

Original Research Articles

Isolation, identification and multilocus sequence typing of seven *Aeromonas veronii* strains

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This study characterized *Aeromonas veronii* isolates recovered from diseased *Micropterus salmoides*, *Procambarus clarkii*, and *Ctenopharyngodon idella* in Guangxi, China. The objective was to assess genetic diversity, virulence gene profiles, and antimicrobial resistance patterns to support effective disease control. Seven strains were confirmed as *A. veronii* using morphological, physiological, biochemical, and molecular methods. Virulence gene screening showed that all isolates carried GcaT, OmpAI, Hly, Fla, and Exu. Artificial challenge tests demonstrated strong pathogenicity, with median lethal dose (LD₅₀) values ranging from 1.43×10⁶ to 3.58×10⁶ CFU/individual. Antimicrobial susceptibility testing indicated sensitivity to ceftriaxone, cefepime, and fosfomycin, but resistance to amoxicillin, teicoplanin, and sulbactam. Multilocus sequence typing (MLST) identified seven novel sequence types (ST2742, ST2740, ST2765, ST2760, ST2770, ST2774, and ST2775). These results confirm that *A. veronii* isolates from Guangxi exhibit high genetic diversity, carry multiple virulence genes, and display multidrug resistance. The findings provide a basis for epidemiological surveillance and targeted control of this pathogen.

INTRODUCTION

Aeromonas veronii belongs to the order Aeromonadales, family Aeromonadaceae, and genus *Aeromonas*. It is a Gram-negative, facultatively anaerobic, motile, mesophilic bacterium commonly found in freshwater and estuarine environments.¹⁻⁴ Numerous studies have shown that this bacterium can infect a wide range of aquatic animals as well as mammals,⁵⁻⁸ including humans, causing diseases such as gastroenteritis, peritonitis, meningitis, septicemia, and wound infections.⁹⁻¹² For instance, Tian et al. reported that *A. veronii* strains isolated in Guizhou province caused symptoms in *Ictalurus punctatus* including body surface ulceration, fin-base hemorrhage, gill erosion, and intestinal hyperemia.¹³ Gao et al. documented that *A. veronii* isolates from Guizhou exhibited strong pathogenicity toward *Megalobrama amblycephala*, *Carassius auratus*, and *Aristichthys nobilis*.¹⁴ It was also shown that *A. veronii* strains isolated in Sichuan could induce ascites, ulceration, and high mortality in fish.¹⁵⁻¹⁸ Collectively, these findings indicate that

A. veronii has significant pathogenic potential, posing a serious threat to both aquaculture and public health. Therefore, investigating the genetic diversity of *A. veronii*, clarifying phylogenetic relationships and evolutionary patterns among strains from different sources, and conducting in-depth identification and typing studies are essential.

Currently, most studies on *A. veronii* have focused on isolation, identification, and preliminary characterization from single host species or limited geographic regions. However, comprehensive analyses of genetic diversity, virulence gene profiles, and antimicrobial resistance patterns among *A. veronii* isolates from representative cultured aquatic species in Guangxi remain limited. Traditional identification methods—such as morphological observation, physiological and biochemical tests, and 16S rRNA gene sequence analysis—are susceptible to environmental interference and often fail to distinguish closely related species.¹⁹⁻²⁵ In comparison, the *gyrB* gene evolves faster, with interspecies divergence reaching up to 15.2%, which partly overcomes the limitations of the 16S rRNA gene for

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identifying closely related taxa.²⁶ Multilocus sequence typing (MLST), which analyzes sequence variations in multiple housekeeping genes, can reveal genetic relationships and evolutionary patterns among bacterial strains with high resolution and excellent reproducibility, and has been widely applied in bacterial molecular epidemiological studies.²⁷ For example, Wei et al. used MLST to identify dominant sequence types in three *Escherichia coli* strains,²⁸ and Wu et al. applied MLST to the epidemiological study of *Aeromonas hydrophila*.²⁹ Robinson and Johnson used MLST to elucidate the evolutionary pathways of MRSA and the correlations between isolates from different periods, respectively.^{30,31}

Virulence genes are key determinants of *A. veronii* pathogenicity. It is known that *A. veronii* carries various virulence factors, including the cytotoxic enterotoxin (*act*), aerolysin (*aerA*), and collagenase gene (*acg*), among others.³² Furthermore, studies by Geng et al. indicate that this bacterium exhibits multidrug resistance, suggesting that conventional antibiotic therapies may have limited efficacy against it.^{33,34} In Guangxi, *Micropterus salmoides*, *Ctenopharyngodon idella*, and *Procambarus clarkii* are important aquaculture species that have been frequently affected by *A. veronii* infections in recent years. Although some progress has been made in the study of *A. veronii* domestically and internationally, systematic research on the genetic diversity, virulence gene profiles, and antimicrobial resistance characteristics of *A. veronii* strains from specific cultured species in Guangxi remains insufficient, making it difficult to provide precise guidance for disease prevention and control in local aquaculture. Therefore, this study focused on *A. veronii* strains isolated from cultured species in Guangxi, including *M. salmoides*, *C. idella*, and *P. clarkii*. The isolates were identified through morphological observation, physiological and biochemical tests, and molecular methods. MLST was used to analyze genetic relationships and evolutionary patterns among the strains. Meanwhile, virulence gene profiles and antimicrobial resistance characteristics were examined to elucidate the genetic diversity, distribution of virulence genes, and resistance features of *A. veronii* from different cultured species in Guangxi, thereby providing a scientific basis for disease prevention and control in local aquaculture.

MATERIALS AND METHODS

2.1. MATERIALS

The samples of diseased *C. idella* and *M. salmoides* were collected from the same fish farm in Guangxi, while the samples of diseased *P. clarkii* were from another different fish farm in Guangxi. All the diseased animals showed typical clinical symptoms of bacterial infection at the time of sampling. *C. idella* and *M. salmoides* presented with external bleeding, abdominal distension, and ascites. The *P. clarkii* showed loss of appetite, listlessness, and deep spots on the local carapace, with some edges ulcerated. Healthy control samples of the same species were collected from the freshwater aquaculture base of the Guangxi Academy of Fish-

ery Sciences. Blood nutrient agar plates used in the experiments were purchased from Guangdong Huankai Microbial Science and Technology Co., Ltd. Antimicrobial susceptibility test discs were obtained from Beijing Tiantan Pharmaceutical Technology Development Company. PCR reagents, including 2×Taq PCR Master Mix, bacterial DNA extraction kits, and agarose gel recovery kits, were all sourced from Beijing Aidlab Biotechnologies Co., Ltd.³⁵ The animal experiments involved in this study were approved by the Animal Ethics Committee of Guangxi Academy of Fishery Sciences (Approval No. 2026-GAFS-08).

2.2. EXPERIMENTAL METHODS

2.2.1. ISOLATION AND PURIFICATION OF PATHOGENIC BACTERIA

Under aseptic conditions, tissues, including the hepatopancreas and kidney, were collected from experimental samples of *M. salmoides*, *C. idella*, and *P. clarkii*. These tissues were inoculated onto both nutrient agar and blood nutrient agar plates, then incubated at 30 °C for 24 h in a constant-temperature incubator. Single colonies were selected and repeatedly streaked for purification over three cycles. This procedure yielded seven purified pathogenic bacterial isolates, which were designated as GX-PC-22041102, GX-MS-22040201, GX-MS-24110416, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, and GX-CI-24110404. The purified strains were stored at -80 °C for subsequent use.²⁸

2.2.2. MORPHOLOGICAL OBSERVATION AND PHENOTYPIC IDENTIFICATION OF PATHOGENS

The isolates were streaked on agar plates for cultivation to observe colonial morphological characteristics. Physiological and biochemical properties of the strains were determined using micro-bacterial biochemical identification tubes.³⁶

2.2.3. EXPERIMENTAL INFECTION

To determine the pathogenicity of the isolated bacterial strains, artificial challenge experiments were conducted on healthy *M. salmoides*, *C. idella*, and *P. clarkii*. Purified strains GX-PC-22041102, GX-MS-22040201, GX-MS-24110416, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, and GX-CI-24110404 were inoculated onto nutrient agar plates and incubated at 30 °C for 18 h. Bacterial lawns were then washed off with sterile physiological saline to prepare bacterial suspensions. Bacterial concentrations were adjusted to three serial dilutions: 9×10^8 , 9×10^7 , and 9×10^6 CFU/mL using the plate counting method.

A total of 720 healthy animals were selected after a two-week acclimatization period. This included 120 *P. clarkii* (body weight 25 ± 2 g), randomly divided into 4 groups (3 challenge groups and 1 control group); 210 *M. salmoides* (body weight 13 ± 2 g), divided into 7 groups (6 challenge groups and 1 control group); and 390 *C. idella* (body weight 30 ± 5 g), divided into 13 groups (12 challenge groups and 1 control group). Each animal received an intraperitoneal

injection of 0.1 mL of bacterial suspension for the challenge groups or 0.1 mL of sterile saline for the control group. Disease onset and mortality in each group were observed and recorded daily for 15 consecutive days. Dead individuals were dissected, and liver and spleen samples were collected for bacterial re-isolation and identification. The median lethal dose (LD₅₀) for each strain was calculated using the modified Karber's method.³⁵ The specific LD₅₀ calculation steps are illustrated with *P. clarkii* as an example. 120 healthy *P. clarkii* were randomly divided into 4 groups (3 dose groups and 1 control group), with 30 individuals in each group. The experimental groups were injected with doses of 9×10^7 , 9×10^6 and 9×10^5 CFU/individual respectively, while the control group was injected with the same volume of sterile physiological saline. The observation period was 15 days, and the cumulative mortality rate of each group was recorded. The injection doses and mortality rates of each experimental group are shown in [Table 4](#). The LD₅₀ was calculated using the formula: $\log_{10}(\text{LD}_{50}) = X_m - i(\sum p - 0.5)$. In this formula, LD₅₀ represents the median lethal dose, with the unit of CFU/individual; X_m represents the logarithmic dose of the highest dose group, that is, $\log_{10}$ of the highest group dose; i represents the difference in logarithmic doses between adjacent groups, and it is required that each dose group forms a geometric sequence; p represents the mortality rate of each group, expressed as a decimal; $\sum p$ represents the sum of the mortality rates of all groups; 0.5 is a constant for formula correction. The specific calculation steps are as follows: First, convert the injection dose per individual of each group into logarithmic form $X = \log_{10}$ dose; Second, determine X_m and the difference in logarithmic doses between adjacent groups i ; Third, calculate the sum of the mortality rates of all groups $\sum p$; Fourth, substitute the above parameters into the formula to calculate $\log_{10}(\text{LD}_{50})$; Fifth, obtain the LD₅₀ value by taking the antilogarithm $10^{\log_{10}(\text{LD}_{50})}$.

2.2.4. 16S RRNA AND GYRB GENE IDENTIFICATION AND PHYLOGENETIC ANALYSIS

Bacterial genomic DNA was extracted using a bacterial DNA extraction kit (Beijing Aidlab Biotechnology Co., Ltd.). PCR primers were synthesized by TaKaRa Bio (Dalian, China). The primers used for amplifying the bacterial 16S *rRNA* gene were 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-ACGGCTACCTTGTACGACTT-3' (reverse), yielding a product of approximately 1441 bp. The primers for the *gyrB* gene were UP1 (5'-GAAGTCATCATGACCGTTCTGCAYGCNNGGNGNAARTTYGA-3') and UP2 (5'-AGCAGGGTACGGATGTGCGAGCCRTC-NACRTCNGCRTCNGTCAT-3'), yielding a product of approximately 1143 bp.⁴ The PCR amplification reaction was performed in a 50 µL system containing 25 µL of 2×F8 FastLong PCR MasterMix, 2 µL each of forward and reverse primers, 5 µL of template DNA, and 18 µL of nuclease-free water. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 10 s; followed by a final extension at 72 °C for 5

min. The PCR products were then analyzed by 2% agarose gel electrophoresis.³⁵ Specific bands were excised and sent to TaKaRa Bio (Dalian) for sequencing. The obtained sequences were subjected to BLAST homology analysis on the NCBI website. Phylogenetic trees based on the 16S *rRNA* and *gyrB* genes were constructed using the neighbor-joining method with 1,000 bootstrap replicates in MEGA 5 software.

2.2.5. ANTIMICROBIAL SUSCEPTIBILITY TESTING

Bacterial suspensions were adjusted to a turbidity of 0.5 McFarland standard and uniformly spread onto the surface of nutrient agar plates. Antimicrobial susceptibility test discs were then aseptically placed onto the inoculated plates. After incubation at 30 °C for 24 h, the diameters of the inhibition zones were measured. The results were interpreted and categorized as susceptible (S), intermediately susceptible (I), or resistant (R) according to the standards established by the Clinical and Laboratory Standards Institute (CLSI).

2.2.6. DETECTION OF VIRULENCE GENES

Eighteen virulence genes in *Aeromonas veronii* were detected by PCR. These included the genes for the ADP-ribosylating toxin (*AexT*), the type III secretion system inner membrane component (*AscV*), glycerophospholipid-cholesterol acyltransferase (*GcaT*), cytotoxic enterotoxin (*Act*), the extracellular virulence factor aerolysin (*aerA*), collagenase (*Acg*), serine protease (*Ahp*), hemolysin (*Hly*), elastase (*AhyB*), lipase (*Lip*), heat-stable enterotoxin (*Ast*), heat-labile enterotoxin (*Alt*), flagellin (*LafA*), temperature sensitive protease (*EprCAI*), outer membrane protein (*OmpAI*), flagella (*Fla*), nuclease (*Exu*), and serine protease (*Ser*). The sequences of the corresponding primers are detailed in [Table 1](#). The PCR reaction system and conditions were the same as those described in section 2.2.4.³⁵

2.2.7. MLST OF STRAINS

In this study, six housekeeping genes (*gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*) were selected for MLST analysis. The sequences of the corresponding primers are detailed in [Table 1](#). The PCR reaction system and conditions were the same as those described in section 2.2.4. The sequences of the six obtained housekeeping genes were subjected to BLAST homology analysis on the NCBI website. These sequences were then submitted to the PubMLST database for comparison and analysis to determine the allele number for each gene and ultimately define the novel sequence type (ST). Additionally, 12 *Aeromonas* strains from the PubMLST *Aeromonas* database were selected as reference strains ([Table 5](#)). A phylogenetic tree based on the different STs was constructed using the neighbor-joining method with 1,000 bootstrap replicates in MEGA 5 software. The sequences have been deposited in the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, and GenBase accession numbers were obtained ([Table 2](#)).

Table 1. Primer information of virulence genes and housekeeping genes.

Gene	Primer sequences(5'-3')	size(bp)	Tm(°C)	Reference
<i>AcsV</i>	F: CTCGAAGTGAAGAGCAGAATG R: GAACATCTGGCTCTCCTTCGATG	537	55	35
<i>AexT</i>	F: ATGCAGATTCAAGCAAACAC R: TTGCCGATCCACTCTTTGAT	226	55	
<i>Act</i>	F: AGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCTTGAATC	232	55.7	
<i>aerA</i>	F: CCTATGGCTGAGCGAGAAG R: CCAGTTCCAGTCCCACCACT	431	64.3	
<i>GcaT</i>	F: CTCCTGGAATCCCAAGTATCAG R: GGCAGGTTGAACAGCAGTATCT	237	55.7	
<i>Acg</i>	F: AACAAAGCACCCGTTAAGCCAC R: ACGTAGTCGAGCCCCCTTGAGG	761	55	
<i>OmpAl</i>	F: GACGATATCATGATGAAAATGGCTCTT R: GCGAAGCTTTTACTTCTGAAGTTCTTG	1026	49.9	
<i>Ahp</i>	F: ATTGGATCCCTGCCTA R: GCTAAGCTTGCATCCG	911	56	
<i>Hly</i>	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGG	597	62	
<i>Ast</i>	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331	63	
<i>Alt</i>	F: TGACCCAGTCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	57	
<i>Fla</i>	F: TCCAACCGTYTGACCTC R: GMYTGGTTGCGRATGGT	608	53	
<i>Exu</i>	F: RGACATGCACAACCTCTTCC R: GATTGGTATTGCCYTGAAS	323	53	
<i>Ser</i>	F: CACCGAAGTATTGGGTCAAG R: GGCTCATGCGTAACTCTGGT	350	57	
<i>AhyB</i>	F: ACACGGTCAAGGAGATCAAC R: CGCTGGTGTGGCCAGCAGG	513	53	
<i>Lip</i>	F: CAYCTGGTKCCGCTCAAG R: GTRCCGAACCAAGTCGGAGAA	247	57	
<i>lafA</i>	F: GGTCTGCGCATCCAATC R: GCTCCAGACGGTTGATG	550	56	
<i>eprCAI</i>	F: GCTCGACGCCAGCTCACC R: GGCTCACCAGCATTGGATTCTG	387	63	
<i>gyrB</i>	F: GGGGTCTACTGCTTCACCAA R: CTTGTCCGGGTTGTACTCGT	669	50.9	
<i>groL</i>	F: CAAGGAAGTTGCTTCCAAGG R: CATCGATGATGGTGGTGTTT	782	50.2	
<i>glfA</i>	F: TTCCGTCTGCTCTCCAAGAT R: TTCATGATGATGCCGAGTA	626	50.2	
<i>metG</i>	F: TGGCAACTGATCCTCGTACA R: TCTTGTGGCCATCTCTTCC	657	54.2	
<i>ppsA</i>	F: AGTCCAACGAGTACGCCAAC R: TCGGCCAGATAGAGCCAGGT	619	55.0	
<i>recA</i>	F: AGAACAAACAGAAGGCACTGG R: AACTTGAGCGCGTTACCAC	640	56.9	

4

Table 2. GenBase accession numbers of the 16S *rRNA*, *gyrB*, and six housekeeping genes of seven strains.

Strains	Gene	GenBase	Strains	Gene	GenBase
GX-PC-22041102	16S <i>rRNA</i>	C_AA131221.1	GX-CI-22041401	16S <i>rRNA</i>	C_AA131173.1
	<i>gyrB</i>	C_AA131222.1		<i>gyrB</i>	C_AA131174.1
	<i>groL</i>	C_AA131224.1		<i>groL</i>	C_AA131176.1
	<i>gyrB</i>	C_AA131225.1		<i>gyrB</i>	C_AA131177.1
	<i>gltA</i>	C_AA131223.1		<i>gltA</i>	C_AA131175.1
	<i>metG</i>	C_AA131226.1		<i>metG</i>	C_AA131178.1
	<i>ppsA</i>	C_AA131227.1		<i>ppsA</i>	C_AA131179.1
	<i>recA</i>	C_AA131228.1		<i>recA</i>	C_AA131180.1
GX-MS-22040201	16S <i>rRNA</i>	C_AA131205.1	GX-CI-22041402	16S <i>rRNA</i>	C_AA131181.1
	<i>gyrB</i>	C_AA131206.1		<i>gyrB</i>	C_AA131182.1
	<i>groL</i>	C_AA131208.1		<i>groL</i>	C_AA131184.1
	<i>gyrB</i>	C_AA131209.1		<i>gyrB</i>	C_AA131185.1
	<i>gltA</i>	C_AA131207.1		<i>gltA</i>	C_AA131183.1
	<i>metG</i>	C_AA131210.1		<i>metG</i>	C_AA131186.1
	<i>ppsA</i>	C_AA131211.1		<i>ppsA</i>	C_AA131187.1
	<i>recA</i>	C_AA131212.1		<i>recA</i>	C_AA131188.1
GX-MS-24110416	16S <i>rRNA</i>	C_AA131213.1	GX-CI-24110403	16S <i>rRNA</i>	C_AA131189.1
	<i>gyrB</i>	C_AA131214.1		<i>gyrB</i>	C_AA131190.1
	<i>groL</i>	C_AA131216.1		<i>groL</i>	C_AA131192.1
	<i>gyrB</i>	C_AA131217.1		<i>gyrB</i>	C_AA131193.1
	<i>gltA</i>	C_AA131215.1		<i>gltA</i>	C_AA131191.1
	<i>metG</i>	C_AA131218.1		<i>metG</i>	C_AA131194.1
	<i>ppsA</i>	C_AA131219.1		<i>ppsA</i>	C_AA131195.1
	<i>recA</i>	C_AA131220.1		<i>recA</i>	C_AA131196.1
GX-CI-24110404	16S <i>rRNA</i>	C_AA131197.1	GX-CI-24110404	<i>gltA</i>	C_AA131199.1
	<i>gyrB</i>	C_AA131198.1		<i>metG</i>	C_AA131202.1
	<i>groL</i>	C_AA131200.1		<i>ppsA</i>	C_AA131203.1
	<i>gyrB</i>	C_AA131201.1		<i>recA</i>	C_AA131204.1

RESULTS

3.1. ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA

3.1.1. MORPHOLOGICAL CHARACTERISTICS OF ISOLATES

Seven bacterial strains were isolated from diseased *M.salmoides*, *C.idella*, and *P.clarkii*. The isolates were designated as GX-PC-22041102, GX-MS-22040201, GX-MS-24110416, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, and GX-CI-24110404. On blood agar plates, all seven isolates formed grayish-white, round, raised colonies with smooth margins, uniform texture, and a moist, glossy surface. They exhibited β -hemolysis, which is consistent with the typical colony characteristics of *A. veronii* (Figure 1).

3.1.2. PHYSIOLOGICAL AND BIOCHEMICAL IDENTIFICATION RESULTS

Seven isolated strains were identified using the VITEK 2 compact fully automatic microbial identification system. As shown in Table 3, the seven isolated strains were consistent with the physiological and biochemical characteristics of *A.veronii*. The identification results indicated that they were *A. veronii*, with a probability of 97%.

3.1.3. MOLECULAR BIOLOGICAL IDENTIFICATION RESULTS

The amplification products of the 16S *rRNA* and *gyrB* genes from the seven isolated strains were analyzed by 2% agarose gel electrophoresis. The results showed specific bands for both the 16S *rRNA* and *gyrB* gene amplification products of all seven isolates, approximately 1441 bp and 1143 bp in size, respectively, which was consistent with the expected outcomes (Figure 2).

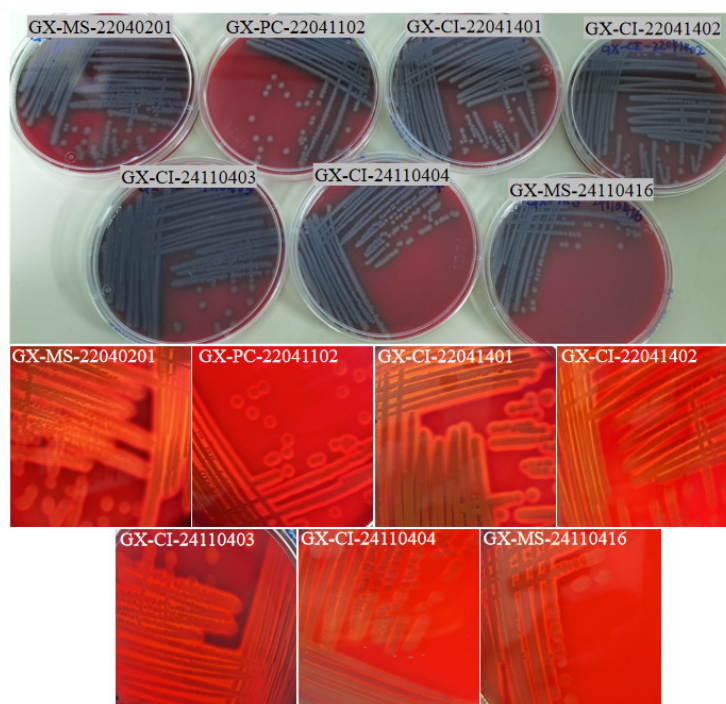


Figure 1. Morphology of strains GX-MS-22040201, GX-PC-22041102, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, GX-CI-24110404, and GX-MS-24110416 in the culture media.

3.1.4. PHYLOGENETIC ANALYSIS

BLAST homology analysis of the 16S *rRNA* and *gyrB* gene sequences from the seven isolates revealed high homology with *A. veronii*. Phylogenetic trees constructed using sequences from different bacterial strains demonstrated that all seven isolates clustered within a distinct clade together with *A. veronii* (Figures 3, 4), confirming their identification as *A. veronii*.

3.1.5. RESULTS OF ARTIFICIAL CHALLENGE INFECTION ASSAY

The artificial challenge infection assay demonstrated that *M. salmoides*, *C. idella*, and *P. clarkii* injected with different concentrations of *A. veronii* suspensions exhibited symptoms similar to those observed in natural infections. These symptoms included hemorrhage, skin ulceration, abdominal swelling, and ascites in *C. idella* and *M. salmoides*. Infected *P. clarkii* showed reduced appetite and lethargy, with post-mortem examination revealing hepatopancreatic atrophy, softening, and pallor. Mortality increased with higher bacterial injection concentrations. Deaths began to occur from the third day post-challenge, with the mortality rates for each group detailed in Table 4. The calculated LD₅₀ values for strains GX-PC-22041102, GX-MS-22040201, GX-MS-24110416, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, and GX-CI-24110404 were 3.58×10^6 , 1.71×10^6 , 1.94×10^6 , 1.66×10^6 , 1.43×10^6 , 1.43×10^6 and 1.66×10^6 CFU/individual, respectively (Table 4). Bacteriological re-isolation and identification from the *M. salmoides*, *C. idella*, and *P. clarkii* subjected to artificial challenge yielded a single dominant bacterium with identical

morphological characteristics to the original challenge strains GX-PC-22041102, GX-MS-22040201, GX-MS-24110416, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, and GX-CI-24110404.

3.2. ANTIMICROBIAL SUSCEPTIBILITY TESTING RESULTS

The antimicrobial susceptibility results of the seven isolated strains against 19 antibiotics are presented in Table 5. All seven isolates were susceptible to ceftriaxone, ceftazidime, ceftazidime sodium, cefepime, fosfomycin, ceftazidime, ceftazidime sodium, and cefuroxime sodium. In contrast, all strains exhibited resistance to clavulanic acid, amoxicillin, teicoplanin, and sulbactam. These results indicate that the seven *A. veronii* isolates exhibited multidrug resistance.

3.3. VIRULENCE GENE DETECTION RESULTS

The detection results of 18 virulence genes in the seven isolated strains are shown in Figure 5. The *GcaT*, *OmpAI*, *Hly*, *Fla*, and *Exu* genes were detected in all isolates. In contrast, the *Acg*, *Ast*, *Alt*, *Ser*, *AhyB*, *lafA*, and *eprCAI* genes were not detected.

3.4. MLST TYPING

The PCR detection results for the six housekeeping genes of the seven isolated strains are shown in Figure 6. All six housekeeping genes from the seven strains amplified specific bands at the expected positions, with consistent fragment lengths.

The gene sequences of 7 isolated bacterial strains were uploaded to the Pubmlst database, resulting in 42 allele numbers and 7 new ST types (Table 6). Using the 7 new ST

Table 3. Identification results of physiological and biochemical characteristics of pathogen.

Items	GX- MS-22040201	GX- PC-22041102	GX- CI-22041401	GX- CI-22041402	GX- CI-24110403	GX- CI-24110404	GX- MS-24110416
Ala-Phe-Pro-arylamidase	-	-	+	-	-	-	-
Adonitol	-	-	-	-	+	-	-
L-pyrrolydonyl- arylamidase	+	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-
D-cellobiose	-	+	-	-	-	-	-
β -galactosidase	+	+	+	+	+	+	+
H ₂ S production	-	-	-	-	-	-	-
β -N-acetyl- glucosaminidase	+	+	+	+	+	+	+
Glutamyl arylamidase pNA	-	-	-	+	-	-	-
D-glucose	+	+	+	+	+	+	+
γ -glutamyl-transferase	-	-	-	-	-	-	-
Fermentation/Glucose	+	+	+	+	+	+	+
β -glucosidase	-	-	-	-	-	-	-
D-maltose	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+
β -xylosidase	-	-	-	-	-	-	-
β -Alanine arylamidase pNA	-	-	-	-	-	-	-
L-proline arylamidas	+	+	+	+	+	+	+
Lipase	+	+	+	+	+	+	+
Palatinose	+	-	-	+	-	-	-
Tyrosine arylamidase	-	+	+	+	+	+	-
Urease	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	+	-	-
Sucrose	+	+	+	+	+	+	+
D-tagatose	-	-	-	-	-	-	-
D-trehalose	+	+	+	+	+	+	+
Citrate (sodium)	-	-	-	-	-	-	-
Malonate	-	+	-	-	-	-	-
5-Keto-D-gluconate	-	-	-	-	-	-	-
L-lactate alkalization	-	-	-	-	-	-	-
α -Glucosidase	-	-	-	-	-	-	-
Succinate alkalization	+	+	+	+	+	+	+
β -N-Acetyl- Galactosaminidase	-	-	-	-	-	-	-
α -Galactosidase	-	-	-	-	-	-	-
Phosphatase	-	-	-	-	-	-	-
Glycine arylamidase	-	-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+	+
Lysine decarboxylase	+	+	+	+	+	+	+
L-histidine assimilation	-	-	-	-	-	-	-
Courmarate	+	+	+	+	-	+	+
β -glucuronidase	-	-	-	-	-	-	-
Glu-Gly-Arg- arylamidase	+	+	-	+	-	-	+
O/129 Resistance	+	+	+	+	+	+	-
L-malate assimilation	+	+	+	+	+	+	+
ELLMAN	+	+	+	+	+	+	+
L-lactate assimilation	-	-	-	-	-	-	-

“+”indicated positive ; “-”indicated negative

types and 12 ST types from the *Aeromonas* MLST database to construct a phylogenetic tree, the results showed that all the new STs clustered with *A.veronii*. Among them, ST2770 (GX-CI-24110403) and ST2774 (GX-CI-24110404) formed a single branch, with the closest genetic relationship; ST2765

(GX-CI-22041401), ST2760 (GX-CI-22041402), and ST2775 (GX-MS-24110416) formed adjacent branches, with a secondary genetic relationship; ST2742 (GX-MS-22040201) was relatively distant. Among them, ST2770 and ST2774 were more closely related to *A.veronii* from freshwater in

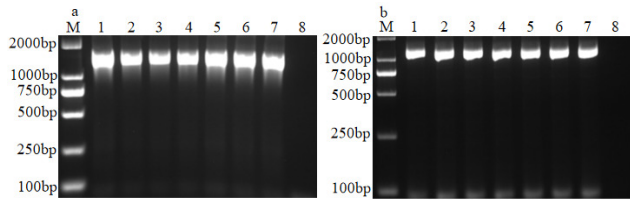


Figure 2. 16S *rRNA*, *gyrB* PCR amplification results of isolates GX-MS-22040201, GX-PC-22041102, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, GX-CI-24110404, GX-MS-24110416. (a) 16S *rRNA*; (b) *gyrB*. (M) DL2000DNA Marker. (1~7) GX-MS-22040201, GX-PC-22041102, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, GX-CI-24110404, GX-MS-24110416. (8) Negative control.

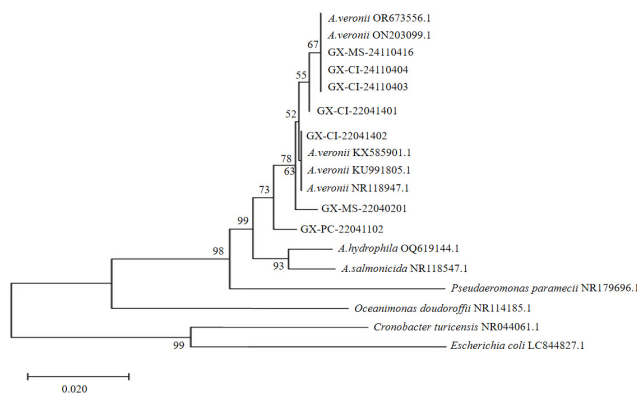


Figure 3. Phylogenetic tree based on 16S *rRNA* gene sequences of the seven isolated strains with reference strains.

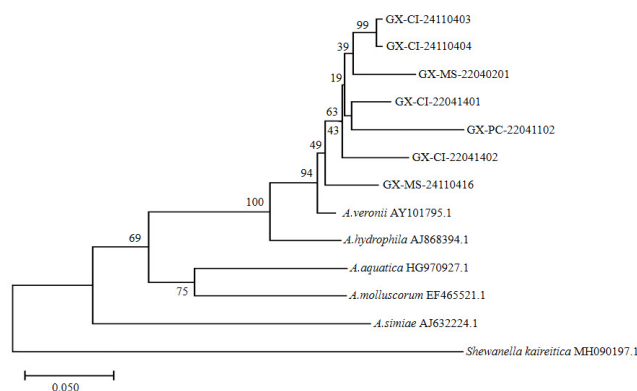


Figure 4. Phylogenetic tree based on *gyrB* gene sequences of the seven isolated strains with reference strains.

China; ST2765 was more closely related to *A. veronii* from Polish *Trachemys scripta elegans*; ST2760 was more closely related to *A. veronii* from Polish *T. scripta elegans*; ST2775 was more closely related to *A. veronii* from Chinese *P. clarkii*. The 7 newly discovered sequence types showed relatively distant genetic relationships with *Cavia porcellus*

and aquatic *Aeromonas*, and the correlation was not significant (Figure 7). The results indicated that the internal typing of the 7 isolated *A. veronii* strains GX-MS-22040201, GX-PC-22041102, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, GX-CI-24110404, and GX-MS-24110416 was rich, further confirming the genetic diversity and complexity of *A. veronii* in Guangxi region.

DISCUSSION

A. veronii is an important zoonotic pathogen that infects fish, crustaceans, and humans, posing substantial risks to global aquaculture and public health.³⁷⁻⁴³ In this study, seven bacterial strains isolated from diseased *P. clarkii*, *M. salmoides*, and *C. idella* in Guangxi were conclusively identified as *A. veronii* using phenotypic and molecular methods. Artificial challenge trials confirmed that these isolates could reproduce typical clinical signs similar to natural outbreaks, with mortality rates increasing in a dose-dependent manner. The LD₅₀ values ranged from 1.43×10^6 to 3.58×10^6 CFU/individual, indicating moderate to high pathogenicity. These results are consistent with previous studies showing that the pathogenicity of *A. veronii* is closely related to infection dose.^{13-15,35}

Studies have shown that the pathogenicity of pathogenic bacteria is closely associated with the virulence factors they carry, with different factors playing distinct roles during the infection process. For example, aerolysin (*aerA*), enterotoxins (*Act/Ast/Alt*), and enzymes such as *GcaT*, *Lip*, and *Exu* can disrupt host cell membranes, nucleic acids, and normal physiological functions, thereby enhancing bacterial adhesion and facilitating attachment and invasion.⁴⁴ In this study, the carriage rates of the *GcaT*, *OmpAI*, *Hly*, *Fla*, and *Exu* genes among the seven isolated *A. veronii* strains were all 100%. The carriage rates of the *Act* and *aerA* genes were both 85.71%, while those of the *Lip* and *Acsv* genes were 71.43% and 57.14%, respectively. In contrast, the *Acg*, *Ast*, *Alt*, *Ser*, *AhyB*, *lafA*, and *eprCAI* genes were not detected (0%). These findings differ from those reported by other researchers. For instance, among the 119 *A. veronii* strains isolated by Liu et al., the carriage rates of *GcaT*, *Fla*, and *Exu* genes were 82.35%, 76.47%, and 92.44% respectively.⁴⁵ Wu et al. found that the carriage rates of *Act* and *Lip* genes in the isolated *A. veronii* strains were 35.0% and 5.0% respectively.¹² Similarly, Kingom et al. found that among 350 *A. veronii* isolates, the carriage rates of the *aerA*, *Act*, and *Hly* genes were 65%, whereas Sen et al. reported a carriage rate of 70% for the *aerA*, *Act*, and *Hly* genes in their isolates.^{46, 47} Analyzing the results of virulence gene carriage in *A. veronii* isolates from other regions, it was found that the five virulence genes, *GcaT*, *OmpAI*, *Hly*, *Fla*, and *Exu*, were not universally present in all *A. veronii* strains, but rather showed differences among the strains. In this study, the 100% carriage rates of virulence genes such as *GcaT*, *OmpAI*, and *Hly* might be due to all strains being isolated from Guangxi or to the relatively small sample size. Among all the strains, only the GX-MS-22040201 strain isolated from largemouth bass did not carry *Act* and *aerA*. The LD₅₀ of this strain was 1.71×10^6 CFU/individual, while the LD₅₀ of the

Table 4. Results of Artificial Challenge Experiments.

Group	Infection concentration CFU/mL	Strains	Death number					Total	Cumulative death rate%
			3d	6d	9d	12d	15d		
1	9×10 ⁸	GX-PC-22041102	19	9	2	0	0	30	100
2		GX-MS-22040201	20	10	0	0	0	30	100
3		GX-MS-24110416	21	8	1	0	0	30	100
4		GX-CI-22041401	21	9	0	0	0	30	100
5		GX-CI-22041402	25	4	1	0	0	30	100
6		GX-CI-24110403	24	6	0	0	0	30	100
7		GX-CI-24110404	22	8	0	0	0	30	100
8	9×10 ⁷	GX-PC-22041102	9	4	3	0	0	16	53.33
9		GX-MS-22040201	11	5	3	1	0	20	66.67
10		GX-MS-24110416	11	6	1	2	0	20	66.67
11		GX-CI-22041401	13	4	2	2	0	21	70
12		GX-CI-22041402	14	5	3	0	0	22	73.33
13		GX-CI-24110403	13	6	2	1	0	22	73.33
14		GX-CI-24110404	12	7	2	0	0	21	70
15	9×10 ⁶	GX-PC-22041102	6	5	0	0	0	11	36.67
16		GX-MS-22040201	8	5	3	0	0	16	53.33
17		GX-MS-24110416	7	5	2	1	0	15	50
18		GX-CI-22041401	9	6	1	0	0	16	53.33
19		GX-CI-22041402	8	6	3	0	0	17	56.67
20		GX-CI-24110403	8	8	1	0	0	17	56.67
21		GX-CI-24110404	9	7	0	0	0	16	53.33
<i>C.idella</i> Control	0	/	0	0	0	0	0	0	0
<i>M.salmoides</i> Control	0	/	0	0	0	0	0	0	0
<i>P.clarkii</i> Control	0	/	0	0	0	0	0	0	0

other six strains ranged from 1.43×10^6 to 3.58×10^6 CFU/individual. There was no significant difference in either LD₅₀ values or host species. This phenomenon might be related to the relatively small sample size of the isolated strains in this study. Such discrepancies in virulence gene profiles may reflect the complexity and regional characteristics of

A. veronii pathogenicity. Strains from different geographical or host origins may adapt to specific environments and exert pathogenic effects through distinct combinations of virulence genes. This variation may be attributed to multiple interacting factors, including environmental growth conditions, host species, horizontal gene transfer, evolution-

Table 5. The antimicrobial sensitivity test of the strain.

Antibacterial agent	Content (µg/tablet)	Standard inhibition zone diameter (mm) /Sensitivities						
		GX-MS-22040201	GX-PC-22041102	GX-CI-22041401	GX-CI-22041402	GX-CI-24110403	GX-CI-24110404	GX-MS-24110416
Ampicillin	10	0(R)	0(R)	0(R)	0(R)	24(s)	0(R)	0(R)
clavulanic acid	20	0(R)	12(R)	0(R)	0(R)	0(R)	0(R)	0(R)
Amoxicillin	10	0(R)	0(R)	8(R)	0(R)	0(R)	0(R)	0(R)
Cefixime	5	44(S)	28(S)	24(S)	18(I)	28(S)	24(S)	28(S)
Ceftriaxone	30	28(S)	42(S)	24(S)	30(S)	26(S)	40(S)	42(S)
Cefoperazone/sulbactam	75/30	19(I)	28(S)	28(S)	14(R)	24(S)	18(I)	24(S)
Cefepime	30	28(S)	28(S)	24(S)	23(S)	24(S)	24(S)	22(S)
Streptomycin	10	20(S)	30(S)	28(S)	0(R)	12(I)	18(S)	12(I)
Kanamycin	30	16(I)	16(I)	16(I)	0(R)	16(I)	16(I)	14(I)
Gentamicin	10	14(I)	18(S)	18(S)	18(S)	20(S)	16(S)	9(R)
Amikacin	30	18(I)	17(I)	18(I)	15(R)	19(I)	14(R)	14(R)
Norfloxacin	10	20(S)	24(S)	18(S)	0(R)	22(S)	0(R)	16(I)
Ofloxacin	5	24(S)	28(S)	22(S)	10(R)	20(S)	20(S)	18(S)
Ciprofloxacin	5	28(S)	24(S)	25(S)	18(I)	24(S)	24(S)	18(I)
levofloxacin	5	26(S)	15(R)	12(R)	14(R)	22(S)	19(S)	15(R)
Tetracycline	30	24(S)	24(S)	24(S)	11(R)	20(S)	14(I)	10(R)
Doxycycline	30	20(S)	22(S)	14(I)	12(R)	16(S)	17(S)	14(R)
Minocycline	30	20(S)	20(S)	20(S)	18(I)	18(I)	20(S)	18(I)
Chloramphenicol	30	26(S)	24(S)	22(S)	26(S)	22(S)	24(S)	11(R)
Cefazolin	30	18(S)	14(R)	14(R)	9(R)	11(R)	11(R)	14(R)
Cefaclor	30	22(S)	17(I)	16(I)	10(R)	18(S)	14(R)	18(S)
Aztreonam	30	40(S)	35(S)	28(S)	34(S)	36(S)	34(S)	34(S)
Roxithromycin	15	14(I)	0(R)	8(R)	0(R)	12(R)	14(I)	0(R)
Clarithromycin	15	14(I)	12(R)	15(I)	0(R)	14(I)	14(I)	0(R)
Vancomycin	30	20(S)	10(R)	8(R)	12(R)	9(R)	12(R)	10(R)
Teicoplanin	30	0(R)	0(R)	10(R)	0(R)	0(R)	0(R)	0(R)
Trimethoprim sulfamethoxazole	23.75/1.25	20(S)	20(S)	15(I)	0(R)	20(S)	12(I)	16(S)
Fosfomycin	200	30(S)	36(S)	28(S)	24(S)	32(S)	30(S)	24(S)
Nitrofurantoin	300	22(S)	19(S)	18(S)	20(S)	20(S)	16(I)	19(S)
Rifampin	5	18(I)	14(R)	18(I)	16(I)	12(R)	16(R)	10(R)
Ceftazidime	30	25(S)	30(S)	30(S)	32(S)	32(S)	34(S)	28(S)
Sulbactam	10/10	10(R)	0(R)	0(R)	0(R)	0(R)	0(R)	9(R)
Ticarcillin-clavulanate	75/10	24(S)	20(S)	22(S)	11(R)	22(S)	20(S)	18(I)
Lomefloxacin	LMF(10)	24(S)	30(S)	22(S)	12(R)	21(I)	20(I)	16(R)
Ceftizoxime	30	36(S)	40(S)	36(S)	32(S)	36(S)	32(S)	36(S)
cefuroxime sodium	30	28(S)	28(S)	26(S)	22(S)	26(S)	26(S)	28(S)

Table 6. Multilocus sequence typing (MLST) profiles, sequence types (STs), and allele numbers of the seven *Aeromonas* isolates obtained in this study, compared with representative reference strains from different hosts and geographic regions available in the PubMLST database. Analysis of the six housekeeping genes (*gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*) identified 42 allele numbers and seven novel ST types among the isolates.

Strains	Source	Area	Species	ST	Allele					
					<i>gyrB</i>	<i>groL</i>	<i>gltA</i>	<i>metG</i>	<i>ppsA</i>	<i>recA</i>
GX-MS-22040201	<i>M. salmoides</i>	China	<i>A. veronii</i>	2742	1216	1095	1229	1259	1406	1319
GX-PC-22041102	<i>P. clarkii</i>	China	<i>A. veronii</i>	2740	1214	1094	1227	65	1404	1318
GX-CI-22041401	<i>C. idella</i>	China	<i>A. veronii</i>	2765	939	859	1230	979	1407	1320
GX-CI-22041402	<i>C. idella</i>	China	<i>A. veronii</i>	2760	1215	584	1228	812	1405	1321
GX-CI-24110403	<i>C. idella</i>	China	<i>A. veronii</i>	2770	68	125	803	1260	1408	1324
GX-CI-24110404	<i>C. idella</i>	China	<i>A. veronii</i>	2774	68	125	803	1260	1409	1325
GX-MS-24110416	<i>M. salmoides</i>	China	<i>A. veronii</i>	2775	212	216	828	213	233	1326
GSH8M-1	<i>C. porcellus</i>	Japan	<i>A. caviae</i>	1250	93	102	92	96	97	98
GSH8-2	<i>C. porcellus</i>	Japan	<i>A. hydrophila</i>	558	402	386	406	284	443	437
WCW1-2	<i>A. contaminata</i>	China	<i>A. caviae</i>	645	404	434	460	458	488	480
X15/0263	<i>T. scripta elegans</i>	Poland	<i>A. hydrophila</i>	660	467	447	476	376	515	522
X15/0279	<i>T. scripta elegans</i>	Poland	<i>A. hydrophila</i>	661	468	448	477	473	299	523
X17/0816	<i>T. scripta elegans</i>	Poland	<i>A. veronii</i>	672	451	71	446	482	499	493
X17/0613	<i>T. scripta elegans</i>	Poland	<i>A. veronii</i>	673	275	453	486	483	241	532
X15/0068	<i>T. scripta elegans</i>	Poland	<i>A. veronii</i>	674	112	219	44	217	384	381
BT06	<i>Oreochromis niloticus</i>	Thailand	<i>A. veronii</i>	656	415	164	160	267	457	160
QD160502	<i>P. clarkii</i>	China	<i>A. veronii</i>	646	453	435	461	459	500	494
C198	<i>Homo sapiens</i>	Thailand	<i>A. veronii</i>	720	126	157	354	16	551	374
D-109	<i>Aqua dulcis</i>	China	<i>A. veronii</i>	2658	340	48	1201	1210	696	49

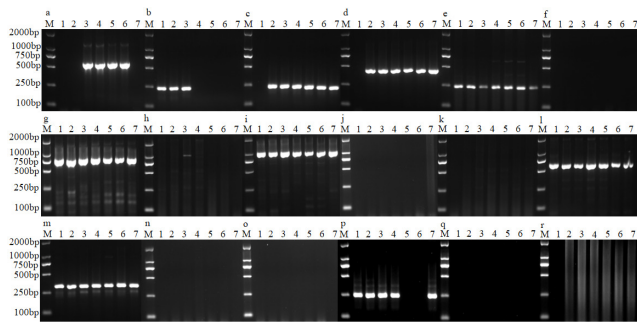


Figure 5. Results of PCR amplification electrophoresis of virulence genes of 7 isolates.(M) DL2000DNA Marker.(1~7) GX-MS-22040201, GX-PC-22041102, GX-CI-22041401, GX-CI-22041402, GX-CI-24110403, GX-CI-24110404, GX-MS-24110416.(a~r) *Acsv*, *AexT*, *Act*, *aerA*, *GcaT*, *Acg*, *OmpAI*, *Ahp*, *Hly*, *Ast*, *Alt*, *Fla*, *Exu*, *Ser*, *AhyB*, *Lip*, *lafA*, *eprCAI*.

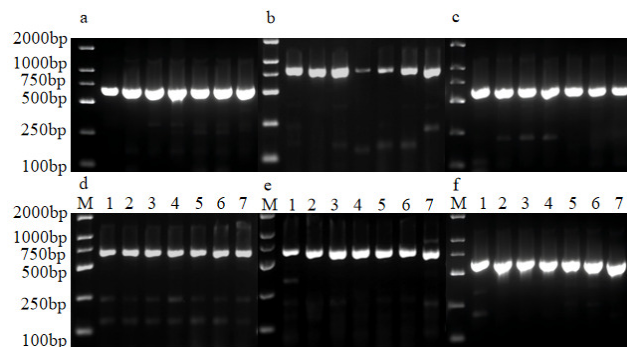


Figure 6. PCR amplification results of *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, *recA* genes of seven isolates.

ary history, ecological niche differentiation, and laboratory cultivation conditions, all of which could contribute to the significant differences in virulence gene carriage among *A. veronii* strains from diverse sources.⁴⁸ Detection of virulence genes can help predict the pathogenic potential of bacterial strains, thereby providing a theoretical basis for formulating targeted prevention and control measures.

Currently, antibiotic therapy remains the primary approach to treating bacterial diseases, making the analysis of antibiotic resistance in *A. veronii* particularly important. The seven *A. veronii* strains isolated in this study exhibited multidrug resistance, which aligns with the multidrug resistance phenomenon reported by Fadel et al.⁴³ However, some strains showed differential responses to the same antibiotic. For instance, strain GX-CI-24110403 was sensitive to ampicillin, whereas the remaining six strains were resistant. This indicates variability in resistance phenotypes among the strains, which may be associated with differences in genetic background, history of antibiotic exposure, or expression of multidrug resistance genes.⁴⁹

MLST typing provides a high-resolution tool for epidemiological studies of bacteria and offers critical insights into bacterial population structure, evolutionary history, and transmission patterns.⁵⁰ In this study, MLST typing re-

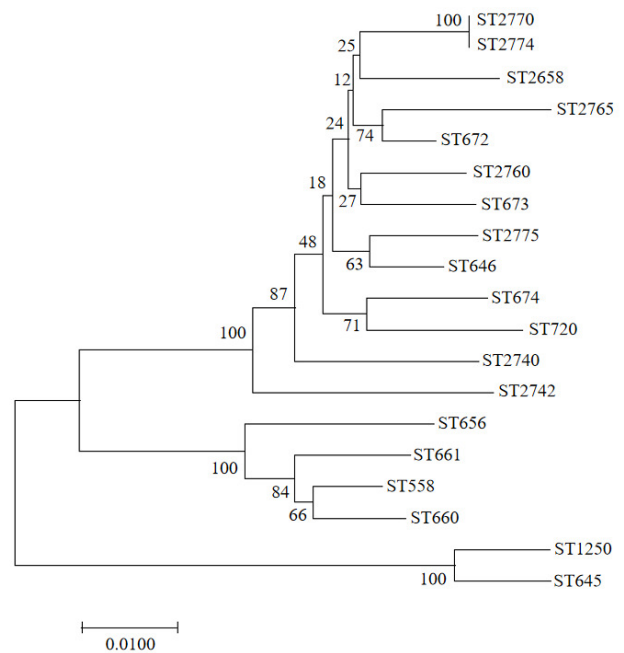


Figure 7. Phylogenetic tree constructed based on tandem gene sequences.

vealed that the seven isolated *A. veronii* strains represented seven novel STs, indicating a high degree of genetic diversity among *A. veronii* populations in the Guangxi region. This considerable genetic diversity may be associated with the complex ecological environment, diverse host species, and genetic recombination among strains in this area. The novel STs were closely related to STs originating from freshwater environments in China and from species such as red-eared sliders in Poland, whereas they were more distantly related to STs derived from other hosts (e.g., guinea pigs) or different species (e.g., *A. hydrophila*). This suggests that strains from different geographic and host origins may exhibit both genetic similarities and differences, which may be attributed to variations in local ecological conditions, host species, and the evolutionary histories of the strains.⁵¹

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AUTHORS' CONTRIBUTION

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COMPETING OF INTEREST – COPE

The authors declare no conflicts of interest.

ETHICAL CONDUCT APPROVAL – IACUC

All procedures involving animals were approved by the relevant ethics committee of Guangxi Academy of Fishery Sciences on 01-05-2026 (Approval No.2026-GAFS-08) and

conducted in accordance with applicable animal welfare guidelines.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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