

## Article

# Isolation, Identification and Characteristics of *Aeromonas caviae* from Diseased Largemouth Bass (*Micropterus salmoides*)

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**Abstract:** The largemouth bass (*Micropterus salmoides*) is one of the most economically valuable fish species in China. In this study, a bacterial pathogen was isolated from the internal organs of diseased *M. salmoides*, and the strain was named WH21406. This isolate was identified as *Aeromonas caviae* on the basis of its morphology, biochemical features and 16S rDNA phylogenetic analysis. Four virulence genes related to pathogenicity, namely, flagella (*fla*), elastase (*ela*), haemolysin (*hly*) and aerolysin (*aer*), were detected in this isolate. The median lethal dosage (LD50) of *A. caviae* WH21406 for *M. salmoides* was calculated to be  $3.46 \times 10^5$  CFU mL<sup>-1</sup>. The histopathological analysis showed obvious tissue damage in the gill, liver, kidney, spleen and gut of the diseased fish. The antibiotic susceptibility test demonstrated that strain WH21406 was highly sensitive to enrofloxacin, norfloxacin, streptomycin and amikacin. The results of this study provide a foundation for the diagnosis, prevention and treatment of *A. caviae* infection in *M. salmoides*.

**Keywords:** *Micropterus salmoides*; *Aeromonas caviae*; virulence genes; antibiotic susceptibility; histopathology



**Citation:** Xue, M.; Xiao, Z.; Li, Y.; Jiang, N.; Liu, W.; Meng, Y.; Fan, Y.; Zeng, L.; Zhou, Y. Isolation, Identification and Characteristics of *Aeromonas caviae* from Diseased Largemouth Bass (*Micropterus salmoides*). *Fishes* **2022**, *7*, 119. <https://doi.org/10.3390/fishes7030119>

Academic Editors: Jee Eun Han and Chorong Lee

Received: 22 April 2022

Accepted: 26 May 2022

Published: 28 May 2022

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## 1. Introduction

The largemouth bass (*Micropterus salmoides*) is indigenous to the Mississippi River Valley in North America, and it was introduced to various countries, including China [1,2]. Owing to its rapid growth and delicious flesh, *M. salmoides* has become one of the most economically valuable fish species in China [3–5]. According to the China Fishery Statistical Yearbook, the annual output of *M. salmoides* already reached 619,519 tons in 2020 (Fisheries and Fisheries Administration Bureau of Ministry of Agriculture and Rural Affairs, 2021). However, the aquatic environment is deteriorating as a result of the rapid expansion of largemouth bass aquaculture, resulting in increasing incidences of serious pathogen outbreaks that threaten the sustainable development of largemouth bass aquaculture [3,6,7]. Outbreaks caused by bacterial pathogens such as *Nocardia seriolae*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas sobria*, *Edwardsiella piscicida*, *Edwardsiella tarda* and *Flavobacterium columnare* are increasing in frequency and causing huge economic losses [8–14].

*Aeromonas* spp. are widely found in aquatic environments; they are the causative agents of major diseases in fish, leading to high mortality and deterioration of product quality [15–17]. *Aeromonas caviae* is a gram-negative bacterium that belongs to the family Aeromonadaceae [18]. *A. caviae* can infect many types of aquatic animals, such as Indian catfish (*Clarias batrachus*), rainbow trout (*Oncorhynchus mykiss*), white shrimp (*Penaeus vannamei*), crayfish (*Procambarus clarkia*), common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idellus*) [19–22]. The typical clinical symptoms of *A. caviae* infection include surface swimming, listlessness, inappetence, haemorrhagic septicemia, ulceration

and abdominal distention [19,23]. The widespread infections of *A. caviae* in fish species seriously threaten the healthy development of aquaculture [24].

The pathogenicity of *Aeromonas* species is complex and confounding. *Aeromonas* produces various toxins that can harm its hosts such as hemolysin, aerolysin and cytotoxic enterotoxins [25]. Dallal et al. found the presence of 5 virulence genes such as *alt*, *ast*, *act*, *aer* and *hly* among 12 strains of *Aeromonas* isolates, including *A. veronii*, *A. hydrophila*, and *A. caviae* [26]. Hence, it is imperative to detect the virulence genes in *Aeromonas* in order to assess its potential pathogenicity.

To the best of our knowledge, this is the first report of *A. caviae* as a causative agent in the largemouth bass (*M. salmoides*). The diseased *M. salmoides* typically showed anabrosis and redness of the body surface, and a large number of deaths were detected. The present study aimed to investigate the etiology of this disease and provide scientific reference for its diagnosis and treatment. An *A. caviae* strain was isolated from the diseased fish and identified through morphological observation, bacterial biochemical identification and 16S rDNA sequence analysis. The pathogenicity of this *A. caviae* strain was confirmed. In addition, histopathological observation of the diseased fish, virulent gene analysis and antibiotic susceptibility screening were performed to obtain information for the healthy breeding of *M. salmoides*.

## 2. Materials and Methods

### 2.1. Fish Specimens

Diseased *M. salmoides* specimens ( $15 \pm 2$  cm in length) were collected from the *M. salmoides* breeding base in Wuhan, Hubei Province, China. The diseased *M. salmoides* showed reduced feeding and slow swimming. The clinical symptoms of diseased *M. salmoides* included lethargy, surface swimming, redness of the skin and mouth, ulcers and turgidity. The illness of *M. salmoides* was lasted for 10 days, and the condition was controlled after drug treatment. The moribund fish were maintained in oxygenated bags and immediately transported to the laboratory for diagnosis and pathogen isolation. Healthy *M. salmoides* specimens ( $15 \pm 2$  cm in length) with no history of disease were obtained from the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. The fish were maintained in recirculating aquaria (300 L) for 14 days to allow them to acclimatize to the environment. During the acclimatization period, the water temperature was  $28 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ , the fish were fed with commercial feed twice a day and the water was renewed daily at a rate of 30%. All experimental procedures were conducted according to the guidelines of the Animal Experimental Ethical Inspection of Laboratory Animal Centre, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (ID Number: YFI2021-zhouyong-05).

### 2.2. Pathogen Detection and Identification

Samples of the gills, skin mucus, liver, kidney and spleen were subjected to standardized virological and parasitological analyses for diagnostic purposes. The fish liver, spleen and kidney were homogenized and filtered with  $0.22 \text{ }\mu\text{m}$  micron membrane for virus examination. The liver cell line of *M. salmoides* was used for virus isolation. The parasites were detected in diseased fish using an inverted microscope. Bacterial isolation was performed in a class II biosafety cabinet (ESCO, Singapore). The moribund fish were anesthetized in  $40 \text{ mg L}^{-1}$  tricaine methane sulfonate (MS-222; Sigma, Saint Louis, MO, USA), and the body surface was disinfected before dissection. The liver and kidney of each moribund fish were removed with an inoculation loop, inoculated onto brain heart infusion (BHI) agar plates (Difco, Detroit, MI, USA) and incubated at  $28 \text{ }^\circ\text{C}$  for 24 h. The colonies were subcultured twice, inoculated into BHI liquid medium and cultured at  $28 \text{ }^\circ\text{C}$  with shaking at 200 rpm. Purified bacterial cultures were used for Gram staining (Jiancheng, Nanjing, China) and observed with a scanning electron microscope (Hitachi, Tokyo, Japan). The obtained strain was designated WH21406.

### 2.3. 16S Ribosomal DNA Sequencing Analysis

The genomic DNA of WH21406 was extracted using a bacterial genomic DNA kit (Tiangen, Beijing, China). Universal primers 27F and 1492R (Table 1) were used to amplify the 16S rDNA. The amplification program was as follows: 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and 72 °C for 10 min. The amplified products were analysed using 1% agarose gel electrophoresis and visualized with an ultraviolet light transilluminator (Bio-Rad, Hercules, CA, USA). The amplified products were collected and sent to Huayu Gene Technology Co., Ltd., Wuhan, China, for sequencing; the results were compared against the NCBI database (<http://blast.ncbi.nlm.nih.gov>, accessed on 19 May 2022).

**Table 1.** Primers for 16S rRNA and virulence gene testing.

| Gene                           | Primer Sequence (5′–3′)                                      | Product Size (bp) | Optimal Annealing Temperature (°C) | References               |
|--------------------------------|--|-------------------|------------------------------------|--------------------------|
| 16SrRNA-F<br>16SrRNA-R         | AGAGTTTGATCATGGCTCAG<br>TACGGTTACCTTGTTACGACTT               | 1500              | 55                                 | Jensen et al. [27]       |
| <i>ahp</i> -F<br><i>ahp</i> -R | ATTGGATCCCTGCCTATCGCTTCAGTTCA<br>GCTAAGCTTGCATCCGTGCCGTATTCC | 911               | 55                                 | Hu et al. [28]           |
| <i>ela</i> -F<br><i>ela</i> -R | ACACGGTCAAGGAGATCAAC<br>CGCTGGTGTGGCCAGCAGG                  | 513               | 55                                 | Sen and Rodgers [25]     |
| <i>act</i> -F<br><i>act</i> -R | ATCGTCAGCGACAGCTTCTT<br>CTCATCCCTTGGCTTGTGT                  | 500               | 55                                 | Fu et al. [29]           |
| <i>fla</i> -F<br><i>fla</i> -R | TCCAACCGTYTGACCTC<br>GMYTGGTTGCGRATGGT                       | 608               | 55                                 | Hu et al. [28]           |
| <i>hly</i> -F<br><i>hly</i> -R | GGCCGGTGGCCCGAAGATACGGG<br>GGCGGCGCCGGACGAGACGGG             | 597               | 65                                 | Heuzenroeder et al. [30] |
| <i>alt</i> -F<br><i>alt</i> -R | TGACCCAGTCTGGCACGGC<br>GGTGATCGATCACCACCAGC                  | 442               | 64                                 | Nawaz et al. [31]        |
| <i>aer</i> -F<br><i>aer</i> -R | CAAGAACAAGTTCAAGTGGCCA<br>ACGAAGGTGTGGTTCCAGT                | 309               | 57                                 | Wang et al. [32]         |
| <i>lip</i> -F<br><i>lip</i> -R | ATCTTCTCCGACTGGTTCGG<br>CCGTGCCAGGACTGGGTCTT                 | 382               | 55                                 | Sen and Rodgers [25]     |

### 2.4. Bacterial Biochemical Identification

The bacterial biochemical characteristics were examined using a Biolog automatic microbial identification system (Biolog, Hayward, CA, USA). The bacterial strains were inoculated onto BUG solid medium at 30 °C for 24 h. A single colony was selected and evenly distributed in IF-A inoculation solution, which was then inoculated into a GEN III identification plate at a dose of 100 µL per well. The GEN III identification plate was placed in the Biolog automatic microbial identification system (Biolog, Hayward, CA, USA) for cultivation and automatic identification [33].

### 2.5. Screening of Virulence Genes

Eight virulence genes, namely, serine protease (*ahp*), flagella (*fla*), cytotoxic enterotoxin (*act*), elastase (*ela*), haemolysin (*hly*), heat-labile cytotoxic enterotoxin (*alt*), lipase (*lip*) and aerolysin (*aer*), were screened using PCR. The primers used for amplification of the virulence genes are listed in Table 1. The amplification programs were similar to those used for 16S rRNA gene amplification reported previously, except that different annealing temperatures were used. The amplified products were analysed with 1% agarose gel electrophoresis and visualized using the ultraviolet light transilluminator (Bio-Rad).

### 2.6. Histopathological Analysis

Tissues of the liver, spleen, kidney and intestine were collected from diseased and healthy *M. salmoides* specimens. The tissues were fixed in 4% paraformaldehyde, dehydrated with an ethanol gradient series, embedded in paraffin and sectioned using a microtome (Thermo, Waltham, MA, USA), stained with haematoxylin-eosin (Solarbio, Beijing, China) and observed under an optical microscope (Olympus, Tokyo, Japan) [34].

### 2.7. Antibiotic Susceptibility Test

The antibiotic susceptibility test for strain WH21406 was performed using the Kirby–Bauer disk diffusion method [35]. WH21406 was incubated in BHI at 28 °C for 24 h. Then, the concentration of bacterial cells was adjusted to  $1 \times 10^8$  CFU mL<sup>-1</sup> with sterile phosphate-buffered saline (PBS; Cytiva, Marlborough, CT, USA). The bacterial solution was pipetted onto Muller Hilton agar plates (Difco, Detroit, MI, USA) and spread evenly. The plates were left for 5 min, and then the drug-sensitive test papers were added. Ten antibacterial drugs, namely, florfenicol (30 µg), enrofloxacin (10 µg), neomycin sulphate (30 µg), doxycycline (30 µg), norfloxacin (10 µg), gentamicin (10 µg), streptomycin (10 µg), trimethoprim-sulfamethoxazole (25:75; 25 µg), tetracyclines (30 µg) and amikacin (30 µg), were selected (Hangwei, Hangzhou, China). The diameter of the inhibition zone was measured after incubation at 28 °C for 24 h [36]. The results were classified as sensitive (S), moderately sensitive (M) and resistant (R), according to the instructions for drug-sensitive test papers (Hangwei, Hangzhou, China).

### 2.8. Pathogenicity Assays

The pathogenicity assay was performed using healthy *M. salmoides* specimens, according to the methodology described in a previous study [37]. The median lethal dose (LD<sub>50</sub>) was calculated using the improved Kohl's method [38]. The bacterial strain WH21406 was cultured in BHI at 28 °C for 20 h, harvested via centrifugation at 4 °C and 6000 rpm for 5 min and washed three times with sterile PBS. The bacteria were re-suspended in sterile PBS at a 10-fold ratio to  $1.0 \times 10^4$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^6$ ,  $1.0 \times 10^7$  and  $1.0 \times 10^8$  CFU mL<sup>-1</sup>. The concentration of the bacterial suspension was determined using the plate colony counting method [38]. One hundred and eighty *M. salmoides* specimens were randomly divided into six groups (30 fish per group). The infected groups were intraperitoneally injected with 0.2 mL of each bacterial suspension ( $1.0 \times 10^4$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^6$ ,  $1.0 \times 10^7$  and  $1.0 \times 10^8$  CFU mL<sup>-1</sup>). The control group was injected with 0.2 mL of sterile PBS. The experiment was repeated twice. The mortality of all groups was recorded daily for 14 days, and the dead fish were subjected to bacterial isolation and identification.

## 3. Results

### 3.1. Clinical Symptoms

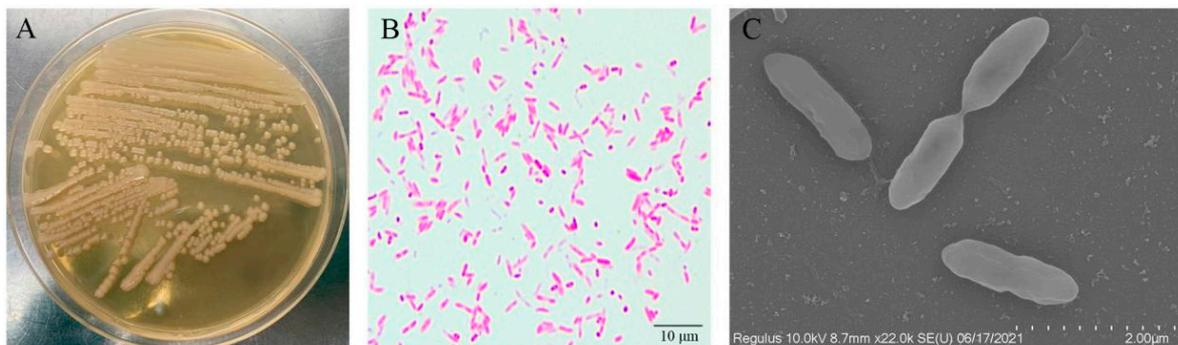
Diseased *M. salmoides* swam on the water surface and swam alone by the pond. Redness of the skin and mouth, ulcers and turgidity were observed (Figure 1A). After dissection, liver yellowing and blood loss were observed. No ascites was found in the abdominal cavity, the kidney was swollen and the muscle was red and bleeding (Figure 1B).



**Figure 1.** Clinical symptoms of diseased largemouth bass. (A) Clinical symptoms of body surface, (B) Clinical symptoms of internal organs.

### 3.2. Pathogen Isolation and Characterization

Buff, translucent, circular and convex bacterial colonies were observed on the BHI agar plates after incubation for 24 h (Figure 2A). No discernible differences in color or form were detected among all colonies on the plates. Additionally, no viruses or parasites were detected in any of the sampled fish. The bacteria from the diseased fish were gram-negative and rod-shaped (Figure 2B). The scanning electron microscope (Hitachi) also demonstrated that the bacteria were nearly rod-shaped (approximately  $2.4 \times 0.8 \mu\text{m}$ ; Figure 2C).



**Figure 2.** Colonial morphology, Gram staining and scanning electron microscopy image of the isolate WH21406. (A) Colonial morphology of WH21406, (B) Gram staining image of WH21406, (C) Scanning electron microscopy image of WH21406.

The bacterial strain WH21406 was identified as *Aeromonas caviae* with the Biolog Automatic Microbial Identification System. According to the test report, the WH21406 matched the *A. caviae* reference strain, and comparison with the reference strain in the database yielded a satisfactory score (Table 2).

**Table 2.** Results of Biolog identification of strain WH21406.

| Reagent             | Result * | Reagent                | Result * |
|---------------------|----------|------------------------|----------|
| A1 Negative Control | N        | E1 Gelatin             | P        |
| A2 Dextrin          | P        | E2 Glycyl-L-Proline    | P        |
| A3 D-Maltose        | P        | E3 L-Alanine           | P        |
| A4 D-Trehalose      | P        | E4 L-Arginine          | P        |
| A5 D-Cellobiose     | P        | E5 L-Aspartic Acid     | P        |
| A6 Gentiobiose      | B        | E6 L-Glutamic Acid     | P        |
| A7 Sucrose          | P        | E7 L-Histidine         | P        |
| A8 D-Turanose       | B        | E8 L-Pyroglutamic Acid | P        |
| A9 Stachyose        | B        | E9 L-Serine            | P        |

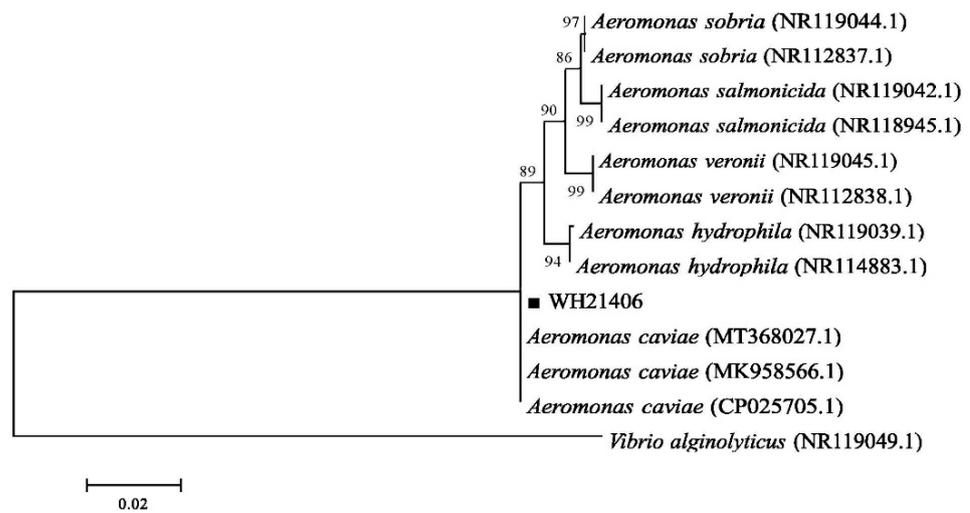
Table 2. Cont.

| Reagent                             | Result * | Reagent                               | Result * |
|-------------------------------------|----------|---------------------------------------|----------|
| A10 Positive Control                | P        | E10 Lincomycin                        | B        |
| A11 PH6                             | P        | E11 Guanidine HCl                     | B        |
| A12 PH5                             | N        | E12 Niaproof 4                        | N        |
| B1 D-Raffinose                      | P        | F1 Pectin                             | P        |
| B2 $\alpha$ -D-Lactose              | P        | F2 D-Galacturonic Acid                | P        |
| B3 D-Melibiose                      | P        | F3 L-Galactonic Acid Lactone          | P        |
| B4 $\beta$ -Methyl-D-Glucoside      | P        | F4 D-Gluconic Acid                    | P        |
| B5 D-Salicin                        | P        | F5 D-Glucuronic Acid                  | P        |
| B6 N-Acetyl-D-Glucosamine           | P        | F6 Glucuronamide                      | N        |
| B7 N-Acetyl- $\beta$ -D-Mannosamine | P        | F7 Mucic Acid                         | P        |
| B8 N-Acetyl-D-Galactosamine         | P        | F8 Quinic Acid                        | P        |
| B9 N-Acetyl Neuraminic Acid         | B        | F9 D-Saccharic Acid                   | P        |
| B10 1% NaCl                         | P        | F10 Vancomycin                        | P        |
| B11 4% NaCl                         | B        | F11 Tetrazolium Violet                | P        |
| B12 8% NaCl                         | N        | F12 Tetrazolium Blue                  | P        |
| C1 $\alpha$ -D-Glucose              | P        | G1 P-Hydroxy-Phenylacetic Acid        | N        |
| C2 D-Mannose                        | P        | G2 Methyl Pyruvate                    | P        |
| C3 D-Fructose                       | P        | G3 D-Lactic Acid Methyl Ester         | P        |
| C4 D-Galactose                      | B        | G4 L-Lactic Acid                      | P        |
| C5 3-Methyl Glucose                 | P        | G5 Citric Acid                        | P        |
| C6 D-Fucose                         | P        | G6 6 $\alpha$ -Keto-Glutaric Acid     | P        |
| C7 L-Fucose                         | P        | G7 D-Malic Acid                       | L        |
| C8 L-Rhamnose                       | P        | G8 L-Malic Acid                       | P        |
| C9 Inosine                          | P        | G9 Bromo-Succinic Acid                | P        |
| C10 1% Sodium Lactate               | P        | G10 Nalidixic Acid                    | P        |
| C11 Fusidic Acid                    | N        | G11 Lithium Chloride                  | P        |
| C12 D-Serine                        | P        | G12 Potassium Tellurite               | N        |
| D1 D-Sorbitol                       | P        | H1 Tween 40                           | P        |
| D2 D-Mannitol                       | P        | H2 $\gamma$ -Amino-Butyric Acid       | P        |
| D3 D-Arabitol                       | B        | H3 $\alpha$ -Hydroxy-Butyric Acid     | P        |
| D4 Myo-inositol                     | B        | H4 $\beta$ -Hydroxy-D, L-Butyric Acid | P        |
| D5 Glycerol                         | P        | H5 $\alpha$ -Keto-Butyric Acid        | P        |
| D6 D-Glucose-6-PO4                  | P        | H6 Acetoacetic Acid                   | P        |
| D7 D-Fructose-6-PO4                 | P        | H7 Propionic Acid                     | P        |
| D8 D-Aspartic Acid                  | P        | H8 Acetic Acid                        | P        |
| D9 D-Serine                         | P        | H9 Formic Acid                        | B        |
| D10 Troleandomycin                  | P        | H10 Aztreonam                         | B        |
| D11 Rifamycin SV                    | P        | H11 Sodium Butyrate                   | B        |
| D12 Minocycline                     | N        | H12 Sodium Bromate                    | N        |

\* Notes: P = Positive, N = Negative, B = Borderline, L = Less than A1 well.

### 3.3. Bacterial 16S rDNA Sequence Analysis

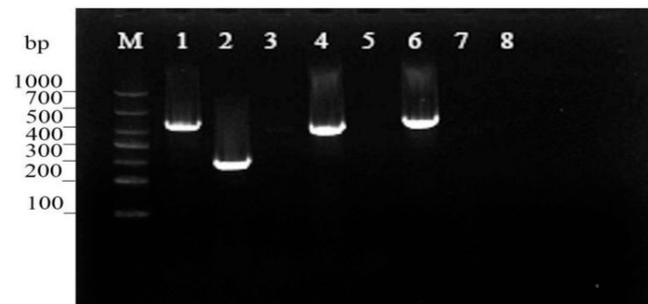
The 16S rDNA of strain WH21406 is 1407 bp in length. BLAST results of the 16S rDNA sequences showed that WH21406 was 100% similar to *A. caviae* (MT368027.1). The phylogenetic was constructed using the neighbor-joining method [39] using MEGA 6.0 software. The phylogenetic analysis results indicated that WH21406 and *A. caviae* (MT368027.1, MK958566.1, CP025705.1) aggregated into a branch (Figure 3).



**Figure 3.** Phylogenetic tree for strain WH21406 on the basis of 16S rDNA sequences (the numbers represent bootstrap values).

### 3.4. Virulence Gene Assessment

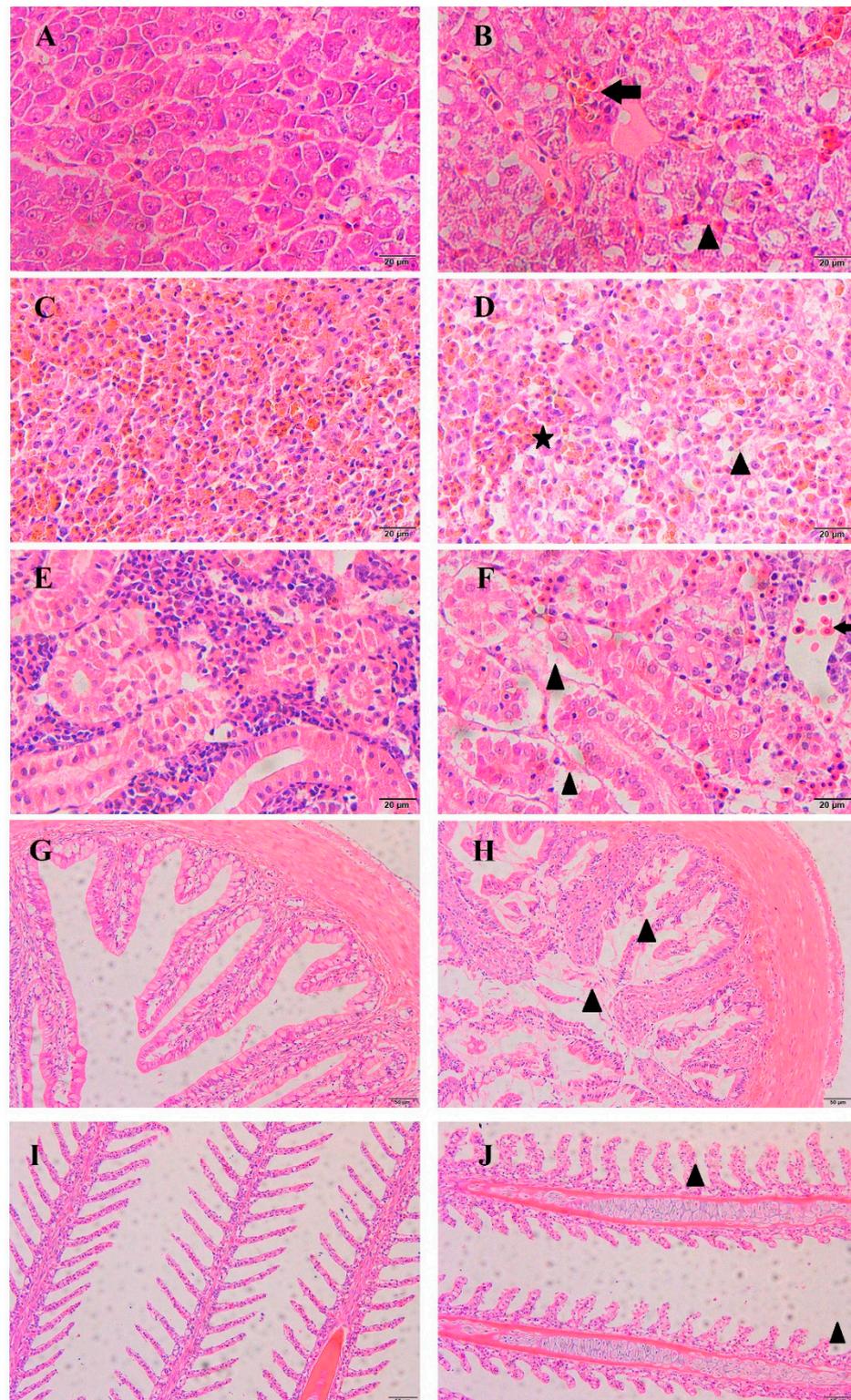
According to the PCR profiles of the eight virulence genes, *fla*, *aer*, *ela* and *hly* were found in WH21406, and *act*, *ahp*, *alt* and *lip* genes were not detected (Figure 4).



**Figure 4.** Agarose gel electrophoresis of PCR products of the virulence genes. M: DL1000 marker, 1: *fla*, 2: *aer*, 3: *act*, 4: *ela*, 5: *ahp*, 6: *hly*, 7: *alt*, 8: *lip*.

### 3.5. Histopathological Observations

The tissues of the diseased fish showed obvious haemorrhaging and necrosis. In the diseased fish, a large number of inflammatory cells had infiltrated the liver (Figure 5B, arrow), and the hepatocyte showed vacuolation (Figure 5B, triangle). The splenic cells of the healthy fish were tightly arranged, but the splenic tissue of the diseased fish showed extensive necrocytosis (Figure 5D, triangle) and hemosiderin deposits (Figure 5D, asterisk). In the diseased fish, a large number of inflammatory cells had infiltrated the kidney (Figure 5F, arrow), and necrosis was observed in the glomerulus (Figure 5F, triangle). In the intestine of the diseased fish, considerable damage and abnormal changes in the villi structure were detected (Figure 5H, triangle). In the gills of the diseased fish, necrosis of the epithelia and swelling were observed (Figure 5J, triangle).



**Figure 5.** Histological observation of the tissues of healthy and diseased largemouth bass specimens. (A) Normal liver tissue; (B) Liver tissue from diseased largemouth bass, inflammatory cell infiltration (arrow), hepatocyte vacuolation (triangle); (C) Normal spleen tissue; (D) Spleen tissue from diseased largemouth bass, necrocytosis (triangle), hemosiderin deposits (asterisk); (E) Normal kidney tissue; (F) Kidney tissue from diseased largemouth bass, inflammatory cell infiltration (arrow), glomerulus necrosis (triangle); (G) Normal gut tissue; (H) Gut tissue from diseased largemouth bass, intestinal villi damage (triangle); (I) Normal gill filaments; (J) Gill tissue from diseased largemouth bass, epithelia necrosis and swelling (triangle).

### 3.6. Antibiotic Susceptibility Test

The antibiotic susceptibility test demonstrated that strain WH21406 was highly sensitive to enrofloxacin, norfloxacin, streptomycin and amikacin and moderately sensitive to gentamicin (Table 3). Additionally, WH21406 was resistant to florfenicol, neomycin sulphate, compound sulfamethoxazole, doxycycline and tetracyclines.

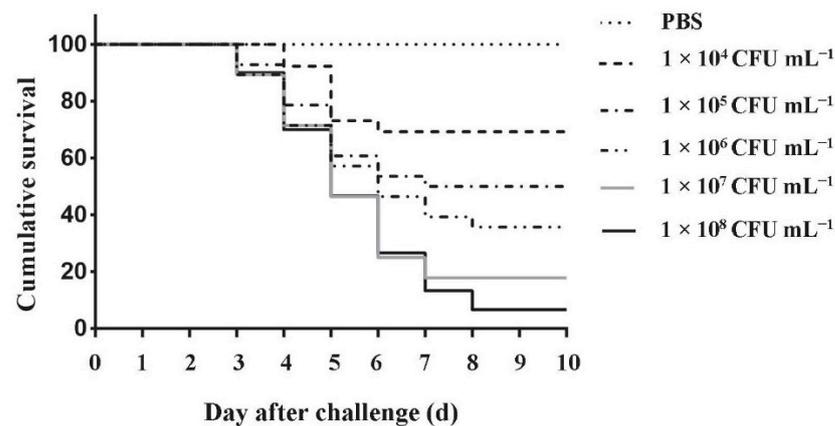
**Table 3.** Antibiotic susceptibility test results.

| Drug Name                 | Inhibition Zone (mm) | Sensitivity * |
|---------------------------|----------------------|---------------|
| Florfenicol               | 6                    | R             |
| Enrofloxacin              | 22                   | S             |
| Neomycin sulfate          | 6                    | R             |
| Doxycycline               | 6                    | R             |
| Norfloxacin               | 18                   | S             |
| Gentamicin                | 12                   | I             |
| Streptomycin              | 22                   | S             |
| Compound Sulfamethoxazole | 6                    | R             |
| Tetracyclines             | 6                    | R             |
| Amikacin                  | 17                   | S             |

\* Notes: S, susceptible; I, intermediate; R, resistant.

### 3.7. Pathogenicity Assays

The cumulative survival rate of *M. salmoides* challenged with the isolate *A. caviae* WH21406 for 10 days is shown in Figure 6. The fish injected with *A. caviae* WH21406 died between the fourth and seventh day post-injection. The LD<sub>50</sub> of *A. caviae* WH21406 injected intraperitoneally was calculated to be  $3.46 \times 10^5$  CFU mL<sup>-1</sup> using the improved Kou's method. The symptoms of the artificially infected fish were consistent with those of the naturally infected fish, which showed ulcers and turgidity on the body surface and fin rot. No death or clinical symptoms were observed in the control group. Furthermore, *A. caviae* was re-isolated from the artificially infected fish. Mortality and symptoms were consistent in the repeated trials. These results indicated that the isolate *A. caviae* WH21406 was the pathogen.



**Figure 6.** Survival rates of largemouth bass specimens challenged with different doses of *A. caviae* WH21406 for 10 days post-infection.

## 4. Discussion

The genus *Aeromonas* contains many opportunistic pathogens that cause infections in many aquatic and terrestrial animals, including human beings [40–42]. *A. caviae* is a mesophilic species, and it is widely distributed in various aquatic ecosystems such as wastewater, aquaculture water and urban drinking water [18,43,44]. Recently, many studies revealed that the incidence of *A. caviae* infection in fish has increased, and the clinical symptoms are in accordance with the typical signs of *Aeromonas* spp. infection [23,44].

In this study, *A. caviae* WH21406 was isolated from diseased *M. salmoides* and identified using morphological characterization, biochemical identification and 16S rDNA sequence analysis. Additionally, the clinical signs of the artificially infected fish were similar to those of the naturally infected fish, and *A. caviae* was re-isolated from the artificially infected fish. These results indicated that *A. caviae* was the pathogen in the diseased fish. To the best of our knowledge, this is the first report of *A. caviae* infection in *M. salmoides*, and this strain caused a large number of deaths.

In this study, the LD<sub>50</sub> of *A. caviae* strain WH21406 was calculated to be  $3.46 \times 10^5$  CFU mL<sup>-1</sup>. According to previous studies on *M. salmoides*, the LD<sub>50</sub> of *A. veronii* HN1903 strain was  $3.72 \times 10^4$  CFU (g fish)<sup>-1</sup> [10], and the LD<sub>50</sub> of *A. hydrophila* LY4 strain was  $5.7 \times 10^5$  CFU mL<sup>-1</sup> [45]. These results demonstrate that *A. caviae* strain WH21406 is highly virulent to *M. salmoides*.

Virulence genes are good indicators of the pathogenicity of a microorganism [46]. We detected virulence genes *fla*, *aer*, *ela* and *hly* in *A. caviae* strain WH21406 (Figure 4). These virulence genes were extensively utilized to investigate the potential pathogenicity of *Aeromonas* spp. [47]. Flagella coded by *fla* are required for cellular propulsion. Motility is key for pathogenic bacteria that adhere to host cells and cause disease [25]. Aerolysin coded by *aer* is a pore-forming toxin that can damage epithelial cells in the intestine [48]. Elastase (*ela*), a secreted protein, can induce epithelial cell apoptosis. Haemolysins (*hly*) produce cytotoxic effects and lysis of erythrocytes, and they contribute to bacterial evasion from host inflammatory responses [49]. The virulence genes present in *A. caviae* WH21406 indicate that the synergistic effects conferred by combinations of these genes are key contributors to its high pathogenicity. And *act*, *ahp*, *alt* and *lip* genes were not detected in *A. caviae* WH21406. Mohamad [50] found that approximately 30 percent of *A. caviae* harbour *ahp* and *lip* genes. Some *Aeromonas* species establish in the gastrointestinal tract and can produce enteritis via elaboration of enterotoxigenic molecules such as cytotoxic enterotoxin (*act*) and cytotoxic heat-labile enterotoxin (*alt*) [40]. No obvious enteritis symptoms occurred in the *M. salmoides*, which may be related to the absence of *act* and *alt* genes in *A. caviae* WH21406.

The histopathological analysis showed that the *A. caviae* isolate can cause obvious tissue damage and hemosiderin accumulation in the gill, liver, kidney, spleen and gut, which was similar to the damage found in *Silurus meridionalis* and *Rhamdia quelen* infected by *A. caviae* [23,51]. Cytopathic changes caused by several bacterial toxins may be an important cause of tissue damage. Previous studies indicated that haemolysin produced by *A. caviae* has a strong virulence and causes rupture and dissolution of red blood cells [23]. This may be the main reason for hemosiderin accumulation in the spleen and kidney.

Few studies were conducted regarding the antibiotic sensitivity of *A. caviae*. Antibiotic susceptibility testing for *A. caviae* WH21406 provided a reference for the treatment of *A. caviae* infection. The isolate WH21406 was highly sensitive to enrofloxacin, norfloxacin, streptomycin and amikacin and resistant to florfenicol, neomycin sulphate, compound sulfamethoxazole, doxycycline and tetracyclines (Table 3). The *A. caviae* strain from *Macrobachium rosenbergii* was highly sensitive to chloramphenicol, florfenicol, tetracycline and doxycycline and resistant to norfloxacin, erythromycin, streptomycin, compound sulfamethoxazole and rifampicin [52]. *A. caviae* strains from different fish exhibit different antibiotic sensitivity characterizations. Therefore, drug sensitivity testing is particularly important for the treatment of diseases in the production process.

## 5. Conclusions

In this study, pathogenic *A. caviae* WH21406 was isolated from diseased *M. salmoides*. The artificial infection test showed that *A. caviae* WH21406 is highly pathogenic to *M. salmoides*, and this isolate is highly sensitive to enrofloxacin, norfloxacin, streptomycin and amikacin. The results of this study provide a reference for the diagnosis and treatment of fish infection with *A. caviae*.

**Author Contributions:** Conceptualization, M.X. and Y.Z.; methodology, M.X.; software, Z.X.; validation, Y.L., N.J. and M.X.; formal analysis, W.L.; investigation, Y.M.; resources, Z.X.; data curation, M.X.; writing—original draft preparation, M.X.; writing—review and editing, Y.Z.; visualization, N.J.; supervision, L.Z.; project administration, Y.F.; funding acquisition, Y.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Key Research Development Program of China, Grant/Award number: 2019YFD0900105; the National Natural Science Foundation of Hubei, Grant/Award number: 2021CFB486; the Central Public-interest Scientific Institution Basal Research Fund, Grant/Award number: 2020TD44; the open project of Agriculture Ministry Key Laboratory of Healthy Freshwater Aquaculture, Grant/Award number: ZJK202210.

**Institutional Review Board Statement:** The study was conducted in accordance with the guidelines of the Animal Experimental Ethical Inspection of Laboratory Animal Centre, and approved by the Institutional Review Board of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (ID Number: YFI2021-zhouyong-05).

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** We thank the anonymous reviewers for their insightful comments and suggestions, and we also thank the National Key Research Development Program of China (2019YFD0900105), the National Natural Science Foundation of Hubei(2021CFB486), the Central Public-interest Scientific Institution Basal Research Fund (2020TD44), and the open project of Agriculture Ministry Key Laboratory of Healthy Freshwater Aquaculture (ZJK202210).

**Conflicts of Interest:** The authors declare that they have no known competing financial interests or personal relationships that may have appeared to influence the work reported in this paper.

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