Original Article

Genetic Diversity, Antibiotic Resistance, and Pathogenicity of *Aeromonas* Species from Food Products in Shanghai, China*

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Abstract

Objective *Aeromonas* has recently been recognized as an emerging human pathogen. *Aeromonas*-associated diarrhea is a phenomenon occurring worldwide. This study was designed to determine the prevalence, genetic diversity, antibiotic resistance, and pathogenicity of *Aeromonas* strains isolated from food products in Shanghai.

Methods *Aeromonas* isolates (*n* = 79) collected from food samples were analyzed using concatenated *gyrB-cpn60* sequencing. The antibiotic resistance of these isolates was determined using antimicrobial susceptibility testing. Pathogenicity was assessed using β-hemolytic, extracellular protease, virulence gene detection, *C. elegans* liquid toxicity (LT), and cytotoxicity assays.

Results Eight different species were identified among the 79 isolates. The most prevalent *Aeromonas* species were *A. veronii* [62 (78.5%)], *A. caviae* [6 (7.6%)], *A. dhakensis* [3 (3.8%)], and *A. salmonicida* [3 (3.8%)]. The *Aeromonas* isolates were divided into 73 sequence types (STs), of which 65 were novel. The isolates were hemolytic (45.6%) and protease-positive (81.0%). The most prevalent virulence genes were *act* (73.4%), *fla* (69.6%), *aexT* (36.7%), and *ascV* (30.4%). The results of *C. elegans* LT and cytotoxicity assays revealed that *A. dhakensis* and *A. hydrophila* were more virulent than *A. veronii*, *A. caviae*, and *A. bivalvium*. Antibiotic resistance genes [*tetE*, *blaTEM*, *tetA*, *qnrS*, *aac(6)-Ib*, mcr-*1*, and *mcr-3*] were detected in the isolates. The multidrug-resistance rate of the *Aeromonas* isolates was 11.4%, and 93.7% of the *Aeromonas* isolates were resistant to cefazolin.

Conclusion The taxonomy, antibiotic resistance, and pathogenicity of different *Aeromonas* species varied. The *Aeromonas* isolates *A. dhakensis* and *A. hydrophila* were highly pathogenic, indicating that food-derived *Aeromonas* isolates are potential risks for public health and food safety. The monitoring of food quality and safety will result in better prevention and treatment strategies to control diarrhea illnesses in China.

Key words: *Aeromonas*; Genetic diversity; Antibiotic resistance; Virulence gene; Cytotoxicity assay; Multidrug-resistance

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INTRODUCTION

Aeromonas spp. are Gram-negative bacteria belonging to the *Aeromonadaceae* family and are widely distributed in the aquatic environment. *Aeromonas* has been isolated from dairy products (4%), vegetables (26%–41%), meat (5%–10%), poultry (3%–70%), and seafood (31%–72%)[1]. *Aeromonas* spp. are opportunistic pathogens that can directly infect or co-infect with other pathogenic bacteria, thus causing sepsis and meningitis in humans and animals[2]. *Aeromonas* spp. cause acute watery diarrhea, dysentery, and chronic gastrointestinal diseases[3], which pose threats to human health and quality of life. China continues to be one of the countries with the highest mortality rate owing to childhood diarrhea. Indeed, > 10,000 deaths from diarrheal diseases occur annually[4].

The *Aeromonas* genus currently consists of > 26 known species[5]. Owing to the limitations of the existing phenotypic identification systems and the complexity of the taxonomy of *Aeromonas* spp., phenotypic identification is deemed reasonably difficult to the species level, especially for *Aeromonas* spp. related to human diseases[6]. Furthermore, conventional methods, such as matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), are time-consuming, labor-intensive, and error-prone[7]. For instance, Morinaga et al.[8] reported that isolates originally recognized as *A. hydrophila* using traditional phenotypic analysis have been re-identified as *A. dhakensis* based on *cpn60* and *gyrB* sequencing methods. David et al.[9] concluded that the universal target (UT) sequence from the *cpn60* gene can be used for phylogenetic and taxonomic studies of the *Aeromonas* genus. The type I chaperone gene, *cpn60*[9], and the single-copy protein-coding gene, *gyrB*[10], are widespread and have been applied in phylogenetic analysis and species identification of *Aeromonas*[10].

The mechanism underlying the pathogenesis of *Aeromonas* is multifactorial[11]. Specifically, the pathogenicity is related to virulence factors, including aerolysin (*aer*), heat-stable cytotoxin (*ast*), hemolysin (*hlyA*), lateral flagella (*laf*), polar flagellum (*fla*), elastase (*ela*), lipase (*lip*), cytotoxic enterotoxin (*act*), and cytotoxic enterotoxin (*alt*). The *Aeromonas* spp. utilize these virulence factors to enable survival within the host, thereby causing extensive cell and tissue destruction, evading the host immune response, and enhancing pathogenic resistance mechanisms, which subsequently help the pathogen to establish an infection[12]. Therefore, further elucidation of the correlation between *Aeromonas* pathogenicity and the associated virulence factors is warranted.

Owing to the ease-of-culture, low cost, short life cycle, and simple genetic background of *Caenorhabditis elegans*[13], wild-type *C. elegans* has been increasingly used as a model to evaluate the virulence of bacteria with pathogenic potential, including *Aeromonas* spp.[14]. Moreover, Wu et al.[15] reported that the virulence manifested in the cytotoxicity assay is correlated with virulence traits demonstrated in a mouse infection model.

Antibiotics are frequently abused in industries to prevent and control *Aeromonas* infections, thus resulting in increased antimicrobial resistance[16]. It has been reported that *Aeromonas* isolates from South Korea were 100% resistant to amoxicillin and nalidixic acid, and 98.5% of the isolates were resistant to ampicillin; all isolates showed multiple antimicrobial resistance phenotypes[17]. Multidrug-resistant (MDR) *Aeromonas* strains from food products can bypass the acidic conditions of the digestive tract of humans and animals[18]. Hence, antimicrobial resistance of *Aeromonas* from food items should be strictly monitored to reduce the risk of food-borne *Aeromonas* infections[19].

In the current study we analyzed the distribution, genetic diversity, antimicrobial resistance, and pathogenicity of *Aeromonas* isolated from food items to assess the risk of disease in humans and animals. Our study provided the basis for clinical treatment of diseases caused by *Aeromonas* spp.

MATERIALS AND METHODS

Aeromonas Isolates

All isolates were obtained from food products, including fish, shrimp, clams, pigs, chickens, cows, and sheep, collected from six supermarkets in Pudong New District, Shanghai in 2019 (Figure 1). An automatic bacteriologic analyzer (Vitek 2 Compact; BioMèrieux, Marcy l'Etoile, France) was used to identify the *Aeromonas* isolates[20], which were then cultured at 28 °C on brain heart infusion agar plates (Oxoid Ltd., Basingstoke, UK).

Multi-locus Sequence Typing (MLST) and Subtyping of Aeromonas Isolates

We extracted total chromosomal DNA from the *Aeromonas* isolates using the QIAamp DNA Mini Kit (Qiagen, Shanghai, China) according to the

manufacturer's instructions. Concatenated *gyrB-cpn60* phylogenetic trees were constructed, and we identified the *Aeromonas* isolates at the species level by comparing two-gene sequences of representative species[21]. The *Aeromonas* MLST scheme (http://pubmlst.org/Aeromonas/), which relies on the amplification and sequencing of six housekeeping genes (*gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*), was applied to identify each *Aeromonas* isolate subtype[22]. PCR was performed using previously described primers and protocols and the sequences of the six loci were compared with the loci hosted on the *Aeromonas* MLST database and sequence types (STs)[23]. A phylogenetic tree was also constructed using the neighbor-joining method in Clustal-W with bootstrap values calculated using 1,000 replicates.

The β-hemolytic Activity and Extracellular Protease Assay

The hemolytic ability and extracellular protease activity of the *Aeromonas* isolates were determined on Luria Bertani (LB) agar plates containing 5% sheep blood (KeMaJia, Shanghai, China) and LB agar plates containing 2% (w/v) skim milk (KeMaJia), respectively. An *Aeromonas* colony was inoculated on blood and milk agar plates and incubated at

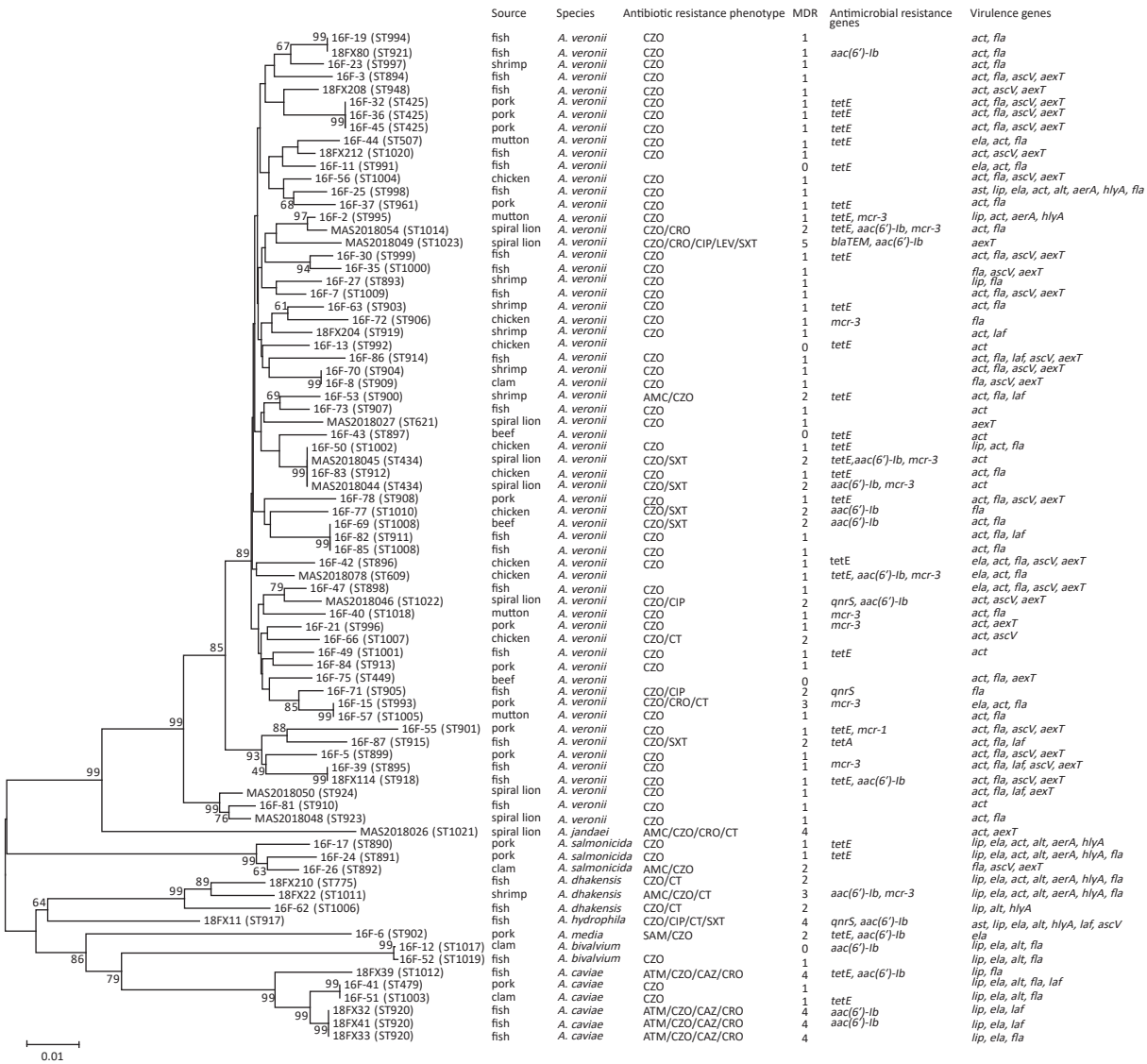

Figure 1. Phylogeny of *Aeromonas* spp. The concatenated sequences of six housekeeping genes were used to analyze the phylogeny of *Aeromonas* spp. The information of source, species, virulence genes, and antibiotic resistance are shown. MDR, multidrug-resistance.

28 °C[20]. The presence of transparent zones surrounding the *Aeromonas* colonies were considered positive reactions for both tests.

C. elegans LT Assay

Aeromonas isolates were selected for the *C. elegans* LT assay[15]. A single colony of *Aeromonas* was incubated in 45 mL of LB broth at 28 °C for 24 h. The *Aeromonas* suspension was adjusted to an OD_{600} of 3.0, which was prepared for the toxicity assay. Nematode growth medium (NGM) agar plates with *Escherichia coli* strain OP50 were used to culture *C. elegans*. The synchronized adult L_4 worms were washed in M_9 buffer (http://www.wormbook.org/). After centrifugation, the worms were re-suspended in 5 µL of S-medium (http://www.wormbook.org/). Approximately 40–50 worms were added to a 48-well plate with 5 µL of fluorodeoxyuridine to prevent reproduction. Finally, 190 µL of the LB broth with *Aeromonas* isolates was added to each lawn to a final volume of 200 µL. The plate was incubated at 20 °C and the survival rate of worms was observed after 1, 2, and 3 days.

Cytotoxicity Assay

The mouse C_2C_{12} fibroblast cell line obtained from the American Type Culture Collection (Manassas VA, USA) was used for cytotoxicity assays[20]. The cells were cultured in complete medium with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a 5% CO_2 incubator for 24 h. The cells were then thrice-washed with DMEM and reacted with *Aeromonas* spp. for 3 h[15]. The *Aeromonas* cultures were incubated with cells at a 100 multiplicity of infection (MOI). The CytoTox 96® Assay (Promega, Madison, WI, USA) was used to measure cell death following treatment with a cytotoxic drug or compound[19]. The CytoTox 96® Assay kit was used to determine the release of lactate dehydrogenase (LDH) from the cell after interacting with *Aeromonas* isolates at 37 °C for 3 h. A group treated with 10× lysis solution (Promega) was used as the positive control, and an untreated group with DMEM (Gibco) was the negative control[15]. Cytotoxicity was expressed as the released LDH level induced by *Aeromonas* isolates compared with that induced by 10× lysis solution (defined as 100% cytotoxicity).

Detection of Virulence-Associated Genes

Virulence genes (*aerA*, *hlyA*, *act*, *ast*, *alt*, *ascV*, *aexT*, *lip*, *ela*, *fla,* and *laf*) were screened as potential toxicity factors of *Aeromonas*[24]. The PCR system (30 µL) contained 15 µL of Taq PCR MasterMix (Takara Bio, Inc., Kyoto, Japan), 1 µL of primer (10 µmol/L), 11.5 µL of ddH_2O, and 1.5 µL of DNA template. The cycling conditions were as follows: pre-denaturation at 96 °C for 5 min; 34 cycles of denaturation at 96 °C for 30 s, annealing at 55–60 °C for 30 s, and extension at 72 °C for 1 min and the final extension was at 72 °C for 5 min. The PCR products were analyzed using sequencing.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing for *Aeromonas* spp. was performed following the 2010 Clinical and Laboratory Standard Institute (CLSI) guideline M45-A2[6]. The minimal inhibition concentrations (MICs) of 10 antibiotics [amoxicillin/clavulanic acid (AMC), ampicillin/sulbactam (SAM), cefazolin (CZO), ceftazidime (CAZ), ceftriaxone (CRO), aztreonam (ATM), ciprofloxacin (CIP), levofloxacin (LEV), oxazin/sulfamethoxine oxazole (SXT), and colistin (CT)] were measured. The quality control strains for all antimicrobial susceptibility tests were *Escherichia coli* ATCC 25922[25].

Evaluation of Antimicrobial Resistance Genes

We detected 19 resistance genes (*tetA*, *tetB*, *tetE*; *blaTEM*, *blaSHV*, *blaCTX*; *armA*, *aphAI-IAB*, *aac(6')-Ib*, *aac(3')-IIa*; *sul1*, *sul2*; *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*; *qnrA*, *qnrB*, and *qnrS*) in the *Aeromonas* isolates. The primer sequences of the antibiotic resistance genes are shown in Table 1. The PCR-positive products were confirmed using sequencing.

Statistics

Data analysis was performed using SPSS software (version 23.0 for Windows; IBM Corp, Armonk, NY, USA). The 95% *CI*s of categorical variables for the distribution of virulence genes and the results of the *C. elegans* LT assay were calculated using Fisher's exact tests. The cytotoxicity test data were analyzed using a nonparametric test to reveal the toxicity differences among different *Aeromonas* isolates.

RESULTS

MLST of Aeromonas Isolates

The 79 isolates of *Aeromonas* were categorized into 73 STs, 65 of which were novel (ST889-ST961

and ST991-ST1023), revealing a high degree of genetic diversity among the *Aeromonas* strains (Figure 1). There was no dominant ST observed in any of the *Aeromonas* isolates.

Diversity and Distribution of Aeromonas

We identified all the *Aeromonas* isolates based on *gyrB-cpn60* sequencing, which were distributed as follows: *A. veronii* (78.5%), *A. caviae* (7.6%), *A. dhakensis* (3.8%), *A. salmonicida* (3.8%), *A. bivalvium* (2.5%), *A. hydrophila* (1.3%), *A. jandaei* (1.3%), and *A. media* (1.3%), Figure 2. In addition, the *Aeromonas* spp. were isolated from a variety of food products, of which the four most frequent foodstuffs were fish, pork, chicken, and spiral shell (Figure 1).

Table 1. Primer sequences used to amplify antimicrobial resistance genes

Targeted genes	Primers	Sequences (5'→3')	Product size (bp)
Extended-spectrum beta-lactamase			
blaTEM	blaTEM-F	ATAAAATTCTTGAAGACGAAA	1,080
	blaTEM-R	GACAGTTACCAATGCTTAATC	
blaSHV	blaSHV-F	TTATCTCCCTGTTAGCCACC	795
	blaSHV-R	GATTTGCTGATTTCGCTCGG	
blaCTX-M	blaCTX-M-F	CGCTTTGCGATGTGCAG	550
	blaCTX-M-R	ACCGCGATATCGTTGGT	
Tetracycline resistance			
tetA	tetA-F	GTAATTCTGAGCACTGTCGC	1,000
	tetA-R	CTGCCTGGACAACATTGCTT	
tetB	tetB-F	CTCAGTATTCCAAGCCTTTG	400
	tetB-R	CTAAGCACTTGTCTCCTGTT	
tetE	tetE-F	GTGATGATGGCACTGGTCAT	1,100
	tetE-R	CTCTGCTGTACATCGCTCTT	
Plasmids mediate quinolone resistance			
qnrA	qnrA-F	AGAGGATTTCTCACGCCAGG	580
	qnrA-R	TGCCAGGCACAGATCTTGAC	
qnrB	qnrB-F	GATCGTGAAAGCCAGAAAGG	496
	qnrB-R	ACGATGCCTGGTAGTTGTCC	
qnrS	qnrS-F	GCAAGTTCATTGAACAGGGT	428
	qnrS-R	TCTAAACCGTCGAGTTCGGCG	
Aminoglycoside resistance			
armA	armA-F	AGGTTGTTTCCATTTCTGAG	591
	armA-R	TCTCTTCCATTCCCTTCTCC	
aphAI-IAB	aphAI-IAB-F	AAACGTCTTGCTCGA GGC	500
	aphAI-IAB-R	CAAACCGTTATTCATTCGTGA	
aac(3)-IIa	aac(3)-IIa-F	ATGGGCATC ATTCGCACA	749
	aac(3)-IIa-R	TCTCGGCTTGAACGAATTGT	
aac(6')-Ib	aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	482
	aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT	
Mobile colistin resistance			
mcr-1	mcr-1-F	CGGTCAGTCCGTTTGTTC	309
	mcr-2-R	CTTGGTCGGTCTGTAGGG	
mcr-2	mcr-2-F	TGTTGCTTGTGCCGATTGGA	567
	mcr-2-R	CAGCAACCAACAATACCATCT	
mcr-3	mcr-3-F	AGTTTGGTTTCGCCATTTCATTAC	1,084
	mcr-3-R	ATATCACTGCGTGGACAGTCAGG	
mcr-4	mcr-4-F	TTACAGCCAGAATCATTATCA	488
	mcr-4-R	ATTGGGATAGTCGCCTTTTT	
Sulfonamide resistance			
sul1	sul1-F	CGGCGTGGGCTACCTGAACG	433
	sul1-R	GCCGATCGCGTGAAGTTCCG	
sul2	sul2-F	GCGCTCAAGGCAGATGGCATT	293
	sul2-R	GCGTTTGATACCGGCACCCGT	

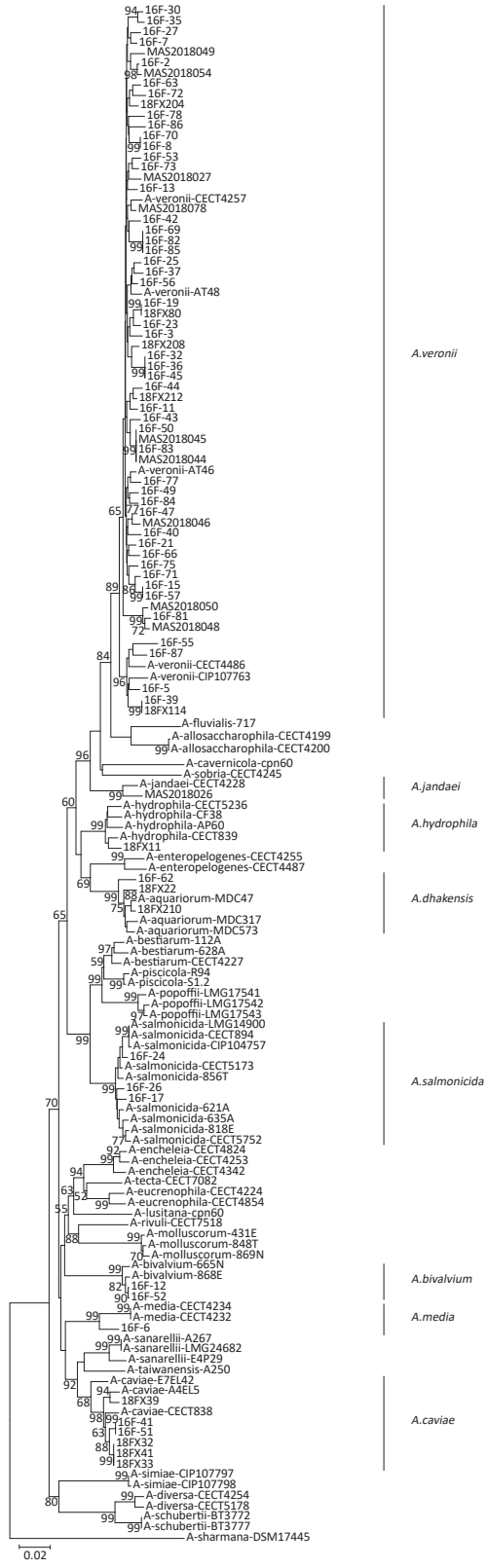

Figure 2. Subtyping of *Aeromonas* isolates. A neighbor-joining phylogenetic tree was constructed using the concatenated sequences of *gyrB* and *cpn60* to reveal the relationships between 79 *Aeromonas* isolates from food products.

The β-hemolytic and Exoprotease Assays

From the 79 *Aeromonas* isolates, 36 (45.6%) exhibited β-hemolytic activity and 64 (81%) exhibited proteolytic activity (Table 2). *A. caviae* lacked hemolytic activity; the positive rate of proteolytic activity was 16.7%. *A. dhakensis* (66.7% and 100%, respectively) and *A. hydrophila* (100% and 100%, respectively) had more hemolytic and exoprotease activities than *A. veronii* (50.0% and 85.5%, respectively), *A. caviae* (0 and 16.7%, respectively) and *A. bivalvium* (0 and 100%, respectively). Moreover, the positive rate of hemolytic and proteolytic activities in *A. salmonicida* strains were 33.3% and 66.7%, respectively. *A. jandaei* showed no signs of β-hemolysis.

C. elegans LT and Cytotoxicity Tests

The pathogenicity of *Aeromonas* isolates was assessed using the *C. elegans* LT test, and the results are shown in Figure 3. The 1, 2, and 3-day survival rates of *C. elegans* fed with *A. dhakensis*, *A. hydrophila*, *A. veronii*, *A. caviae,* and *A. bivalvium* were as follows: 26.1%, 23.2%, and 17.4%; 26.7%, 11.1%, and 11.1%; 41.7%, 33.8%, and 29.6%; 43.8%, 35.8%, and 31.0%; and 47.7%, 20.9%, and 15.1%, respectively. Thus, *A. dhakensis* and *A. hydrophila* were more pathogenic than other *Aeromonas* spp. Overall, the *C. elegans* survival rates when cultured with *A. dhakensis* and *A. hydrophila* were significantly lower than *A. veronii*, *A. caviae*, and *A. bivalvium* ($P < 0.05$).

The pathogenicity of *Aeromonas* isolates was assessed using cytotoxicity tests. Compared with LDH release of the 10× lysis solution, the volume of LDH release induced by *A. dhakensis*, *A. hydrophila*, *A. veronii*, *A. caviae,* and *A. bivalvium* were 75.9%, 74.5%, 46.4%, 11.9%, and 5.2%, respectively. Our results indicated that *A. dhakensis* and *A. hydrophila* were more virulent than *A. veronii*, *A. caviae,* and *A. bivalvium*, which was in agreement with the results of the *C. elegans* LT assay. The LDH release level varied between *Aeromonas* spp. ($P < 0.05$).

Distribution of Virulence Genes

The distribution of the 11 virulence genes in the *Aeromonas* strains is summarized in detail (Table 3). In particular, *act* and *fla* were the two most frequent virulence genes among all the isolates, accounting for 73.4% and 69.6%, respectively. The *ast* and *aerA* genes were detected in < 10% of all isolates. The detection rates of *lip*, *ela*, *act*, *alt*, *aerA*, and *hlyA* were significantly different among the eight species of *Aeromonas* [$P < 0.05$ (Fisher's exact test)]. Apart

Table 2. Prevalence of hemolytic and proteolytic activities in *Aeromonas* isolates

Assay	*A. veronii*, *n* (%)	*A. caviae*, *n* (%)	*A. dhakensis*, *n* (%)	*A. hydrophila*, *n* (%)	*A. salmonicida*, *n* (%)	*A. bivalvium*, *n* (%)	*A. jandaei*, *n* (%)	*A. media*, *n* (%)
Hemolysis	31 (50.0)	0	2 (66.7)	1 (100)	1 (33.3)	0	0	1 (100)
Proteolysis	53 (85.5)	1 (16.7)	3 (100)	1 (100)	2 (66.7)	2 (100)	1 (100)	1 (100)
Total	62	6	3	1	3	2	1	1

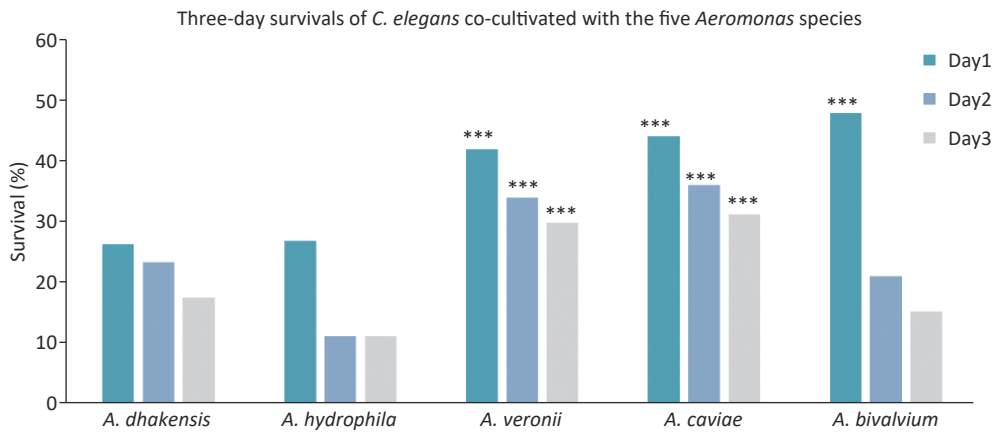

Figure 3. *C. elegans* liquid toxicity assay. Three-day survival of *C. elegans* co-cultivated with the following five *Aeromonas* species: *A. dhakensis*; *A. hydrophila*; *A. veronii*; *A. caviae*; and *A. bivalvium* in the liquid-toxic assay. $^{***}P < 0.05$, as compared with *A. dhakensis*.

from the three genes (*ast*, *aexT,* and *ascV*), all other virulence genes were detected in *A. dhakensis*. Only *act*, *fla*, *laf*, *aexT*, and *ascV* were more prevalent in *A. veronii* than other *Aeromonas* spp.

In addition to three genes (*fla*, *ascV*, and *aexT*), the prevalence of other genes differed significantly among the five *Aeromonas* spp. [$P < 0.05$ (Fisher's exact test); Table 4]. Only *hlyA* was more frequent in *A. dhakensis* and *A. hydrophila* than *A. veronii*, *A. caviae,* and *A. bivalvium*. The genes, *act* and *aerA*, were abundant in *A. dhakensis* and *A.veronii*, but neither was detected in the other three *Aeromonas* spp. The gene, *alt*, was most commonly identified in *A. dhakensis*, *A. hydrophila,* and *A. bivalvium*. Moreover, the genes, *lip* and *ela,* were less prevalent in *A.veronii* than *A.dhakensis*, *A. hydrophila*, *A. caviae*, and *A. bivalvium.*

Antimicrobial Resistance

Resistance to CZO was demonstrated in 74 (93.7%) *Aeromonas* isolates. The antibiotic resistance rates of *A. veronii* to AMC, CRO, CIP, LEV, trimethoprim-sulfamethoxazole, and CT were 1.6%, 3.2%, 4.8%, 1.6%, 9.7%, and 1.6%, respectively. *A. veronii* was susceptible to SAM, CAZ, and ATM. *A. dhakensis* showed 100% resistance to CT and CZO, and 33.3% to AMC and CRO. *A. caviae* was shown to be 100% resistant to CZO and 66.7% against CAZ, CRO, and ATM. In addition to CZO, *A. bivalvium* was susceptible to other antibiotics. The antimicrobial resistance rates of *Aeromonas* isolates against 10 common antibiotics are presented in (Table 5).

In this study, 9 (11.4%) *Aeromonas* isolates were multidrug-resistant, and the high MDR rates of *A. dhakensis*, *A. hydrophila*, *A. veronii*, and *A. caviae* were 33.3%, 100%, 3.2%, and 66.7%, respectively, which is an enormous challenge for treatment options.

Detection of Antimicrobial Resistance Genes

The tetracycline resistance gene, *tetE,* was found in *A. veronii* (35.5%), *A. caviae* (33.3%), *A. salmonicida* (66.7%), and *A. media* (100%). The aminoglycoside resistance gene, *aac(6')-Ib,* was detected in *A. veronii* (16.1%), *A. caviae* (50.0%), *A. bivalvium* (50.0%), *A. dhakensis* (33.3%), *A. media* (100%), and *A. hydrophila* (100%). Moreover, the CT resistance gene, *mcr-3,* accounted for 16.1% of *A. veronii* and 33.3% of *A. dhakensis*. The *PMQR* gene, *qnrS,* was present in 3.2% of *A. veronii* and 100% of *A. hydrophila*. In addition, *blaTEM* (1.6%), *tetA* (1.6%), and *mcr-1*(1.6%) were present in *A. veronii*. No drug resistance gene was observed in *A. jandaei*. The following genes were not detected in any *Aeromonas* isolates: the *ESBL* genes, *blaSHV* and *blaCTX-M*; the tetracycline resistance gene, *tetB*; the *PMQR* genes, *qnrA* and *qnrB*; the aminoglycoside resistance genes, *aphAI-IAB*, *aac (3')-IIa,* and *armA*; the CT resistance genes, *mcr-2* and *mcr-4*; and the sulphonamide resistance genes, *sul1* and *sul2* (Figure 1).

DISCUSSION

Common clinical manifestations of *Aeromonas* infections include gastrointestinal infections, diarrhea, bacteremia, and localized soft tissue infections[11]. The clinical *Aeromonas* isolates (96.5%) that have been identified to date belong to one of the following species: *A. caviae* (29.9%); *A. dhakensis* (26.3%); *A. veronii* (24.8%); and *A. hydrophila* (15.5%)[26]. Escarpulli et al.[27] detected 82 species of

Table 3. Distribution of putative virulence factors in all *Aeromonas* isolates

Isolate	*ast*, *n* (%)	*lip*, *n* (%)	*ela*, *n* (%)	*act*, *n* (%)	*alt*, *n* (%)	*aerA*, *n* (%)	*hlyA*, *n* (%)	*fla*, *n* (%)	*laf*, *n* (%)	*ascV*, *n* (%)	*aexT*, *n* (%)
	n = 2	*n* = 18	*n* = 21	*n* = 58	*n* = 11	*n* = 6	*n* = 8	*n* = 55	*n* = 11	*n* = 24	*n* = 29
A. veronii	1 (1.6)	4 (6.5)	8 (13)	53 (85.5)	1 (1.6)	2 (3.2)	2 (3.2)	45 (72.6)	7 (11.3)	22 (35.5)	26 (41.9)
A. caviae	0	6 (100)	5 (83.3)	0	2 (33.3)	0	0	4 (66.7)	3 (50)	0	1 (16.7)
A. dhakensis	0	3 (100)	2 (66.7)	2 (66.7)	3 (100)	2 (66.7)	3 (100)	2 (66.7)	1 (33.3)	0	0
A. hydrophila	1 (100)	1 (100)	1 (100)	0	0	0	1 (100)	0	0	1 (100)	0
A. salmonicida	0	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	0	1 (33.3)	1 (33.3)
A. bivalvium	0	2 (100)	2 (100)	0	2 (100)	0	0	2 (100)	0	0	0
A. jandaei	0	0	0	1 (100)	1 (100)	0	0	0	0	0	1 (100)
A. media	0	0	1 (100)	0	0	0	0	0	0	0	0
P value	0.105	< 0.001	< 0.001	< 0.001	< 0.001	0.003	< 0.001	0.310	0.215	0.155	0.486

Aeromonas in 250 frozen edible fish in Mexico City, Mexico and emphasized the risks associated with the consumption of the *Aeromonas* isolated from frozen edible fish. In our study, 29 (36.7%) strains of *Aeromonas* were isolated from fish samples. Consumption of food infected with *Aeromonas* poses a potential threat to human health. Accordingly, we analyzed *Aeromonas* isolates obtained from food samples from Shanghai Agricultural Market and evaluated the species distribution, antibiotic resistance, and pathogenicity.

Among the 73 STs identified from the 79

Table 4. Distribution of virulence factors among isolates of *A. dhakensis*, *A. hydrophila*, *A. veronii A. caviae*, and *A. bivalvium*

Gene	*A. dhakensis*, *n* (%)	*A. hydrophila*, *n* (%)	*A. veronii*, *n* (%)	*A. caviae*, *n* (%)	*A. bivalvium*, *n* (%)	*P* value
	n = 3	*n* = 1	*n* = 62	*n* = 6	*n* = 2	
ast	0	1 (100)	1 (1.6)	0	0	0.047
lip	3 (100)	1 (100)	4 (6.5)	6 (100)	2 (100)	< 0.001
ela	2 (66.7)	1 (100)	8 (13)	5 (83.3)	2 (100)	< 0.001
act	2 (66.7)	0	53 (85.5)	0	0	< 0.001
alt	3 (100)	1 (100)	1 (1.6)	2 (33.3)	2 (100)	< 0.001
aerA	2 (66.7)	0	2 (3.2)	0	0	0.027
hlyA	3 (100)	1 (100)	2 (3.2)	0	0	< 0.001
fla	2 (66.7)	0	45 (72.6)	4 (66.7)	2 (100)	0.603
laf	1 (33.3)	1 (100)	7 (11.3)	3 (50)	0	0.020
ascV	0	1 (100)	22 (35.5)	0	0	0.121
aexT	0	0	26 (41.9)	1 (16.7)	0	0.360

Table 5. Prevalence of resistance to different antibiotics

Drug	Resistance isolates, *n* (%)							
	A. veronii	*A. caviae*	*A. dhakensis*	*A. hydrophila*	*A. salmonicida*	*A. bivalvium*	*A. jandaei*	*A. media*
	n = 62	*n* = 6	*n* = 3	*n* = 1	*n* = 3	*n* = 2	*n* = 1	*n* = 1
Penicillins								
Ampicillin/sulbactam	0	0	0	0	0	0	0	1 (100)
Amoxicillin/clavulanic acid	1 (1.6)	0	1 (33.3)	0	1 (33.3)	0	1 (100)	0
Cephems								
Cefazolin	58 (93.5)	6 (100)	3 (100)	1 (100)	3 (100)	1 (50)	1 (100)	1 (100)
Ceftazidime	0	4 (66.7)	0	0	0	0	0	0
Ceftriaxone	2 (3.2)	4 (66.7)	1 (33.3)	0	0	0	1 (100)	0
Monobactams								
Aztreonam	0	4 (66.7)	0	0	0	0	0	0
Quinolones								
Ciprofloxacin	3 (4.8)	0	0	1 (100)	0	0	0	0
Levofloxacin	1 (1.6)	0	0	0	0	0	0	0
Folate pathway inhibitors								
Trimethoprim-sulfamethoxazole	6 (9.7)	0	0	1 (100)	0	0	0	0
Polymyxins								
Colistin	1 (1.6)	0	3 (100)	1 (100)	0	0	1 (100)	0

Aeromonas isolates, 65 were novel, which indicated that the isolates obtained from food products had high genetic diversity. Lau et al.[28] also found 36 STs in 47 isolates in Malaysia, 34 of which were novel STs, which suggested that the isolates obtained from food products often had high genetic diversity. The phylogenetic relationship of 79 *Aeromonas* isolates was evaluated using the housekeeping gene, *gyrB-cpn60*[28]. *A. veronii* was the most dominant *Aeromonas* species, comprising 78.5% of all isolates, and was isolated from meat (43.5%) and fish (33.9%). *A. caviae* (7.6%) was the second most prevalent *Aeromonas* species and was distributed in various foods, such as fish and calms. Elala et al.[29] isolated *A. caviae* from chickens, suggesting that this species could survive in both aquatic and terrestrial environments. The third most abundant species was *A. dhakensis*, which has been reported[5] to have a higher virulence potential and is a notable human pathogen. In our study, *A. dhakensis* was isolated from fish and shrimp samples at a 2:1 ratio. Similarly, Wu et al.[15] reported bacteria isolated from fish and clinical isolates in Tainan to be *Aeromonas* spp.; the most prevalent species were *A. caviae*, *A. dhakensis,* and *A. veronii*.

In this study, we analyzed the virulence of *Aeromonas* isolates using β-hemolysis and proteolytic, *C. elegans* LT, and cytotoxicity assays. The positivity rates of *A. dhakensis* and *A. hydrophila* based on the β-hemolysis and proteolytic assays were higher than *A. veronii*, *A. caviae,* and *A. bivalvium*, which was confirmed by the survival rate of *C. elegans* fed with *Aeromonas* isolates, as well as the LDH release from mouse fibroblasts C_2C_{12} infected with *Aeromonas* spp. Wu et al.[15] reported that the pathogenicity of *A. veronii* and *A. caviae* were less virulent than *A. dhakensis* and *A. hydrophila*, which was consistent with our findings. The virulence of *A. dhakensis* against *C. elegans* was shown to be more evident than that of *A. hydrophila*[20], in contrast to the pathogenicity of *A. dhakensis*, which differed little from *A. hydrophila* in our investigation.

Cody et al.[30] reported that the virulence phenotype of *Aeromonas* was the result of a combined effect of its virulence factors. We detected several virulence genes from the *Aeromonas* spp. In all *Aeromonas* isolates, *act* was the most prevalent virulence gene, followed by *fla*, *aexT*, and *ascV*. The cytolytic enterotoxin gene, *act,* was the most frequently detected among all isolates, and the result corresponded with the data of *act* (63%) and *alt* (57%), which were higher than *ast* (6%) in *Aeromonas* from water and fish sourced in India[31]. Furthermore, *act* was mainly distributed in *A. veronii* (85.5%), and *fla* was observed in most *Aeromonas* spp. except *A. jandaei*, *A. hydrophila*, and *A. media*. The *aexT* gene was present in all *A. jandaei*, 41.9% of *A. veronii*, 33.3% of *A. salmonicida*, and 16.7% of *A. caviae*. Another study[15] suggested that compared to other *Aeromonas* pathogens, the lack of *aerA* or *hlyA* may be associated with low virulence of *A. caviae* and *A. bivalvium*. These results represented the distribution of virulence genes in food products and indicated the potential pathogenicity of *Aeromonas* isolates, which could help enhance food quality and hygiene in this region, and aid in preventing and controlling the outbreak of *Aeromonas*-related diseases.

The results of this study indicated that *Aeromonas* isolates displayed 93.7% resistance to the first-generation cephalosporin (CZO) and < 11% resistance to third-generation cephalosporins (10.1% for CRO and 5% for CAZ). Several studies[32,33] reported that the majority of isolates had 100% susceptibility to third-generation cephalosporins (CAZ and cefotaxime), while there was a different degree of resistance to first-generation cephalosporins, ranging from 79.3% to 91.8%. Generally, our findings were in agreement with other studies[32,33]. In addition, *A. bivalvium* had resistance to CZO (50%), and this finding corroborated the finding that all isolates from clinical and environmental samples were 80% resistant to CZO[33], which indicates that resistance to first-generation cephalosporins could be prevalent[34]. We found that isolates of *A. dhakensis* exhibited resistance to CZO (100%), CT (100%), CRO (33.3%), and AMC (33.3%), confirming multiple drug resistance of this species, which is in agreement with previous findings[6]. This study demonstrated that *A. veronii* had various types of drug resistance, with 100% susceptibility to SAM, CAZ, and ATM. Absolute (100%) sensitivity to CIP was reported in a previous study in which several *A. veronii* strains were isolated from clinical patients[33]; however, this differed from the CIP resistance rate of 5% determined in the current study. Moreover, we found that the resistance rate of *Aeromonas* spp. to CT was 7.6%, which was approximately the same as that of *Aeromonas* spp. isolated from Australian fish. CT is the last line of antibiotic treatment used for clinically severe infections caused by multidrug-resistant gram-negative bacteria[33]. In the present study, nine strains of *Aeromonas* showed a multidrug resistance rate of 11.4%, which was lower

than that reported in a previous study[6].

Consistent with the findings of a study conducted by Dahanayake et al.[35], *tetE* was detected in 27 strains (34.2%) and *tetA* was detected in 1 strain (1.3%); However, *tetB* was not detected in the present study. Moreover, the *tetE* detection rates varied among *A. veronii*, *A. caviae*, *A. media*, and *A. salmonicida*, ranging from 33.3% to 100%. Recently, aminoglycosides have been extensively used for treating severe infections caused by *Aeromonas*, and Dahanayake et al.[35] reported the presence of *aac(6')-Ib* in all *Aeromonas* spp. isolated from cockles in Korea. In the present study, 21.5% of the isolates of *Aeromonas* spp. harbored *aac(6')-Ib,* except for *A. salmonicida* and *A. jandaei*.

Our study indicated that approximately 13.9% of *Aeromonas* spp. harbored *mcr*-3 (16.1% of *A. veronii* and 33.3% of *A. dhakensis*). These data support the discovery of *mcr-3* in *A. jandaei* isolated from fish sold in Beijing, China[36]. Studies have reported the presence of *qnrS* not only in *Enterobacteriaceae,* but also in *Aeromonas*, suggesting that this gene is distributed across different bacterial species[37]. We found that *Aeromonas* strains collected from food products had a resistance rate of 8.9% to SXT, and the same strains exhibited a detection rate of 3.8% for *qnrS*. This gene was detected in 3.2% and 100% of *A. veronii* and *A. hydrophila* isolates, respectively. Previous studies have reported different rates of detection of *qnrS* in Lake Lugano (Switzerland)[37] and China[6]. Moreover, the rate of detection of bla_{TEM} in *A. veronii* was 1.6%, confirming the presence of bla_{TEM} in *A. veronii* isolated from German water sources[38]. Nonetheless, these findings should be further assessed in future studies.

CONCLUSIONS

Aeromonas was an important zoonotic pathogen that is mainly distributed in fish and other aquatic products. Studies have demonstrated that these isolates had a high degree of genetic diversity; the 79 isolates in the current study were shown to produce 73 independent STs. The isolates were divided into eight species by *gyrB-cpn60* sequencing. The distribution, taxonomy, drug resistance, and pathogenicity varied among different *Aeromonas* spp. The percentage of *Aeromonas* isolates shown to be MDR was 11.4%. Food-derived *Aeromonas* isolates (93.7%) were resistant to CZO. *Aeromonas* carried some virulence and antibiotic resistance genes, which posed a threat and challenge to food safety and clinical treatment monitoring. A few isolates, such as *A. dhakensis* and *A. hydrophila*, had high pathogenicity and could cause extensive infections and posed risks to human and animal health.

CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

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