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Molecular Characterization and Virulence Genes of *Aeromonas hydrophila* Isolated from the Chinese Giant Salamander (*Andrias davidianus*)

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Abstract The Chinese giant salamander (*Andrias davidianus*) is the largest living amphibian in the world. *Aeromonas hydrophila* strain L602 was isolated from *A. davidianus.* The 16S rDNA gene of this isolate was amplified using PCR, and the phylogenetic tree was constructed by the neighbor-joining method. Four virulence genes (aerA, aha1, hly and alt) of *A. hydrophila* were amplified by PCR and drug resistances were tested using Kirby-Bauer disk diffusion method. The results showed that the length of this 16S rDNA sequence was 1453 bp, which showed 99% homology with *A. hydrophila*. The GenBank accession number was JX155398. Phylogenetic analysis indicated it grouped together with *A. hydrophila.* Four virulence genes were all detected, indicating that strain L602 was highly virulent. This stain was resistant to four antibiotics (vibramycin, furazolidone, ampicillin and erythromycin), while it was insensitive to streptomycin. Furthermore, this strain was susceptible to six antibiotics (sulfafurazole, ciprofloxacin, penbritin, norfloxacin, florfenicol and enrofloxacin). This study will help to validate the classification and virulence of pathogenic bacteria in amphibians.

Keywords Chinese giant salamander, 16S rDNA, virulence gene, drug resistance, *Aeromonas hydrophila*

1. Introduction

The Chinese giant salamander *(Andrias davidianus)* is a member of the Order Caudata (Amphibia) in the family of Cryptobranchidae. Currently, there are only three species in this family: the Chinese giant salamander (*A. davidianus*), the Japanese giant salamander (*A. japonicus*) and the Hellbender (*Cryptobranchus alleganiensis*) in North America. The Chinese giant salamander is the largest living amphibian in the world, reaching 1.7 m in length and exceeding 60 kg in weight. *A. davidianus* is entirely aquatic and endemic to Mainland China. The species was once widely distributed in central and southern China. However since the 1950s, its populations have declined sharply in both range and number, due to habitat loss and fragmentation, infectious disease and hunting for food trade (Fu, 1993). Most wild populations are now threatened and some are already extinct. *A. davidianus* has been protected by the state since 1973, and is currently listed as a State Specially Protected Animal (Category II) by the Chinese Conservation Law, in Appendix I of CITES and as Data Deficient in the IUCN Red List (Wang *et al*., 2004). Recently, many studies on the Chinese giant salamander have been done, mainly focusing on its physiology, reproduction, genetics and artificial culture (Zhang *et al*., 2006; Guo *et al*., 2012; Yang *et al*., 2010; Murphy *et al*., 2000). However, little information

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can be obtained on the diseases of *A. davidianus*, especially bacteria-caused diseases (Geng *et al.*, 2011; Wang *et al.*, 2010; Li *et al.*, 2008).

Aeromonas hydrophila is a ubiquitous Gram-negative rod and belongs to the family Aeromonadaceae. It is widely distributed in fresh and salt water, and is also found in food, treated drinking water, domestic water supplies and hospital water supply systems (Janda and Abbott, 2010; Figueras, 2005). *A. hydrophila* has been implicated as a causative agent of motile aeromonad septicemia, which is associated with disease conditions mainly in fish and humans and causes gastrointestinal and extraintestinal infections in humans, such as septicemia, wound infections, gastroenteritis, hepatobiliary tract infections, and hemolytic uremic syndrome (Kang *et al*., 2005). Although this pathogen can infect healthy individuals, most infections are found in immunocompromised hosts, especially those with liver cirrhosis and malignancies (Lay *et al*., 2010). Identification of strains of *A. hydrophila* capable of causing illness in apparently healthy individuals, by infecting open wounds and possibly by ingestion of the microorganisms in food or water, has generated immense interest in this organism. So far, *A. hydrophila* has been isolated from humans, Chinese soft-shelled turtle, farmed Nile tilapia, bovine, channel catfish, rainbow trout and so on (Lukkana *et al*., 2012; Zhou *et al*., 2008; Moro *et al*., 1999; Xu *et al*., 2012; Liu *et al*., 2010).

Presently, there are some studies on the isolation and pathogenicity of *A. hydrophila* in fish, humans and other animals (Esteve *et al.*, 2012; Lukkana *et al.*, 2012; Okumura *et al.*, 2011; Zhou *et al.*, 2008; Fang *et al.*, 2005). However, little attention has been given to the involvement of *A. hydrophila* infections in Cryptobranchidae (Geng *et al.*, 2011). The purpose of the present study was to evaluate the 16S rDNA gene, virulence genes and antibiotic-resistant phenotype of *A. hydrophila* isolated from a sick Chinese giant salamander.

2. Materials and Methods

2.1 Animal culture and bacterial isolates Chinese giant salamanders were kept in the Chengdu Aquarium. The sick Chinese giant salamander was 1.8 kg in weight and measured 56 cm in length. The symptoms of this Chinese giant salamander included anorexia, gastric disorder, skin ulcers in legs and tail. It then ceased eating and died six days later. The sample of heart was immediately inoculated onto blood plates under aseptic processing. Then, the sample was subcultured onto general nutrition agar plate followed by incubation at 28ºC for 20 h under aerobic conditions. Single colonies of bacterial strains were picked, and further grown and subcultured several times to obtain a pure culture. The colony morphology of the pure isolates grown on nutrition agar plate for overnight at 28°C was observed directly and microscopically examined after Gram staining.

2.2 DNA Isolation of bacteria After an overnight cultivation in liquid lysogeny broth (LB) medium at 28ºC, bacterial DNA was extracted using genomic mini kit (Tiangen, China) according to the manufacturer's instructions. The integrity of DNA was checked using 1.0 % (w/v) agarose gel in TAE buffer. Concentration and purity of DNA were estimated spectro photometrically at 260 nm and 280 nm, respectively, for further assay.

2.3 PCR amplification of 16S rDNA gene The ribosomal 16S rDNA gene was partially amplified in PCR reactions using the universal primers 27F and 1492R. The 27F primer was 5'-AGA GTT TGA TCC TGG CTC AG-3' and the 1429R primer was 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The PCR reaction mixture included 2 μL bacterial DNA, 21 μL ddH_2O, 23 μL rtaq Mix (TaKaRa, Japan), and 2 μL each primer (10 umol) for a final reaction volume of 50 μL. Amplifications were performed at 94°C for 4 min, 94°C for 30 s, 60°C for 30 s, 72°C for 4 min (5 cycles); 94°C for 30 s, 55°C for 30 s, 72°C for 4 min (5 cycles); and 94°C for 30 s, 50°C for 30 s, 72°C for 4 min (30 cycles). PCR products were examined for size and yield using 1.2% (w/v) agarose gel in TAE buffer. After successful amplification, the obtained product was sequenced on both strands using an ABI PRISM 3730 automated sequencer (Applied Biosystem Inc.).

2.4 Sequence analysis DNA sequences were edited and assembled using the programs SeqMan and Edit Seq (DNA Star, Laser Gene 6, USA). Sequence similarity analysis was performed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were generated using Clustal W. Phylogenetic tree was constructed using neighbour-joining method MEGA 5 package (The Biodesign Institute, USA). The reliability of the neighbor joining tree was estimated by bootstrap analysis with 1000 replicates.

2.5 Detection of virulence gene Four virulence genes (aerA, aha1, hly and alt) of *A. hydrophila* were amplified by PCR. The primers were designed according to the sequences in GenBank database and references (Fang *et al*., 2005). These primers are

Table 1 Primers of virulence genes for *A. hydrophila* strain L602.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Tmperature (°C)	Size (bp)
hly	gga tcc atg atg aat aga ata	aag ctt tta ttg aac cgg aac	55	1470
alt	atg acc cag tcc tgg cac gg	gcc gct cag gcc gaa gcc gc	60	480
aerA	tgt cgg gga tga cat gaa cgt g	cca gtt cca gtc cca cca ctt ca	53	720
aha1	gcc gct aac gct gca gtg gtt tac gac	cgc aga ggc tag att aga agt tgt attg	60	1087

shown in Table 1. The PCR reaction mixture included rtaq Mix (TaKaRa, Japan) 12 μL, bacterial DNA 1 μL, ddH_2O 11 μL, and each primer (10 μmol) 1 μL in a 25 μL final volume. A negative control (PCR mixture without DNA) was included in all PCR amplifications. Amplifications were performed in a thermal cycler with the following PCR parameters: 94°C for 3 min; 94°C for 30 s, 53–60°C for 30 s, 72°C for 60 s (35 cycles); and 72°C for 10 min. PCR products were examined for size and yield using 1.2% (w/v) agarose gel in TAE buffer.

2.6 Antibiotic susceptibility test Susceptibility of the bacterial isolate to eleven antibiotics was determined by Kirby-Bauer disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) Guidelines. The test organism was picked up with sterile loop, suspended in peptone water and incubated at 37°C for 2 h. Standard 0.5 McFarland saline suspensions of bacteria were used to inoculate Mueller Hinton agar media confluently with a cotton swab. Antibiotic disks were placed on the agar, and incubated at 37°C overnight. The zone of inhibition was measured and interpreted as per the CLSI Guidelines. *Escherichia coli* (ATCC25922) was used as quality control strain for the susceptibility testing method.

3. Results

3.1 Bacterial isolates A single strain was isolated from the heart of a sick Chinese giant salamander and named L602. This strain exhibited *β*-hemolysis on sheep blood agar. The *β*-hemolysis was a broad zone, or a narrow zone of *β*-hemolysis just under the colony edge. In nutrient agar, strain L602 produced smooth and transparent colonies with an entire edge. The isolate displayed morphologic characteristics typical of the *Aeromonas*, appearing as Gram-negative and short rods.

3.2 PCR amplification and analysis of 16S rDNA gene The amplified band was about 1500 bp, which was accordance with the anticipated objective strap size (Figure 1). The length of this 16S rDNA sequence was 1453 bp, which showed 99% homology with *A. hydrophila* (AB473005.1). This 16S rDNA gene was submitted to NCBI, and its GenBank accession number was JX155398. A phylogenetic tree to assess the relationship of *A. hydrophila* with other known bacteria was performed (Figure 2). The bacteria were largely clustered into two major groups, *Aeromonas* and *Vibrio*. The isolate belonged to the group of *Aeromonas*, and grouped together with *A. hydrophila*, as the closest neighbor. According to the morphological characteristics, sequence homology and phylogenetic tree, it is confirmed that this strain is *A. hydrophila.*

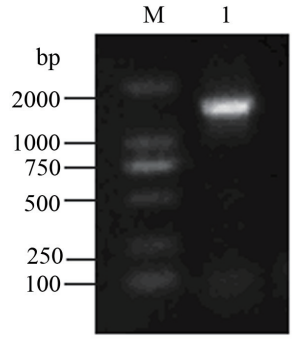

Figure 1 The 16S rDNA gene of *A. hydrophila* strain L602. 1: PCR product; M: DNA marker DL2000.

3.3 Detection of virulence gene Four virulence genes (aerA, aha 1, hly and alt) of *A. hydrophila* strain L602 were amplified by PCR. The result is shown in Figure

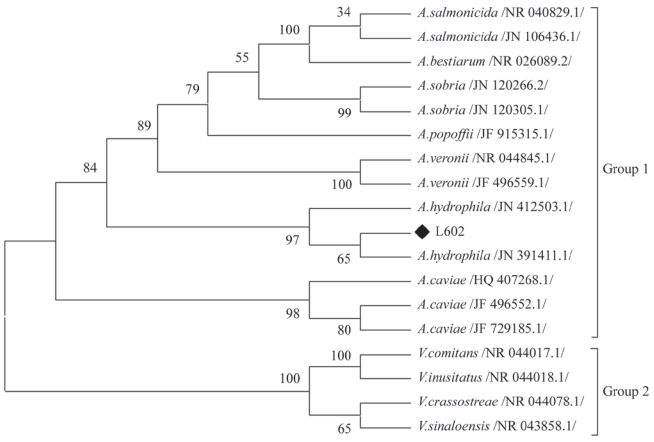

Figure 2 Phylogenetic tree of *A. hydrophila* strain L602 (shown by ◆) and other bacteria.

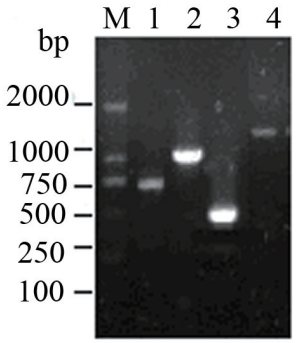

Figure 3 The virulence genes of *A. hydrophila* strain L602. 1: aerA; 2: aha1; 3: alt; 4: hly; M: DNA Marker DL2000.

3. The electrophoresis results of PCR production were satisfactory with clear straps, correct place and non-specificity amplification. The lengths of aerA, aha1, alt and hly genes were about 720 bp, 1087 bp, 480 bp and 1470 bp, respectively, which conformed to the anticipated objective strap sizes.

3.4 Antibiotic susceptibility test *Aeromonas hydrophila* strain L602 was screened for antibiotic resistance using the Kirby-Bauer disk diffusion method. The result is shown in Table 2. This stain was resistant to four antibiotics (vibramycin, furazolidone, ampicillin and erythromycin), while it was insensitive to streptomycin. Furthermore, this strain was susceptible to six antibiotics, that is, sulfafurazole, ciprofloxacin, penbritin, norfloxacin, florfenicol, and enrofloxacin.

4. Discussion

Aeromonas hydrophila is a ubiquitous Gram-negative bacterium present in aquatic environments, which has been implicated in illness in humans and animals. 16S rRNA gene sequence analysis has become the "gold standard" method for definitive species identification. Primers targeting the conservative regions of the rDNAs were used to generate amplicons of variant regions that are informative in taxonomic assignment. Furthermore, the identity of 16S rRNA gene sequences collected from environment can be related to the taxonomic identity of sequences obtained from cultivated, characterized strains (Vondracek *et al*., 2011). Eight *A. hydrophila*-like arabinose-negative isolates from diverse sources (i. e., river water, cooling-system water pond, diseased wild European eels, and human stools) sampled in Valencia (Spain) during 2004–2005, were characterized by 16S rRNA gene sequence and extensive biochemical test along with reference strains of most *Aeromonas* species. These isolates and all reference strains of *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* showed a 16S rRNA sequence similarity of 99.8%–100%, and they all shared an identical phenotype (Esteve *et al*., 2012). In this paper, strain L602 was identified by a molecular method based on 16S rDNA species specific gene amplification and subsequent sequence analysis. According to the cultural, morphological and 16S rDNA gene analyses, this isolate is confirmed to be *A. hydrophila*.

Aeromonas species are significant human pathogens that caused both gastrointestinal and nonintestinal diseases in children and adults. Epidemiological studies implicated *Aeromonas* species in causing food-borne outbreaks and travelers' diarrhea (Chopra and Houston, 1999). In immunocompromised individuals, *A. hydrophila* could cause substantial mortality from a wide spectrum of infections. Severe sepsis was caused by *A. hydrophila* in an elderly patient using tocilizumab (Okumura *et al*., 2011). An extracellular lethal toxin produced by *A. hydrophila* strain RT860715K originally isolated from diseased rainbow trout was purified by using fast protein liquid chromatography system. Both

Table 2 The result of susceptibility test of *A. hydrophila* strain L602.

Antibiotic	Concentration of tablet μg/slice	S I R (mm)	Inhibition zone (mm)	Sensitivity
Furazolidone	300	≥17 15-16 ≤14	11	R
Sulfafurazole	300	≥17 13-16 ≤12	18	S
Penbritin	10	≥17 14-16 ≤13	19	S
Norfloxacin	10	≥17 13-16 ≤12	20	S
Streptomycin	10	≥15 12-14 ≤11	12	I
Florfenicol	30	≥18 13-17 ≤12	19	S
Vibramycin	30	≥16 13-15 ≤12	12	R
Enrofloxacin	5	≥21 16-20 ≤15	23	S
Ciprofloxacin	5	≥21 16-20 ≤15	22	S
Erythromycin	15	≥23 14-22 ≤13	5	R
Ampicillin	10	≥17 14-16 ≤13	11	R

S: Susceptible; I: Insensitive; R: Resistant

the extracellular products of *A. hydrophila* RT860715K and the purified protease were lethal to rainbow trout (Liu *et al*., 2010). Based on both LD50 and LD95 values of intraperitoneal injection assays, the virulences of three *A. hydrophila* isolates from infected channel catfish were not significantly different from each other, indicating that the three west Alabama isolates of *A. hydrophila* were highly virulent to channel catfish (Pridgeon and Klesius, 2011). Parasitism by protozoan *Ichthyophthirius multifiliis* enhanced invasion of *A. hydrophila* in tissues of channel catfish (Xu *et al.*, 2012).

The pathogenesis of *Aeromonas* infection was complex and multifactorial (Chopra and Houston, 1999). Some studies concentrated on the characterization of virulence factors in different animal models by using different strains. A number of virulence factors were identified in *A. hydrophila*, including cytotoxic and cytotonic enterotoxins, aerolysins, haemolysins, proteases, haemagglutinins, lipases, lectins, adhesins, and flagella (Singh *et al*., 2009; 2010; Sha *et al*., 2004; Laohachai *et al.*, 2003; Yu *et al*., 2005). Furthermore, the type 6 secretion system (T6SS) of *A. hydrophila* played an important role in bacterial virulence in a mouse model, and immunization of animals with the T6SS effector haemolysin co-regulated protein protected them against lethal infections with wild type bacteria (Suarez *et al*., 2010). Virulence properties of *A. hydrophila* correlated well with the presence of virulence genes tested. The aerA (+) alt (+) ahp (+) was more frequent virulence genotype in *A.hydrophila* isolates from clinical diseases than from healthy fish and water environment, and the aerA (+) alt (+) ahp (+) isolates were more virulent to zebrafish compared to the other six genetic profiles. The detection for aerA, alt and ahp could be used for virulence typing of *A. hydrophila* isolates (Li *et al*., 2011). In this study, aerA, alt, hly and aha1 were checked out, which indicated that this stain was highly virulent. It should have some virulence to Chinese giant salamander and other amphibians.

The prevention and treatment of both human and fish diseases by the extensive use of antimicrobial agents had undoubtedly contributed to an increase in frequency of resistant strains (Rhodes *et al*., 2000). Most of the Kolkata *A. hydrophila* strains (clinical) showed resistance to ampicillin, nalidixic acid, cephalothin, streptomycin and furazolidone (Sinha *et al*., 2004). *A. hydrophila* strain isolated from a cirrhotic patient's fecal sample in a Budapest hospital was resistant to ceftazidime, cefepime, piperacil-in-tazobactam, and imipenem, and it remained susceptible to aztreonam (Libisch *et al*., 2008). The environmental isolates of *A. hydrophila* were resistant or had reduced susceptibility to ampicillin (93%), furazolidone (93%), tetracycline (93%), neomycin (71%), cephalothin (62%), streptomycin (18%) and nalidixic acid (14%) (Bhowmik *et al*., 2009). Eight *A. hydrophila*-like arabinose-negative isolates were all resistant to ticarcillin, amoxicillin-clavuranic acid, cefoxitin, and imipenem, regardless of their sources (Esteve *et al*., 2012).

The reported emerging resistance of *Aeromonas* to quinolonesand carbapenems, as well as aztreonam, cephalosporins and aminoglycosides, might influence physicians to employ polytherapy for empirical treatment of severe *A. hydrophila* infections (Sahin and Barut, 2010; Libisch *et al*., 2008; Shak *et al*., 2011). In this study, *A. hydrophila* stain L602 was resistant to vibramycin, furazolidone, ampicillin and erythromycin. The differences between these reports might be due to the diversity in bacterial strains, degree of infections, different medications, and so on. Emergence of multiple resistances was a serious clinical problem in the treatment and containment of diseases. Comprehensive studies were needed to examine broader trends in *Aeromonas* antibiotic resistance.

In summary, *A. hydrophila* strain L602 was isolated from a sick Chinese giant salamander. The 16S rDNA gene, virulence genes and antibiotic-resistant phenotype of this strain were studied by molecular biological technology and pharmacological method. This study will be useful for data-mining for the development of effective diagnosis and treatment against animal and human infections caused by *Aeromonads*. Future studies will be targeted at defining the pathogenicity and prophylaxis of *A. hydrophila* infection in amphibians.

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