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Cloning and Sequence Analysis of *aha1* Gene Encoding Major Adhesin Protein from *Aeromonas* sp. Isolated from Aquaculture Animals with Haemorrhagic Septicemia

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Abstract: Nine bacterial strains (LA1, LA2, LA4, JA1, CA3, CA4, BA1, BA18 and EA9) were isolated from different aquaculture animals with haemorrhagic septicemia and then they were identified as *A. hydrophila* (LA4, JA1, CA3 and BA1), *A. sobria* (LA1 and EA9), *A. caviae* (BA18) and *A. veronii* (CA4 and LA2), respectively by morphological and biochemical characterization. All isolates were found to be pathogenic to experimental zebrafish (*Danio rerio*) by artificial infection test. The outer membrane protein Aha1 is a major adhesin of *A. hydrophila* and also highly conserved in different serotypes of *A. hydrophila*. In order to ascertain the conservation of aha1 protein among mesophilic motile aeromonads, full length *aha1* gene from all isolates was detected, cloned and sequenced. As the results show, the *aha1* genes were amplified in all strains and the ORF size of the *aha1* gene from *A. hydrophila* and other phenotypic species of aeromonas isolates was 1,068 and 1,038 bp, respectively. Four Anhui *A. hydrophila* isolates and six *A. hydrophila* reference strains formed a cluster together with 91.4-99.7% nucleotide identity and 91.9-99.7% amino acid identity of the *aha1* gene. Five Anhui other phenotypic species isolates formed another cluster, they shared 79.5-81.1% nucleotide identity and 79.6-81.6% amino acid identity of the *aha1* gene compared with *A. hydrophila* and the major sequence variations were observed between amino acids 85-134, 176- 227, 243-263, 280-295 and 321-336.

Key words: *Aeromonas* sp., identification, *aha1* gene, cloning, sequence analysis

INTRODUCTION

Aeromonas sp. are Gram-negative, oxidase-positive, rod-shaped bacteria of the family Aeromonadaceae (Janda and Abbott, 2010). According to growth temperature and motile, aeromonads could be broken down into mesophilic motile group and psychrophilic non-motile group. Among mesophilic motile group, *A. hydrophila*, *A. sobria*, *A. caviae* and *A.veronii* have been recognized as important pathogens. They can infect a variety of aquatic animals and results in great economic losses to aquaculture (Liao and Zhong, 2009). Therefore, finding a conserved and protective antigen among mesophilic motile aeromonads for vaccine development is very important for the prevention of bacterial disease in aquaculture animals.

The adherence of pathogen on the host cell surface is usually mediated by adhesins which plays an essential role in causing infection. In earlier study, an outer membrane protein designated Aha1 has been confirmed to be a major adhesin of *A. hydrophila* which is also a highly conserved antigen in different serotypes of *A. hydrophila* and could confer immune protection to fish against experimental *A. hydrophila* challenge (Fang *et al*., 2004; Khushiramani *et al*., 2008; Pan *et al*., 2010). However, it is not clear whether Aha1 major adhesion protein is conserved among other phenotypic species of mesophilic motile aeromonads. In this study, 9 different phenotypic species of mesophilic motile aeromonads were isolated and identified from different aquaculture animals suffering from haemorrhagic septicemia in Anhui province of China and the gene encoding Aha1 protein designated *aha1* from Anhui isolates was also cloned, sequenced and characterized which might be potentially useful in vaccine development of mesophilic motile aeromonads.

MATERIALS AND METHODS

Bacterial strains, plasmid and experimental fish: *E. coli* DH5α strain, *A. hydrophila* ATCC7966 strain and pMD18-T vector were purchased from China Institute of Veterinary Drug Control. *E. coli* DH5α grew in the Luria-Bertani medium (LB medium, Oxoid, UK) supplemented with 100 μg mL^{-1} ampicillin when needed, and *A. hydrophila* ATCC7966 grew in brain heart infusion broth (BHIB, Oxoid, UK). About 80 days old AB line inbred zebrafish (*Danio rerio*) were provided by Fisheries Research Institute of Chinese Academy of Fishery.

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Isolation and idetification of bacteria: Bacteria were isolated from the liver samples of fish, *Trionyx sinensis* and *Eriocheir sinensis* with haemorrhagic septicemia in Anhui province of China. The samples were streaked on BHI agar plates and incubated at 28°C for 24 h. Suspected colonies were restreaked on fresh media to obtain pure cultures which were then identified with the Gram staining test for microscopic analysis and with the aid of the API-20NE System (BioMerieux, France) for biochemical analysis. The accuracy of API-20NE System was good for *A. hydrophila* and *A. veronii* but not for *A. caviae*. So, additional tests like Voges-Proskauer reaction and lysine decarboxylase production are necessary for confirmation of *Aeromonas* species identified by the commercial systems.

Experimental infection: Zebrafish (*Danio rerio*) is a good infection model for studying pathogens. About 150 healthy 80 days old AB line inbred zebrafish which were divided into ten groups on average were used for evaluation of pathogenicity of nine isolates in present study. Experimental fish were challenged with 30 μL of the 9 isolates suspension by intraperitoneal injection, respectively. The final dose of injections was approximately 10^8 cfu mL^{-1}. The control fish were injected with 30 μL of sterile saline. After injection, these fish were observed for 2 weeks and were maintained at 25°C with aeration during the course of the experiment. Dead fish were sampled from the livers for isolation of bacteria.

PCR amplification, cloning and sequencing of the *aha1* gene: According to the *aha1* gene sequence of *A. hydrophila* ATCC7966 (accession number CP000462), a pair of primers was designed to amplify the full length *aha1* gene. The primer sequences are as follows: 5'-CTA TGAAAAAGACAATTCTGGCT-3' and 5'-TTAGAAGT TGTATTGCAGGGC-3'. PCR was performed in 25 μL reaction volume containing 10×PCR buffer 2.5 μL, 10 mmol L^{-1} dNTPs 0.5 μL, 25 mmol L^{-1} $MgCl_2$ 1.5 μL, 25 mmol L^{-1} each primer 0.5 μL, 1 μL DNA template of nine Anhui isolates, 5 U μL^{-1} Taq enzyme 0.3 μL, ddH_2O 18.2 μL. Amplification conditions were as follows: initial denaturation at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR amplification products were checked on 0.8% agarose gels and purified using Gel extraction kit (Takara). Purified PCR products were cloned into the pMD18-T vector and transformed into competent *E. coli* DH5α. Positive clones were sent to Shanghai Sangon Bioloical Engineering Technology and Services Co., Ltd. for sequencing.

Table 1: Information on all sequences of the *aha1* genes from *Aeromonas* sp. used in this study

Strain No.	Species	Isolated place	Accession No.
ATCC7966	*A. hydrophila*	USA	CP000462
PPD134/91	*A. hydrophila*	Singapore	AY165026
961004	*A. hydrophila*	Guangdong, China	AF276639
BA17	*A. hydrophila*	Anhui, China	DQ302124
BSK-10	*A. hydrophila*	Zhejiang, China	EU518465
WC10-1	*A. hydrophila*	Fujian, China	EF189590
LA4	*A. hydrophila*	Anhui, China	JQ946885
JA1	*A. hydrophila*	Anhui, China	JQ946884
CA3	*A. hydrophila*	Anhui, China	JQ946883
BA1	*A. hydrophila*	Anhui, China	JQ946882
LA1	*A. sobria*	Anhui, China	JQ946887
EA9	*A. sobria*	Anhui, China	JQ946886
BA18	*A. caviae*	Anhui, China	JQ946880
CA4	*A. veronii*	Anhui, China	JQ946881
LA2	*A. veronii*	Anhui, China	JQ946888

Sequence and phylogenetic tree analysis: To analyze the *aha1* gene sequences of Anhui isolates, six *A. hydrophila* reference strains from GenBank were included in the study (Table 1). The phylogenetic Neighbor-Joining (NJ) tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) Version 4.1 by neighbor-joining strategy. Alignment was performed using CLUSTAL W Method in MEGA Version 4.1.

RESULTS AND DISCUSSION

Characterization of the *Aeromonas* sp. isolates: Nine strains were isolated from the liver samples of fish, *Trionyx sinensis* and *Eriocheir sinensis* with hemorrhagic septicemia and were provisionally numbered LA1, LA2, LA4, JA1, CA3, CA4, BA1, BA18 and EA9, respectively. They were all non-sporing, gram-negative rods and produced colonies in diameter 1.5-2 mm, convex, moist, translucent and colorless with a shiny surface and entire edge on BHI agar plates. The biochemical characteristics of the nine isolates were shown in Table 2. According to the result of API 20NE and supplementary test, strains LA4, JA1,CA3 and BA1 were identified as *A. hydrophila*, strains LA1 and EA9 were identified as *A. sobria*, strain BA18 was identified as *A. caviae*, strains CA4 and LA2 were identified as *A. veronii*.

Pathogenicity of Anhui isolates of *Aeromonas* sp: All Anhui isolates were found to be pathogenic to the experimental zebrafish. These pathogens started to cause lesions in the experimental fish at the 18 h post-injection and the mortality rate was 100% after 36 h. No mortality was observed in the control group. The bacteria inoculated could be reisolated from the dead fish.

PCR amplifications of the *aha1* gene: Using specific primers, approximately 1068 bp DNA strip of *aha1* gene was successfully amplified in all Anhui isolates (Fig. 1).

Table 2: The biochemical properties of the Anhui isolates

Characteristics	Strain LA4, JA1, CA3, BA1	LA1, EA9	BA18	CA4, LA2
API 20E system				
Nitrate reduction to nitrite	+	+	+	+
Indole	+	+	+	+
Glucose acid	+	+	+	+
Arginine dihydrolase	+	+	+	+
Urea	-	+	-	+
Esculin	+	-	+	-
Gelatin	+	+	+	+
Nitrobenzene-A-D-pyran galactoside	+	+	+	-
Glucose assimilation	+	+	+	+
L-Arabinose	+	-	+	-
Mannose	+	+	+	+
Mannitol	+	+	+	+
N acetyl glucosamine	+	+	+	+
Malt sugar	+	+	+	+
Gluconic acid salt	+	+	+	+
Capric acid	+	+	+	+
Adipic acid	-	-	-	-
Malic acid	+	+	+	+
Citric acid	-	+	-	-
Phenyl acetic acid	-	-	-	-
Oxidase	+	+	+	+
Supplementary test				
Voges-Proskaue	+	-	-	+
Lysine decarboxylase	+	+	-	+
	A. hydrophila	*A. sobria*	*A. caviae*	*A. veronii*

+: Positive; -: Negative

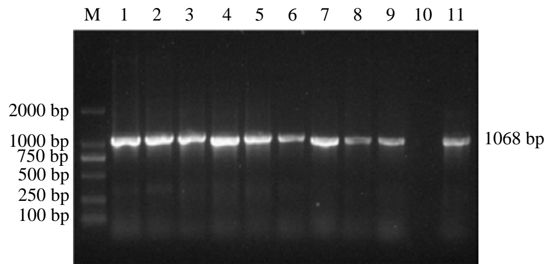

Fig. 1: PCR amplification of the *aha1* gene from Anhui isolates. Lane M: DNA Molecular weight standard. Lane 1: JA1 product ; Lane 2: BA3 product; Lane 3: BA18 product; Lane 4: CA3 product; Lane 5: CA4 product; Lane 6: LA1 product; Lane 7: LA2 product; Lane 8: LA4 product; Lane 9: EA9 product; Lane 10: Negative control; Lane 11: ATCC7966 product

Homology analysis of nucleotide sequences of the *aha1* gene: After PCR-based cloning, the complete sequences of the *aha1* genes from Anhui isolates were identified and submitted to GenBank under accession numbers JQ946880 to JQ946888. The ORF size of the *aha1* gene from Anhui *A. hydrophila* isolates (LA4, JA1, CA3 and BA1) and other phenotypic species isolates (LA1, EA9, BA18, CA4 and LA2) were 1,068 and 1,038 bp, respectively. The nucleotide identity of the *aha1* gene between the Anhui *A. hydrophila* isolates and *A. hydrophila* reference strains from GenBank varied 91.4-99.7%. The *aha1* nucleotide sequences from five other phenotypic species isolates are 94.2-99.3% identical and they shared 79.5-81.1% identity to the *aha1* gene sequences from *A. hydrophila* (Table 3).

Comparison of deduced amino acid sequence from the *aha1* gene: As well, the deduced Aha1 amino acid sequences from reference and isolated strains of *A. hydrophila* showed 91.9-99.7% identity. The amino acid sequences of Aha1 protein from five other phenotypic species isolates are 97.1-100% identical and they shared 79.6-81.6% amino acid identity compared with *A. hydrophila* (Table 3). Five other phenotypic species isolates had 5, 6 and 2 amino acids deletion between residues 47-51, 74-79 and 285-286 respectively and 3 amino acids insertion between residues 118-120 and the major sequence variations were observed between amino acids 85-134, 176-227, 243-263, 280-295 and 321-336 compared with the *A. hydrophila* (Fig. 2).

Phylogenetic analysis of the *aha1* gene: Phylogenetic analysis suggested that four Anhui *A. hydrophila* isolates formed a cluster together with six *A. hydrophila* reference strains. Five Anhui other phenotypic species isolates formed another cluster, they showed similar evolutionary distances each other (Fig. 3).

In this study, 9 isolates of *Aeromonas* sp. comprising *A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii* were isolated from moribund fish, *Trionyx sinensis* and *Eriocheir sinensis* with hemorrhagic septicemia and were identified by morphological and biochemical characterization in Anhui province of China. Zebrafish were used as experiment animals to evaluate pathogenicity of the *Aeromonas* sp. isolates. After experimentally infected with the isolates via intraperitoneal inoculation, zebrafish died of hemorrhagic septicemia, similar to naturally infected aquatic animals. The results indicated that *A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii* are all the causative agent of hemorrhagic septicemia in aquatic animals which is consistent with previous reports (Zhang *et al*., 2000; Nielsen *et al*., 2001; Fang *et al*., 2008; Zhao *et al*., 2011; Chuang *et al*., 2011; Majtan *et al*., 2012).

Hemorrhagic septicemia in aquatic animals caused by *Aeromonas* sp. is one of the most significant economic concerns in the aquaculture industry. Therefore, it is important to search for a conserved protective antigen among mesophilic motile aeromonads and to develop a

Table 3: Nucleotide and amino acid identity of the *Aha1* genes among the Anhui isolates and *A. hydrophila* reference strains

Aeromonas sp. strains	Amino acid identity (%) LA4	BA1	JA1	CA3	LA1	EA9	CA4	LA2	BA18	BA17	ATCC7966	PPD134/91	961004	BSK-10	WC10-1
LA4		99	100	99	81	81	81	81	81	99	99	94	99	92	93
BA1	99		99	100	82	82	81	81	81	100	100	94	99	92	94
JA1	100	99		99	81	81	81	81	81	99	99	94	99	92	93
CA3	99	100	99		82	82	81	81	81	100	100	94	99	92	94
LA1	81	81	81	81		100	100	99	97	81	81	81	82	80	81
EA9	81	81	81	81	99		100	99	97	81	81	81	82	80	81
CA4	81	81	81	81	99	99		99	97	81	81	81	81	80	80
LA2	81	81	81	81	99	99	99		98	81	81	81	81	80	81
BA18	81	81	81	81	95	95	94	95		81	81	81	81	80	80
BA17	99	100	99	100	81	81	81	81	81		100	94	99	92	93
ATCC7966	99	100	99	100	81	81	81	81	81	100		94	99	92	93
PPD134/91	93	92	92	92	81	81	81	81	80	92	92		94	93	98
961004	99	99	99	99	81	81	81	81	81	99	99	93		92	93
BSK-10	92	91	92	91	81	81	81	81	81	91	91	92	92		91
WC10-1	92	92	92	92	80	80	80	80	80	92	92	95	92	90	

Nucleotide identity (%)

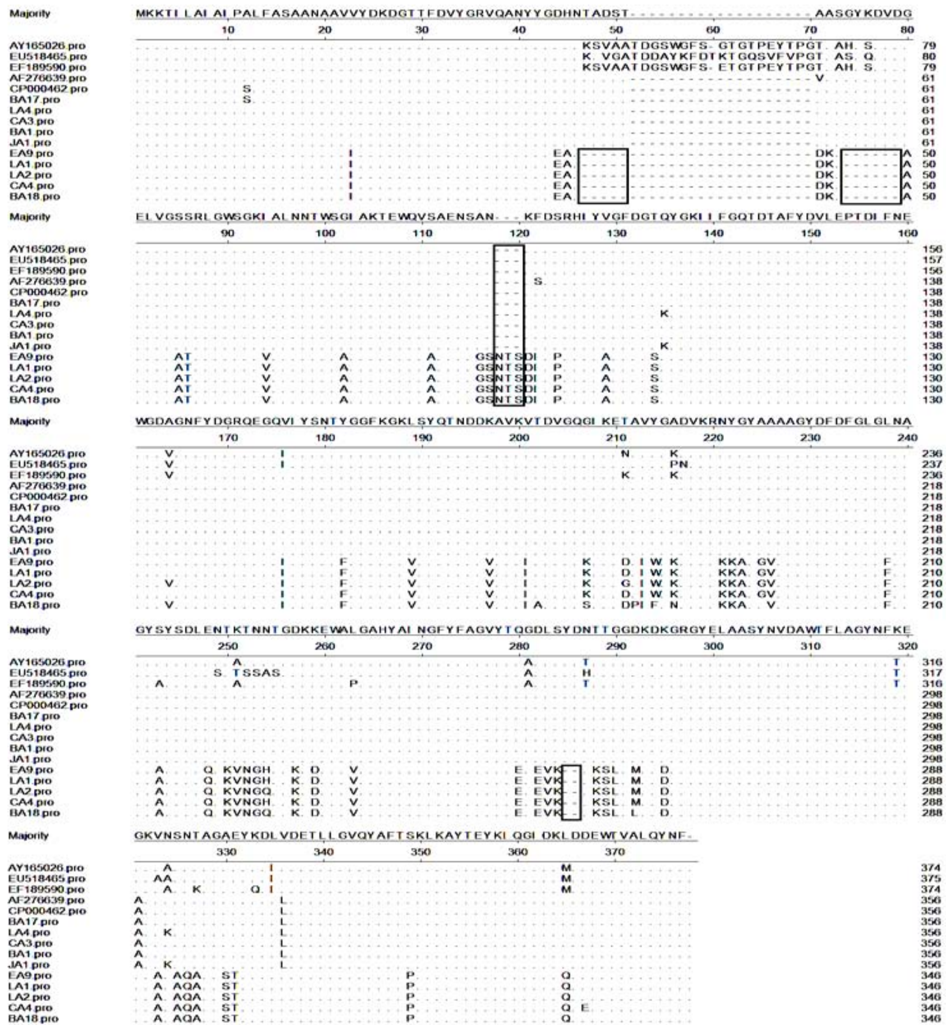

Fig. 2: Deduced amino acid sequences of the *aha1* genes from *Aeromonas* sp. Deletion sites of amino acid are marked by the black box. The major sequence variations were observed between amino acids 85-134, 176-227, 243-263, 280-295 and 321-336

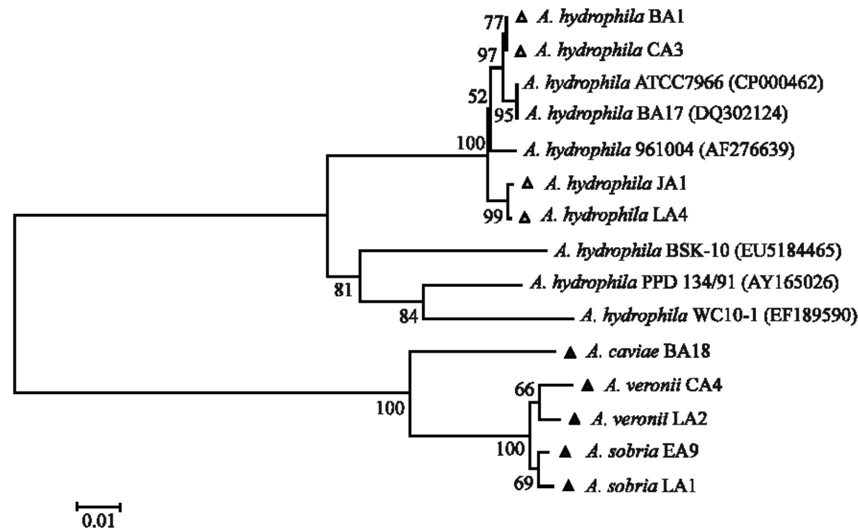

Fig. 3: Phylogenetic tree analysis of the *aha1* gene sequences from *Aeromonas* sp. NJ tree of nine Anhui isolates with six reference strains from GenBank was generated by the MEGA4.1 Software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. The scale bar represents 0.01 substitutions per site. Bootstrap values above 50% are shown at the nodes. The Anhui isolates in this study are indicated by the triangle, respectively, red for the *A. hydrophila* isolates, black for other phenotypic species isolates

vaccine against the disease. The outer membrane protein Aha1 was reported as a major adhesin in *A. hydrophila* which was a highly conserved antigen in different serotypes of *A. hydrophil* (Lee *et al*., 1997). Recombinant Aha1 protein's antiserum has the cross reaction to mesophilic motile aeromonads (Fang *et al*., 2004). In the present study, this major adhesion gene from Anhui isolates was cloned, sequenced and analyzed in detail so as to ascertain the conservation of aha1 protein among mesophilic motile aeromonads. Homology comparison revealed that four Anhui *A. hydrophila* isolates and reference strains formed a cluster together with 91.4-99.7% nucleotide identity and 91.9-99.7% amino acid identity of *aha1* gene. Five Anhui other phenotypic species isolates formed another cluster, their *aha1* gene nucleotide sequences are 94.2-99.3% identical with each other and they shared 79.5-81.1% nucleotide identity and 79.6-81.6% amino acid identity of the *aha1* gene compared with *A. hydrophila*. The findings indicate that Aha1 protein is relatively conserved among mesophilic motile aeromonads.

Comparing the deduced aha1 amino acid sequences of mesophilic motile aeromonads, the major sequence variations were observed between amino acids 85-134, 176-227, 243-263, 280-295 and 321-336. Furthermore, researchers predicted the linear B-cell epitopes of the aha1 protein using the ABCpred Software's artificial neural network method (Saha and Raghava, 2006). The results showed that the potential epitope peptides at amino acid positions 24-43 and 143-163 were also the conserved regions of *aha1* gene in different phenotypic species of mesophilic motile aeromonads which is valuable for the development of epitope-based vaccine.

CONCLUSION

The study clearly demonstrates that *A. hydrophila*, *A. sobria*, *A. caviae* and *A. veronii*, all can cause bacterial haemorrhagic septicemia in aquaculture animals. Aha1 protein is relatively conserved antigen among mesophilic motile aeromonads and might be developed as a vaccine candidate.

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