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FULL PAPER

Bacteriology

Molecular characteristics and virulence analysis of eight *Aeromonas hydrophila* isolates obtained from diseased Amur sturgeon *Acipenser schrenckii* Brandt, 1869

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ABSTRACT. *Aeromonas hydrophila* is an opportunistic pathogen of a variety of aquatic animals that displays extreme diversity in drug resistance, phenotypes, virulence genes, and virulence. In this study, eight pathogenic *A. hydrophila* strains were isolated from diseased Amur sturgeons and investigated for their sensitivity to select antibiotics, their phenotype, virulence genes, and virulence. According to the phylogenetic analysis of the DNA gyrase subunit B protein, the eight isolates formed a single branch in the *A. hydrophila* group. The antibiotics ceftazidime, cefuroxime, cefoperazone, cefotaxime, ceftriaxone, aztreonam, and cefepime appeared effective against them. All of the isolates possessed the virulence genes for aerolysin, flagellin, heat-stable cytotonic enterotoxin, heat-labile cytotonic enterotoxin, hemolysin, and elastase, while only one isolate, HZ8, possessed the gene for lateral flagella. The cytolytic enterotoxin and lipase genes were present in all isolates, except in ZJ10 and ZJ12. Enterobacterial repetitive intergenic consensus sequence PCR indicated that the eight *A. hydrophila* isolates could be divided into four types. Isolates YW2, TR3, HZ8 and ZJ10, each representing a different type, were selected for challenge experiments. The challenge tests revealed that isolate HZ8 had the lowest lethal dose, causing 50% mortality at 2.30×10^4 colony forming units (cfu)/m*l*. The isolate ZJ10 had the highest LD_{50}, 1.25×10^6 cfu/m*l*. Knowledge of the characteristics of the *A. hydrophila* isolates obtained from Amur sturgeon will be beneficial in developing potential disease control strategies.

KEY WORDS: *Aeromonas hydrophila*, Amur sturgeon, antibiotic sensitivity, phenotype, virulence genes

Aeromonas hydrophila belongs to the family Aeromonadaceae and is an important opportunistic pathogen that is widely distributed in aquatic environments [6]. It represents one of the main pathogenic bacteria of aquatic animals [1]. Reportedly, many species of fish can be infected by *A. hydrophila*, including grass carp [27], common carp [24], crucian carp [37], goldfish [10], catfish [23], Siberian sturgeon [33], and Amur sturgeon [18]. *A. hydrophila* displays extreme diversity in antibiotic sensitivity, phenotypes, virulence genes, and virulence [1, 17].

The sturgeon is one of the economically most important fish in China because of its excellent dietary properties [35]. China is responsible for approximately 80% of the total farmed sturgeon production of the world [14]. However, frequent outbreaks of infectious diseases pose a serious threat to the further development of the rapidly expanding sturgeon production industry [35]. *A. hydrophila* is a dangerous pathogen for the sturgeon and causes hemorrhagic septicemia that is characterized by symptoms such as ascites, swelling of the cloaca and ventral abdomen and/or cloacal hemorrhaging, a digestive tract devoid of food, digestive tract hemorrhaging, liver and kidney hemorrhaging, and a fragile spleen [18].

In this study, eight isolates of *A. hydrophila* obtained from diseased Amur sturgeon were investigated for their sensitivity to select antibiotics, their phenotype, virulence genes, and virulence. The study provides a basis for understanding the epidemiology of *A. hydrophila* infection of the sturgeon.

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Table 1. Information on the *Aeromonas hydrophila* isolates used in this study

Numerical order	Isolate	Source (Province)
1	YW2	Hubei
2	TR3	Guizhou
3	TH5	Hubei
4	HZ8	Zhejiang
5	ZG9	Hubei
6	ZJ10	Jiangsu
7	QJ11	Hubei
8	ZJ12	Jiangsu

Table 2. Primers used to detect the genes in this study

Name	Primers, 5′→3′	Product size (bp)	Reference
gyrB3F	TCCGGCGGTCTGCACGGCGT	1,111	[34]
gyrB14R	TTGTCCGGGTTGTACTCGTC		
*aer*F	CAAGAACAAGTTCAAGTGGCCA	309	[31]
*aer*R	ACGAAGGTGTGGTTCCAGT		
*fla*F	TCCAACCGTYTGACCTC	608	[25]
*fla*R	GMYTGGTTGCGRATGGT		
*laf*F	GGTCTGCGCATCCAACTC	550	[19]
*laf*R	GCTCCAGACGGTTGATG		
*act*F	GAGAAGGTGACCACCAAGAACA	232	[15]
*act*R	AACTGACATCGGCCTTGAACTC		
*ast*F	TCTCCATGCTTCCCTTCCACT	331	[25]
*ast*R	GTGTAGGGATTGAAGAAGCCG		
*alt*F	TGACCCAGTCCTGGCACGGC	442	[25]
*alt*R	GGTGATCGATCCACCAGC		
*hly*F	GGCCGGTGGCCCGAAGATACGGG	595	[11]
*hly*R	GGCGGCGCCGGACGAGACGGGG		
*lip*F	ATCTTCTCCGACTGGTTCGG	382	[25]
*lip*R	CCGTGCCAGGACTGGGTCTT		
*ahy*BF	ACACGGTCAAGGAGATCAAC	513	[25]
*ahy*BR	CGCTGGTGTTGGCCAGCAGG		
ERIC3F	ATGTAAGCTCCTGGGGATTCAC		[17]
ERIC14R	AAGTAAGTGACTGGGGTGAGCG		

MATERIALS AND METHODS

Fish

Apparently healthy Amur sturgeon, 22 ± 2 cm in total length, were obtained from the experimental farm of the Yangtze River Fisheries Research Institute for *A. hydrophila* challenge. The sturgeon were maintained in 500-*l* plastic aquaria at 23 ± 1°C and fed daily a commercial sturgeon feed for 2 weeks during the maintenance period. All experimental procedures were conducted according to guidelines of the appropriate Local Ethics Commission for Experiments on Animals.

Bacterial isolates and growth conditions

Eight *A. hydrophila* isolates identified from moribund Amur sturgeon in our laboratory (Table 1) were cultured in Luria-Bertani (LB) broth and incubated at 28°C for 24 hr.

DNA isolation

The genomic DNA of each isolate was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, U.S.A.) per the manufacturer's instructions.

DNA gyrase subunit B amino acid sequence analysis

The DNA gyrase subunit B (*gyr*B) gene provides higher phylogenetic resolution than 16S rDNA [34]. The *gyr*B of *A. hydrophila* was amplified by PCR with the primers gyrB3F and gyrB14R (Table 2). All PCR tests were performed using a PCR amplification kit (Promega). Phylogenetic trees were constructed using the neighbor-joining method in MEGA 5 software [22, 30].

Analysis of virulence genes

Nine genes were amplified as potential markers of virulence: the genes for aerolysin (*aer*), heat-stable cytotonic enterotoxin (*ast*), heat-labile cytotonic enterotoxin (*alt*), cytolytic enterotoxin (*act*), hemolysin (*hly*), elastase (*ahy*B), lipase (*lip*), flagellin (*fla*) and lateral flagella (*laf*). Primers were designed based on these virulence genes, and the virulence genes were amplified from the respective isolates (Table 2). The PCR products were confirmed by DNA sequencing.

Enterobacterial repetitive intergenic consensus sequence PCR

Enterobacterial repetitive intergenic consensus sequence (ERIC) PCR was used for genotypic analysis of the eight *A. hydrophila* isolates as previously described, using the primers ERIC3F and ERIC14R [17]. Phosphate-buffered saline (PBS) was set as the negative control.

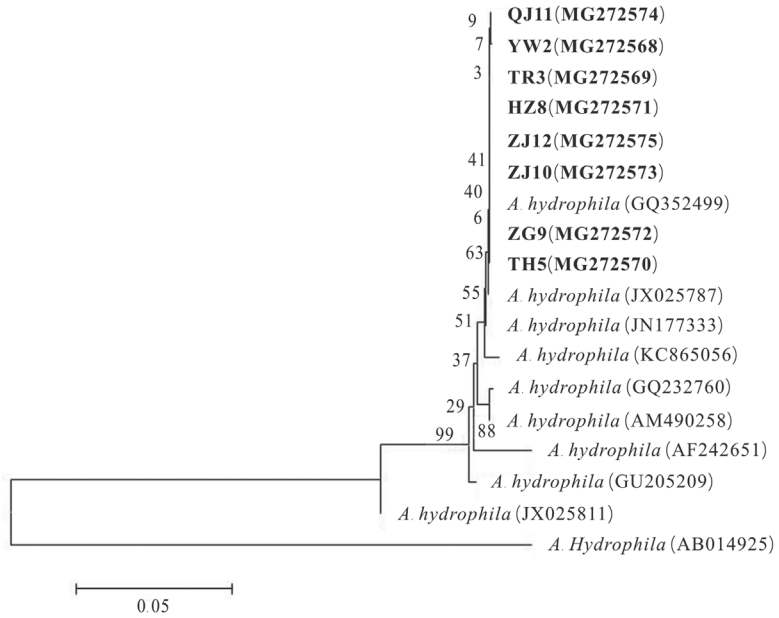

Fig. 1. *gyrB* amino acid sequence-based neighbor-joining phylogenetic tree of *A. hydrophila* strains isolated from sturgeon. Isolates are shown in bold. Accession numbers are shown in parentheses. Values at nodes indicate bootstrap values for 1,000 replicates. The scale bar represents the average number of amino acid substitutions per site.

Antibiotic sensitivity tests

Antibiotic sensitivity testing was performed using the Kirby-Bauer disc diffusion method in Mueller-Hinton (MH) agar (Solarbio, Beijing, China) according to the standards outlined by the Clinical and Laboratory Standards Institute [12]. The *A. hydrophila* isolates were grown overnight in LB broth, and the turbidity of the cell suspensions was adjusted with PBS until the optical density reached 0.15 ± 0.02 at 600 nm. The cell suspensions were plated on MH agar and antibiotic disks (Oxoid, Basingstoke, U.K.) were placed on the plates before incubation at 30°C for 24 hr. Thirty-five antimicrobial agents were tested. Antibiotic sensitivity was determined by measuring the diameter of the inhibition zone.

Virulence of four representative A. hydrophila isolates

Four *A. hydrophila* isolates were selected, one from each of the four different genotypes determined by ERIC-PCR. Overnight cultures were collected by centrifugation at 3,000 rpm for 10 min. The bacterial pellets were diluted to 10^3–10^8 colony forming units (cfu)/m*l* in PBS. Each group of 30 Amur sturgeon was inoculated intraperitoneally with 0.1 m*l* of serially 10-fold diluted bacterial suspension. Thirty other Amur sturgeon were inoculated intraperitoneally with 0.1 m*l* of PBS as controls. The mortality was monitored and recorded for 7 days. According to Koch's postulates, the bacteria were isolated and identified from dead or moribund Amur sturgeon. LD_{50} values were calculated by the statistical methods of Reed and Muench [21].

RESULTS

gyrB-based phylogeny

Phylogenetic trees based on the *gyrB* amino acid sequences of *A. hydrophila* strains were analyzed by the neighbor-joining method using the MEGA 5 program (Fig. 1). The eight *A. hydrophila* strains isolated from diseased Amur sturgeon formed a single branch in the *A. hydrophila* group.

Distribution of virulence genes

Nine virulence genes of the *A. hydrophila* isolates were detected by PCR. The virulence genes *aer*, *fla*, *ast*, *alt*, *hly* and *ahy*B were detected in all eight *A. hydrophila* isolates (Table 3), whereas *laf* was only detected in the isolate HZ8. *Act* and *lip* were detected in all isolates, except in ZJ10 and ZJ12.

ERIC-PCR

Eight *A. hydrophila i*solates were fingerprinted by ERIC-PCR. Four genotypes were identified using the method (Fig. 2). The results of electrophoresis showed all isolates had a very distinct band, about 750 bp long. ERIC fingerprinting indicated a high similarity in the banding patterns of isolates YW2, TH5, ZG9 and QJ11. The banding patterns of ZJ10 and ZJ12 were identical, but differed from the banding patterns of the four isolates mentioned above. A <1,000 bp amplicon was obtained only from the isolate TR3. Additionally, a 760 bp amplicon was only found in the isolate HZ8.

Table 3. Distribution of virulent genes detected in *Aeromonas hydrophila* isolates assessed in this study

Virulence gene	Isolate							
	YW2	TR3	TH5	HZ8	ZG9	ZJ10	QJ11	ZJ12
aer	+	+	+	+	+	+	+	+
fla	+	+	+	+	+	+	+	+
laf	-	-	-	+	-	-	-	-
act	+	+	+	+	+	-	+	-
ast	+	+	+	+	+	+	+	+
alt	+	+	+	+	+	+	+	+
hly	+	+	+	+	+	+	+	+
lip	+	+	+	+	+	-	+	-
*ahy*B	+	+	+	+	+	+	+	+

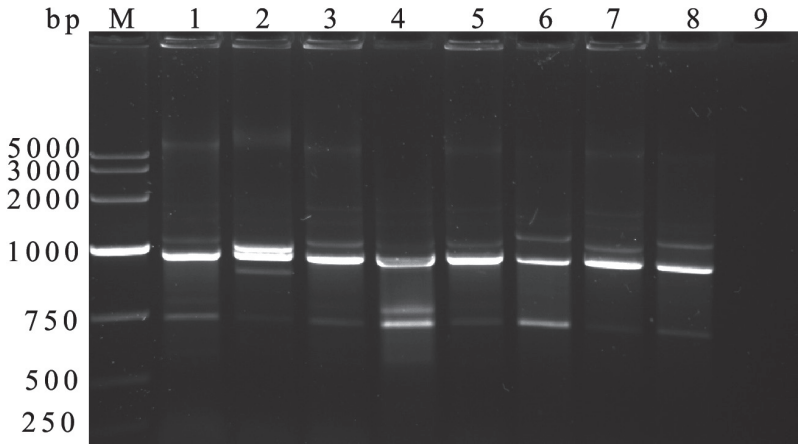

Fig. 2. ERIC profiles of the *A. hydrophila* isolates. Lane M: 5 kb DNA ladder marker; Lane 1: YW2; Lane 2: TR3; Lane 3: TH5; Lane 4: HZ8; Lane 5: ZG9; Lane 6: ZJ10; Lane 7: QJ11; Lane 8: ZJ12; Lane 9: negative control.

Antibiotic sensitivity

The antibiotic sensitivity of all of the *A. hydrophila* isolates was analyzed using 35 antimicrobial agents. All of the *A. hydrophila* isolates exhibited sensitivity against ceftazidime, cefuroxime, cefoperazone, cefotaxime, ceftriaxone, aztreonam, and cefepime and resistance against clindamycin, oxacillin, penicillin-G, ampicillin, medemycin and cefalotin. Furthermore, the isolates showed intermediate sensitivity to 22 antimicrobial agents: ofloxacin, clarithromycin, piperacillin, vancomycin, norfloxacin, sulfamethoxazole, amikacin, gentamicin, cefoxitin, macrodantin, chloramphenicol, levofloxacin, minocycline, erythrocin, spectinomycin, ciprofloxacin, tobramycin, kanamycin, streptomycin, cefazolin, polymyxin B, and tetracycline.

Virulence of the A. hydrophila isolates

To assess the virulence of the *A. hydrophila* isolates in terms of Amur sturgeon mortality, LD_{50} measurements were made after intraperitoneal injection. The highest LD_{50} value was 1.25×10^6 cfu/m*l*, for the ZJ10 isolate. The lowest LD_{50} value was 2.30×10^4 cfu/m*l*, for the HZ08 isolate. The LD_{50} values of YW2 and TR3 were 3.52×10^5 cfu/m*l* and 5.13×10^5 cfu/m*l*, respectively. Most dead or moribund Amur sturgeon showed typical clinical symptoms of hemorrhagic septicemia (Fig. 3). *A. hydrophila* could be isolated from the kidneys of all dead or moribund Amur sturgeon in the bacteria-infected group.

DISCUSSION

Certain virulence genes are very important for the pathogenicity of *Aeromonas* spp. [13]. Two hemolytic toxins, hly and aer, are secreted by most virulent *A. hydrophila* isolates [31, 32]. All of the *A. hydrophila* isolates used in this study possessed both *hly* and *aer*. This result supports the finding that the occurrence of hemolytic factors in aeromonads is widespread [38]. In addition, there are other important virulence genes in aeromonads [5], some of which were detected by PCR in this study. The enterotoxin genes *act*, *alt* and *ast* are key virulence genes in diarrheal disease [26]. The *ahy*B gene product contributes the most to the elastolytic activity of this bacterium, which plays an important role in the invasiveness and establishment of infection [5]. The combined effect of lip itself and the products of its enzymatic activity contribute to the virulence of *A. hydrophila* [28]. Both lateral polar flagella

Fig. 3. Clinical signs of dead or moribund sturgeon. The Amur sturgeon were inoculated intraperitoneally with *A. hydrophila.* Small petechial hemorrhages were present in the digestive tract of dead and moribund Amur sturgeon.

(*laf* and *fla*, respectively) are essential for its adherence to the gastrointestinal epithelium [5]. All of the isolates in this study were found to possess the genes *aer*, *fla*, *ast, act*, *hly* and *ahy*B, while only HZ8 possessed *laf*. Additionally, *act* and *lip* were found in all isolates, except in ZJ10 and ZJ12. Thus, the different *A. hydrophila* isolates have different distributions of virulence genes.

Thanks to the fast throughput, high resolution, and cost-effectiveness of the ERIC-PCR technique [36], it has been used to study *A. hydrophila* etiology and molecular epidemiology [3, 4, 7, 29]. In this study, the eight *A. hydrophila* isolates were divided into four types by ERIC-PCR (Fig. 1). The results of the distribution of virulence genes divided the isolates into the same types as ERIC-PCR (Table 3). These results support the finding that the distribution of virulence genes could be used for identifying the genotypes of *A. hydrophila* isolates in epidemiological studies [15, 25].

Resistance to antimicrobials helps bacteria to survive and spread. In this study, the response of the eight *A. hydrophila* isolates to 35 antimicrobial agents was evaluated. Our results indicated that some antimicrobials, such as ciprofloxacin, norfloxacin, amikacin, piperacillin, and ampicillin, which were once effective against *A. hydrophila* infection [8, 9, 16], exhibited low levels of activity against the isolates used in this study. However, there are still seven antimicrobials the isolates were sensitive to. These drugs should be used preferentially for the treatment of sturgeon infected with *A. hydrophila*.

In this study, four isolates were used in bacterial pathogenicity challenge tests. The LD_{50} values of *A. hydrophila* ranged from 2.30×10^4 to 1.25×10^6 cfu/m*l*, which is similar to the LD_{50} values determined in previous studies [2, 20, 36]. The virulence of the *A. hydrophila* isolates decreased with a decreasing number of virulence genes: the isolate HZ8, which contained all nine virulence genes, was the most virulent isolate. This result supports the finding that the characterization of virulence genes can significantly assist in determining the virulence of a particular isolate. In addition, the highly virulent isolate HZ8 could be used to produce a vaccine against *A. hydrophila* for the Amur sturgeon.

In conclusions, eight *A. hydrophila* isolates obtained from Amur sturgeon clustered into four types based on the results of ERIC-PCR. Seven effective antibiotics were identified for these isolates. Isolate HZ8 was the most virulent and had the highest number of virulence genes among the eight isolates. We expect that the results will provide a theoretical basis for further studies on drug resistance, prevention, and control of *A. hydrophila* in China.

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