced and shown to be immunogenic in rohu carp[11]. The present study was undertaken to isolate virulent strain of *A. hydrophila* from the infected gold fish and clone the OMP gene of that strain.

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Materials and Methods

Isolation of A. hydrophila

Infected goldfish were collected from J.J. fish farm, Nagercoil, Tamil Nadu, India and aseptically bagged in sterile polythene bags and transported to Lab. Infected fish samples such as muscle tissue, intestine, body fluid and gills were dissected out and homogenized in 10ml sterile alkaline peptone water. Homogenates were serially diluted up to 10^{-6} in sterile normal saline solution and 100il aliquots of each dilution were plated on *Aeromonas* isolation media using spread plate technique in duplicate. The plates were incubated at 37°C for 16-18hr. Typical green colonies of 2-5mm in diameter were subjected to biochemical tests for identification of *A. hydrophila*. The cultures were stored at -80ºC in 15% (v/v) glycerol for further studies.

Identification of A. hydrophila

A. hydrophila were identified biochemically to species level by using 14 chosen tests and such as motility, Kovac's oxidase, oxidation and fermentation, catalase, indole, methyl red, urease, haemolysin production, sugar fermentation, Voges-Prokauer, nitrate reduction, H_2S production, lysine decarboxylase and arginine dihydrolase.

Virulent studies

LD_{50}

C. auratus having the body weight ranged between 30 to 40g were used for LD_{50} studies. *A. hydrophila* of a concentration ranged between 10^5 to 10^9cfu/ml in PBS (pH 7.2) injected intraperitoneally to 0.1ml/fish using 3 test group for each concentration and the control test group were injected intra peritoneally with 0.1ml of PBS (pH 7.2). Fish groups were observed for a period of 7 days and the lethal dose (LD_{50}) was determined.

Proteolytic and Haemolytic activity

Proteolytic activity of *A. hydrophila* was determined by production of a clear zone of proteolysis around the colonies on skim milk agar plates incubated at 37°C for 24 hr. Haemolytic activity was determined by producing a zone of haemolysis around the colonies on blood agar plates containing 2% (v/v) human blood.

Challange test

In order to study the virulence of the isolated strains, fresh culture of different *A. hydrophila* isolates were diluted into 0.5 % NaCl and injected intraperitoneally to *C. auratus* in a dose of 3×10^7cfu/ml. Each group had 10 fishes in triplicate and a control group is also maintained. The control group was injected with 100µl sterile 0.5 % NaCl without bacteria. The fish were observed up to 7 days and any dead specimens were removed for routine bacteriological examination.

Cloning of OMP gene

Genomic DNA was extracted from 24 hr old cultures of *A. hydrophila* which was grown in Tryptone soya broth. In short, 1ml of culture was taken in an eppndorf tube, spun at 3000rpm and extracted the DNA using miniprep DNA easy kit (Qiagen, Hamburg, Germany) as per the manufacturer's protocol. DNA was quantified by Nanodrop 1000 (Thermoscientific) and samples were stored at -20º C until further use.

Primer designing and PCR amplification

The OMP-gene primer was designed and synthesized (Sigma–Aldrich, Bangalore) following the NCBI database sequence. The sequence details of the primers are OMP-TS F 5' CCCAAGCTTATGGCAGTGGTTTATGACAAA 3'; OMP TS R 5'AACTGCAGTTAGAAGTTGTATTGCAGGGC 3'. The OMP TS gene was amplified from the genomic DNA of *A. hydrophila*. The PCR protocol was, denaturation of DNA at 94º C for 5 mins followed by 33 cycles consisting of 94ºC for 30, 50º C for 1 min and 72º C for 1.5 min. After completion of 33 cycles, an extended time of 10 min at 72º C was maintained. The amplified PCR product had 1008 bp, resolved on a 1% (w/v) low melting point agarose gel in 1X TAE buffer, with a 1kb ladder (fermentas) and visualized with ethidium bromide staining in Gel documentation.

Restriction and digestion

The PCR products were excised from the agarose gels using sterile blade and purified using gel extraction kit (Qiagen). The purified PCR products of OMP TS were double digested with the restriction enzymes *Nde* I and *Xho* I. The digested PCR products were run on an agarose gel, excised and purified using gel extraction kit again.

Ligation and transformation

The purified OMP TS products were ligated to T Easy Vector, pTZ57R/T. The ligation reaction was performed by preparing a 5 ìl of ligation mix containing 0.5 µl of vector and 3.5 µl of purified PCR product. The insert and vector concentration maintained at the ratio of 3:1 respectively and incubated the mix overnight at 4º C. Five micro liter of ligated plasmid was transformed to *E. coli* DH_5 α super competent cells and the positive clones were amplified using the OMP TS Primers.

Plasmid isolation, Sequencing and submission

Recombinant Plasmid (OMP TS-pTZ57R/T) was isolated from the positive DH_5 α transformants using mini prep plasmid isolation kit (Qiagen, Hamburg, Germany) as per the manufacturer's instructions. The recombinant plasmids were sequenced by the primers, M13+ and M13- (Macrogen 3730XL7, South Korea). The Nucleotide sequence of OMP gene was submitted to NCBI GenBank database (accession no: HQ331525).

Results and Discussion

The isolated bacteria from body fluid, intestine, gills, and muscle of infected goldfish were confirmed as *A. hydrophila.* The isolates from body fluid (AHV1), intestine (AHV2) of highly infected fish and muscle of low infected (AH3) fish were found to be motile, Gram negative, citrate, oxidase and catalase positive. The AHV1 and AHV2 showed strong proteolytic and haemolytic activities

(Table 1) *A. hydrophila* has received particular attention because of its association with soft tissue, wound, and blood infections[12]. Boulanger *et al.*[13] reported that *A. hydrophila* could be isolated from cutaneous lesions and kidneys of diseased fish, whereas both *A. hydrophila* and *A. sobria* could be recovered from the intestine of normal fish. Motile aeromonads that have been taken from lesions on diseased fish have been shown to have a greater chemotactic response to skin mucus than isolates that were obtained as free-living organisms from pond water[14]. Nordmann and Poirel[15] reported that *Aeromonas spp*. are Gram-negative, rod shaped, mainly motile, facultative anaerobic, oxidase positive and glucose fermenting bacteria. They have recently been transferred from Vibrionaceae to their own family Aeromonadaceae[16].

The relationship between the distribution of three *A. hydrophila* strains and their pathogenicities against *C. auratus* are shown in the Table 2. The LD_{50} data revealed that, the fish *C. auratus* highly susceptible to AHV1 and AHV2. The AH3 and MTCC strains had less virulence of 45 and 25 % lethal rate respectively due to their low level of extra cellular products expression. Kou[17] found that many of the virulent, avirulent, and attenuated aeromonads that he studied possessed hemorrhagic factors and lethal toxins. The virulent bacteria had quantitatively more toxic potential than did their avirulent or attenuated counterparts. *A. hydrophila* causes infections in food and ornamental fishes, there by posing a threat to the development of the aquaculture enterprise. Several strains of *A. hydrophila* release extracellular toxins for its pathogenicity[18,19]. Chopra *et al.*[20] reported that, the haemolysins released by *Aeromonas* are cytotoxic and cause lysis of erythrocyte and play important roles in pathogenesis. The role of protease enzyme is to provide nutrients by breaking down host proteins into small molecules capable of entering the bacterial cell[21,22]. De Figuerirredo and Plumb[23] have found that environmental and clinical isolates differed in virulence when injected into channel catfish. LD_{50} values from diseased fish were $6.4x10^4$ cfu, compared with $1.5x10^6$ cfu for environmental isolates. In general, virulent isolates of *A. hydrophila* has an LD_{50} values of 10^4 to 10^5 cfu, while strains which do not kill fish at 10^7 cfu are considered non-virulent[24]. The higher lethal rate in *C. auratus* due to the AHV1 and AHV2 was also reflected in the proteolytic and haemolytic activities. These activities were higher in AHV1 followed by AHV2, AH3 and MTCC strains. This may due to the higher amount of extra cellular products secretions (Table 3).

Table 1. *Morphological and biochemical confirmation of A. hydrophila (AHV1, AHV2 and AH3) isolated from infected Gold fish*

S.No			Confirmative Test	*A. hydrophila* isolates	
			AHV1	AHV2	AH3
1	Motility		motile	motile	motile
2	Gram staining		-	-	-
3	Cell shape		rod	rod	rod
4	Indole		+	+	+
5	Methyl red		+	+	+
6	Voges proskauer		+	+	+
7	Citrate		+	+	+
8	Oxidase		+	+	+
9	Catalase		+	+	+
10	Nitrate reduction		+	+	+
11	Lipase		+	+	+
12	Protease		++	++	+
13	Hemolysin		++	++	+
14	Starch hydrolysis		+	+	+
15	Urea	-	-	-	
16	Carbohydr-ate fermentati-on	D-Glucose	+	+	+
		Sucrose	+	+	+
		Lactose	+	+	+
		Maltose	+	+	+
		Galactose	+	+	+

AHV: *A. hydrophila* Virulent; AH: *A. hydrophila*

Table 2. *Relationship between the distribution of three virulent A. hydrophila strains and their pathogenicities to Carassius auratus*

S.No	Test groups	Strains	Lethal rate (%)	Virulence
1	Fish group 1	Control	0	-
2	Fish group 2	AHV1	100	Strong
3	Fish group 3	AHV2	100	Strong
4	Fish group 4	AH3	45	Medium
5	Fish Group 5	MTCC	25	Low

Table 3. *Proteolytic and Haemolytic activity of virulent A. hydrophila isolated from infected gold fish in comparison with MTCC strain*

S. No	Strains	Proteolytic activity	Haemolytic activity
1	AHV1	++++	++++
2	AHV2	+++	++++
3	AH3	++	++
4	MTCC	+	+

The survival of gold fish *C. auratus* against *A. hydrophila* challenge was given in the Fig 1. The fish group survived 95 % after 5th day of post challenge in the blank control had no *A. hydrophila* challenge. The survival was significantly ($P<0.05$) decreased to 70% after 5th day of post challenge in the MTTC challenged groups. The groups that, succumbed to death 100 % mortality (0% survival) in the AHV1 and AHV2 challenged fishes after 4th day of post challenge. The AH3 challenged group had only 40 % survival due to less virulence. The preparation of ECP of different *A. hydrophila* strains were injected to the fingerlings of hybrid catfish[25]. The ECP of highly virulent strain resulted in 100% mortality within 18 hours while the low virulent strains showed 100% mortality within 96 hours.

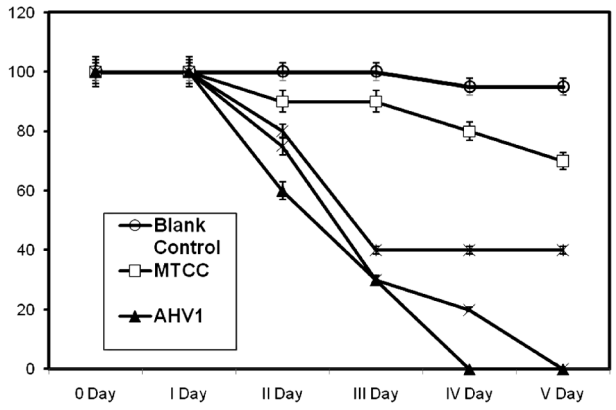

Figure 1. *Percentage survival of C. auratus after injecting A. hydrophila at different intervals*

The OMP gene was successfully amplified from the genomic DNA of virulent *A. hydrophila* AHV1 strain, having a size of 1008 base pairs (Fig 2). Further, the amplified gene was successfully cloned to pTZ57R/T - vector (Fig 3) and sequenced (Fig 4). The sequence was submitted to NCBI database (Accession number HQ331525) and this sequence resemble to various *A. hydrophila* isolates. In gram negative pathogenic

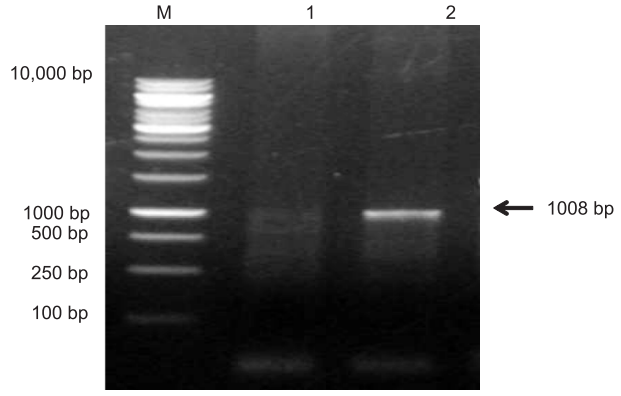

Figure 2. *PCR amplification of OMP gene from Genomic DNA of virulent A. hydrophila AHV1, Lanes 1: Marker; 2. Negative control and 3: AHV1*

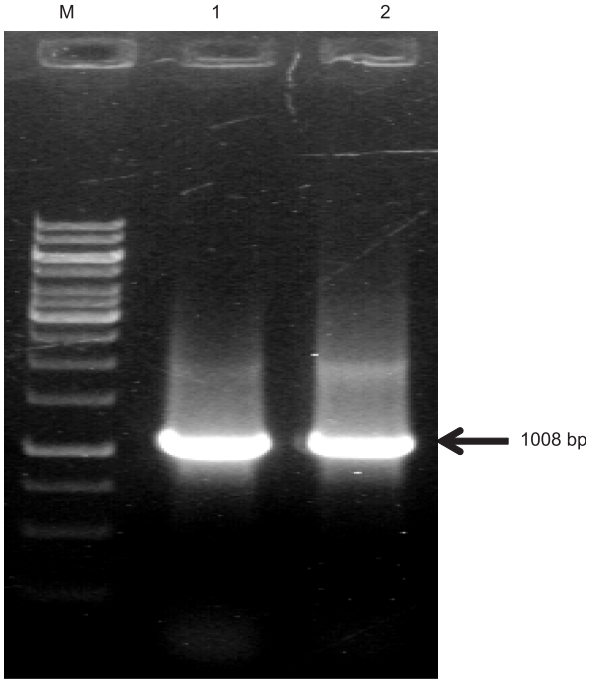

Figure 3. *PCR amplification of OMP gene from recombinant plasmid (OMP- pTZ57R/T) Lanes 1: Marker; 2&3: OMP –AHV1 positive clones*

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1   CNNNANNNGTCCGGTCTCTCTATTGANTTGCTTTGATGTT 40
41  GGCCCAACNCCCCTTCCTTTTATATCGCAACAGCTCTATA 80
81  CCCCCCAGATTGAATGCCAGCGGTTCGGCATCGCCTCGAC 120
121 AAACATGTGTGCATGGGCATGGATCTGCCGATGATGCTGG 160
161 ACAACGCTTTCGGTCCTCCACTGCTGCAGAAGAGCGACCC 200
201 GTGCCCGGAGACGGAGAAAACCGCTTTTGGCTTTGTCATT 240
241 CTGGCGCTGCCGGTGTTCCTGATGGAGCGTATCATCGGTG 280
281 ACGTCTGGGGTATGTGCCTGTGGGCAATGCTCGGGGTAGC 320
321 GTTCTTTACCTGGGCGTTTATCGTCAGCCTGGGGGCGAAA 360
361 AAAGCGTGGATGCGTCTGGTATCCTCCTGCTGCTGGCTGC 400
401 CGCGCTGGTAAGCGTGCGTCCTCTTCAGGACTGGGCGTTC 440
441 GGCTCGCCGGTCGGCCAGACGCAGGCGCATTTGAACTTCA 480
481 TCCAGATTAAGATCGTTGATGAGCTCAACAGCGCGCTCTT 520
521 ACAGGCAAAAGGCAAACCGGTGATGCTCGATCTCTATGCC 560
561 GACTGGTGCGTCGCCTGCAAAGAGTTTGAAAAATACACCT 600
601 TTTAGCGATCCACANNCACAAAATGCCCTGAAAGAGACTG 640
641 TTCTGCTCCAGGCAAACGTGACACCCAACAAGCACACGAT 680
681 ACGCTCTGCTGAAGCAGCTTAACGTCCTCAGACTGCAACC 720
721 ATTCTTTTTCTTCAATCAACAGGGCCAGTAGCAGCCAGAA 760
761 AAGCGTGTAACCGTCGTTTATAGGATGCTAGCGGCATTAG 800
801 CGCGCATTTGCGCAATCCGCCAGACGTAAACTACACTTTA 840
841 ACGGGACAAACCCGTGGGTATTAGCGAACGACATAACCGT 880
881 GCAAGCTGAAGACGTACTGTCACAAGCCCCTCCAATCACC 920
921 TCTCTACTGCAGTTTATCGGATCCGGGNCC          950
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Figure 4. *Sequence analysis of A. hydrophila OMP gene (HQ331525) from AHV1 after cloning*

bacteria, the outer membrane proteins play an important role in infection and pathogenicity to the host[26]. The outer membrane proteins of the warm water fish pathogen, *Aeromonas hydrophila* have a role in the virulence of the organism and are potential candidates for vaccine development[27]. In the present study, the 1008 bp outer membrane protein, which had been shown to be an OMP TS in virulent AHV1 strain, was cloned. Fang *et al.*[10] found significant protection against two isolates of *A. hydrophila* in blue gourami, *Trichogaster trichopterus* (75 and 87.5 % RPS) immunized with a recombinant 43kDa OMP, while a recombinant 37 kDa OMP of *A. hydrophila* has been shown to be immunogenic in rohu carp[11]. Ebanks *et al.*[28] recorded 10 outer membrane proteins in the same region which included homologues of OMPC, OMPAI/AII, OMPK, OMPTolC, OMPD and other OMPs involved in nutrient acquisition in *A. salmonicida* by proteomics. The major drawbacks in ornamental goldfish industry are lack of availability of precise disease diagnostic tools and vaccines against deadly haemorrhagic ulceration. The present work we conclude that the PCR amplification of OMP gene as a diagnostic tool in infected/ non-infected ornamental fishes. Also the OMP clone will be used for developing vaccines against ornamental/ fresh water fish species against A. *hydrophila* infection.

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