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ORIGINAL PAPER

Phylogenetic characteristics, virulence properties and antibiogram profile of motile *Aeromonas* spp. isolated from ornamental guppy (*Poecilia reticulata*)

Sabrina Hossain[1] · B. C. J. De Silva[1] · P. S. Dahanayake[1] · Mahanama De Zoysa[2] · Gang-Joon Heo[1]

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Abstract

Aeromonas spp. are opportunistic pathogenic bacteria related to an assembly of infectious diseases in ornamental fish. In the present study, virulence properties and antibiotic susceptibility of 52 guppy-borne *Aeromonas* spp. were investigated. The isolates were identified as *A. veronii* ($n=34$), *A. dhakensis* ($n=10$), *A. hydrophila* ($n=3$), *A. caviae* ($n=3$) and *A. enteropelogenes* ($n=2$) by *gyrB* gene sequencing. The *gyrB* sequence deviation within and among the species ranged from 0 to 2.6% and 2.7–9.2%. Each species formed a distinct group in the unrooted neighbor-joining phylogenetic tree. The phenotypic virulence factors such as β-hemolysis, slime, caseinase, DNase, gelatinase and lipase production were observed in 28 (53.9%), 33 (63.5%), 28 (53.9%), 42 (80.8%), 37 (71.2%) and 42 (80.8%) isolates, respectively. The virulence genes were detected by PCR assay in the following proportions- *act* (84.6%), *hly* (80.8%), *aer* (73.1%), *lip* (73.1%), *gcaT* (73.1%), *ascV* (53.8%), *ahyB* (53.8%) *fla* (51.9%), *alt* (48.1%), *ast* (36.5%) and *ser* (34.6%), respectively. The amoxicillin, ampicillin, imipenem, nalidixic acid, oxytetracycline and rifampicin were resistant to more than 70.0% of the isolates in antibiotic susceptibility test. Our study suggests that the ornamental guppy can be a potential reservoir of virulent and multi-drug resistant *Aeromonas* spp.

Keywords *Aeromonas* spp. · Phylogenetic tree · Virulence markers · Antibiotic susceptibility · Guppy

Introduction

Ornamental fish culture is a powerful income and employment source in the world. As a non-food activity, ornamental fish trade provides an excellent opportunity in the aquaculture sector. More than 1 billion ornamental fish are traded internationally, making the industry the most important in global trade (John and Hatha 2013; Sreedharan et al. 2013).

Guppy (*Poecilia reticulata*) is one of the most popular tropical freshwater aquarium fish in many countries, including Korea. Like other ornamental fish, guppies are prone to various types of viral, bacterial and fungal infections. Bacterial diseases especially caused by Gram-negative species are commonly found in ornamental fish (Lewbart 2001). Environmental factors such as stress, poor water quality, unhygienic handling and polluted feeding can increase the chance of infections among the fish in the captive condition (Kigigha et al. 2012). Furthermore, the deficiency in the immune system of fish increases morbidity and mortality rates (Toranzo et al. 2005).

Aeromonas spp. or aeromonads are the natural microflora of freshwater or brackish water environment (Krishnakumar et al. 2009). To date, there are 36 recognized species in the genus *Aeromonas* (Navarro and Martínez-Murcia 2018). Psychrophilic or non-motile *A. salmonicida* is mainly associated with furunculosis in fish while mesophilic or motile *Aeromonas* spp. such as *A. veronii*, *A. hydrophila*, *A. dhakensis* and *A. caviae* are particularly documented in humans and animals (Teunis and Figueras 2016). They are regarded as important pathogenic bacteria in fish and may cause motile aeromonad septicemia (MAS). In Korea, several studies have been reported *Aeromonas* infection in farmed Israeli carp (*Cyprinus carpio*), cyprinid loach (*Misgurnus*

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✉ Gang-Joon Heo
gjheo@cbu.ac.kr

[1] Laboratory of Aquatic Animal Medicine, Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Chungdae-ro 1, Seowon-gu, Cheongju, Chungbuk 28644, Korea

[2] College of Veterinary Medicine, Chungnam National University, Daejeon, Korea

anguillicaudatus) and mud loach (*Misgurnus mizolepis*) (Yu et al. 2010, 2015; Jun et al. 2010). *Aeromonas* infection is a serious threat for freshwater fish production which brings enormous loss to the ornamental fish industry (John and Hatha 2013; Krishnakumar et al. 2009).

The identification of virulence factors is a major constituent to determine the potential pathogenicity due to the multifunctional roles of those virulence factors in *Aeromonas* pathogenesis (Nawaz et al. 2010). The existence of an assortment of virulence factors allows them to colonize, establish, replicate and damage the host tissues (Yu et al. 2005). The pathogenicity of aeromonads is related to several virulence factors including various extracellular enzymes, cytotoxic and cytotonic enterotoxins, hemolytic toxins and type 3 secretion system (T3SS) (Janda and Abbott 2010).

In the ornamental fish industry, antibiotics have been used since 1970s for controlling bacterial diseases by the ornamental fish producers, retailers as well as fish owners (Trust and Whitby 1976). The uncontrolled antibiotic uses the result in the emergence of antibiotic-resistant bacteria and the expansion of multi-drug resistant *Aeromonas* spp. (Rowe-Magnus et al. 2002).

As far as we know, there is no report about the identification of both phenotypic and genotypic virulence markers as well as antibiotic susceptibility patterns of *Aeromonas* spp. isolated from ornamental guppy in Korea. Thus, the objective of our study was to characterize *Aeromonas* isolates from guppy based on phylogenetic grouping, virulence potential and multi-drug resistance profile.

Materials and methods

Isolation and phenotypic identification of *Aeromonas* spp.

A total of 52 *Aeromonas* spp. were isolated from 62 healthy guppies with an average length of 2.20–4.77 cm which were randomly purchased from the pet shops in Seoul, Korea. The live guppies were transported from the pet shops to the laboratory in separate sterile polyethylene bags within 2 h.

Each fish was humanely euthanized with an overdose of MS222 (250 mg/l, buffered) in separate test tubes (AVMA 2013). The whole body of the fish was homogenized with 3 ml of sterile physiological saline. One ml of sample was enriched in 9 ml of alkaline peptone water at 35 °C for 24 h. One loopful from each homogenate was streaked onto a plate of *Aeromonas* agar base (MB Cell, LA, CA) and incubated overnight at 35 °C. The typical green colonies with dark green center assuming as *Aeromonas* spp. were subcultured on tryptic soy agar (MB Cell, LA, CA) and tested for indole, oxidase, H_2S and starch hydrolysis tests (Hossain et al. 2018a). The genomic DNA from phenotypically identified *Aeromonas* species was extracted using Exgene Cell SV kit (GeneAll, Seoul, Korea). For molecular identification, PCR amplification and sequencing of the *gyrB* gene were performed. The primers selection, PCR amplification and sequencing were done according to a previous report (Yáñez et al. 2003). Taxonomic identification of the DNA sequences was done by means of the BLAST option in GenBank (http://blast.ncbi.nlm.nih.gov/).

Genetic distance and phylogenetic analysis of *gyrB* sequences

Multiple sequence alignments were carried out using ClustalW in MEGA 7.0 software program (Kumar et al. 2016). The *gyrB* sequences from all isolates and their corresponding reference sequences were included in the alignment to determine the genetic distance within and between the species. The genetic distances were obtained using Kimura's two-parameter model.

To construct an unrooted phylogenetic tree, *gyrB* reference sequences of *A. veronii* (Accession no.: AF417626.1), *A. hydrophila* (Accession no.: AF417622.1), *A. caviae* (Accession no.: AJ868400.1), *A. dhakensis* (Accession no.: AM262163.1) and *A. enteropelogenes* (Accession no.: AJ868392.1) were obtained from NCBI database. The evolutionary tree was created by the neighbor-joining method with 1000 bootstrap replicates.

Phenotypic characterization of virulence factors

To determine the phenotypic virulence factors, several phenotypic pathogenicity tests were carried out at 35 °C for 24 h. β-hemolysis activity was assessed using sheep blood agar (MB Cell, LA, CA). Slime production was assessed by means of tryptic soy agar supplemented with 5% sucrose and 0.08% Congo red (Freeman et al. 1989). Caseinase production was perceived in nutrient agar supplemented with 10% skim milk (Abd-El-Malek, 2017). DNase activity was tested by DNase agar plates adding 1 N HCl. The gelatin media prepared by 3% beef extract, 5% peptone and 15% gelatin with a final pH 7.0 to detect gelatin production. Lipase production was screened using Tryptic soy agar plates supplemented with 1% Tween 80 (*v*/*v*) (Harley and Prescott 2002).

Genotypic characterization of virulence factors

PCR was carried out to determine the presence of 11 virulence genes including *act, alt, ast, aer, hly, ser, lip, fla, ascV, ahyB* and *gcaT*. The PCR amplification of the virulence genes was done by means of PCR primers and conditions described in Table 1.

The PCR mixture of 20 µl contained 2 µl Taq buffer, 1.6 µl dNTP, 14.2 µl nuclease-free water, 0.2 µl AmpOne

Table 1 Primers used for the detection of virulence genes

Target gene	Nucleotide sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Function	Reference
act	F: AGAAGGTGACCACCACCAAGAACA	232	64	Cytotoxic enterotoxin	Nawaz et al. (2010)
	R: AACTGACATCGGCCTTGAACTC				
alt	F: TGACCCAGTCCTGGCACGGC	442	63	Heat-labile cytotonic enterotoxin	Nawaz et al. (2010)
	R: GGTGATCGATCACCACCAGC				
ast	F: TCTCCATGCTTCCCTTCCACT	331	63	Heat-stable cytotonic enterotoxin	Nawaz et al. (2010)
	R: GTGTAGGGATTGAAGAAGCCG				
aer	F: CCTATGGCCTGAGCGAGAAG	431	62	Aerolysin	Nawaz et al. (2010)
	R: CCAGTTCCAGTCCCACCACT				
hlyA	F: GGCCGGTGGCCCGAAGATACGGG	597	62	Hemolysis A	Sen and Rodgers (2004)
	R: GGCGGCGCCGGACGAGACGGG				
fla	F: TCCAACCGTYTGACCTC	608	55	Flagellin	Nawaz et al. 2010)
	R: GMYTGGTTGCGRATGGT				
ser	F: CACCGAAGTATTGGGTCAGG	350	56	Serine protease	Nawaz et al. (2010)
	R: GGCTCATGCGTAACTCTGGT				
ahyB	F: ACACGGTCAAGGAGATCAAC	513	58	Elastase	Nawaz et al. (2010)
	R: CGCTGGTGTTGGCCAGCAGG				
lip	F: ATCTTCTCCGACTGGTTCGG	382	62	Lipase	Carvalho-Castro et al. (2010)
	R: CCGTGCCAGGACTGGGTCTT				
gcaT	F: CTCCTGGAATCCCAAGTATCAG	237	65	Gycerophospholipid-cholesterol acyltransferase	Nawaz et al. (2010)
	R: GGCAGGTTGAACAGCAGTATCT				
ascV	F: AGCAGATGAGTATCGACGG	891	58	Type III secretion system	Wong et al. (1998)
	R: AGGCATTCTCCTGTACCAG				

Taq DNA polymerase (GeneAll, Seoul, Korea), 1 µl template and 1 µl of 10 µM of both forward and reverse primers. The PCR products were observed by electrophoresis on 2% (*w*/*v*) agarose gel. A 100-bp DNA ladder (Invitrogen, San Jose, CA) was used as a molecular size reference.

Antibiotic susceptibility testing

The antibiotic susceptibility patterns of the isolates were determined by the antibiotic disk diffusion test using commercially available 17 antibiotic disks of 10 antibiotic groups.

The following antibiotics were used: Penicillins; amoxicillin (30 µg), ampicillin (10 µg), Cephalosporins; cephalothin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), Carbapenem; imipenem (10 µg), Quinolones; nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), Tetracyclines; tetracycline (30 µg), oxytetracycline (30 µg), Folate pathway inhibitor; trimethoprim/sulfamethoxazole (1.25/23.75 µg), Ansamycin; rifampicin (5 µg), Phenicol; chloramphenicol (30 µg) and Aminoglycosides; kanamycin (30 µg), amikacin (30 µg), gentamicin (10 µg). *Escherichia coli* ATCC 25922 was used as a quality control strain.

The resistance phenotype was interpreted according to the Clinical and Laboratory Standards Institute, CLSI guidelines (CLSI 2014).

Results

Aeromonas spp. identification

The sequences of *gyrB* gene demonstrated that the isolates could be identified as 5 species, namely *A. veronii* ($n=34$), *A. dhakensis* ($n=10$), *A. hydrophila* ($n=3$), *A. caviae* ($n=3$) and *A. enteropelogenes* ($n=2$). The *gyrB* gene sequences of the isolates were deposited in Genbank.

Percentage of nucleotide substitutions were calculated for a continuous segment of 917 nt. The sequence similarities of the *Aeromonas* species ranged from 97 to 99% in

BLAST comparison of the NCBI database. The nucleotide substitution rates in the intraspecies levels varied from 0 to 2.6%. The inter-species sequence divergence ranged from 2.7 to 9.2%.

Phylogenetic analysis

An unrooted neighbor-joining phylogenetic tree was constructed from the nucleotide sequence alignment (Fig. 1). The phylogenetic tree was established which divides into two major clusters. One subsection included *A. veronii* whereas

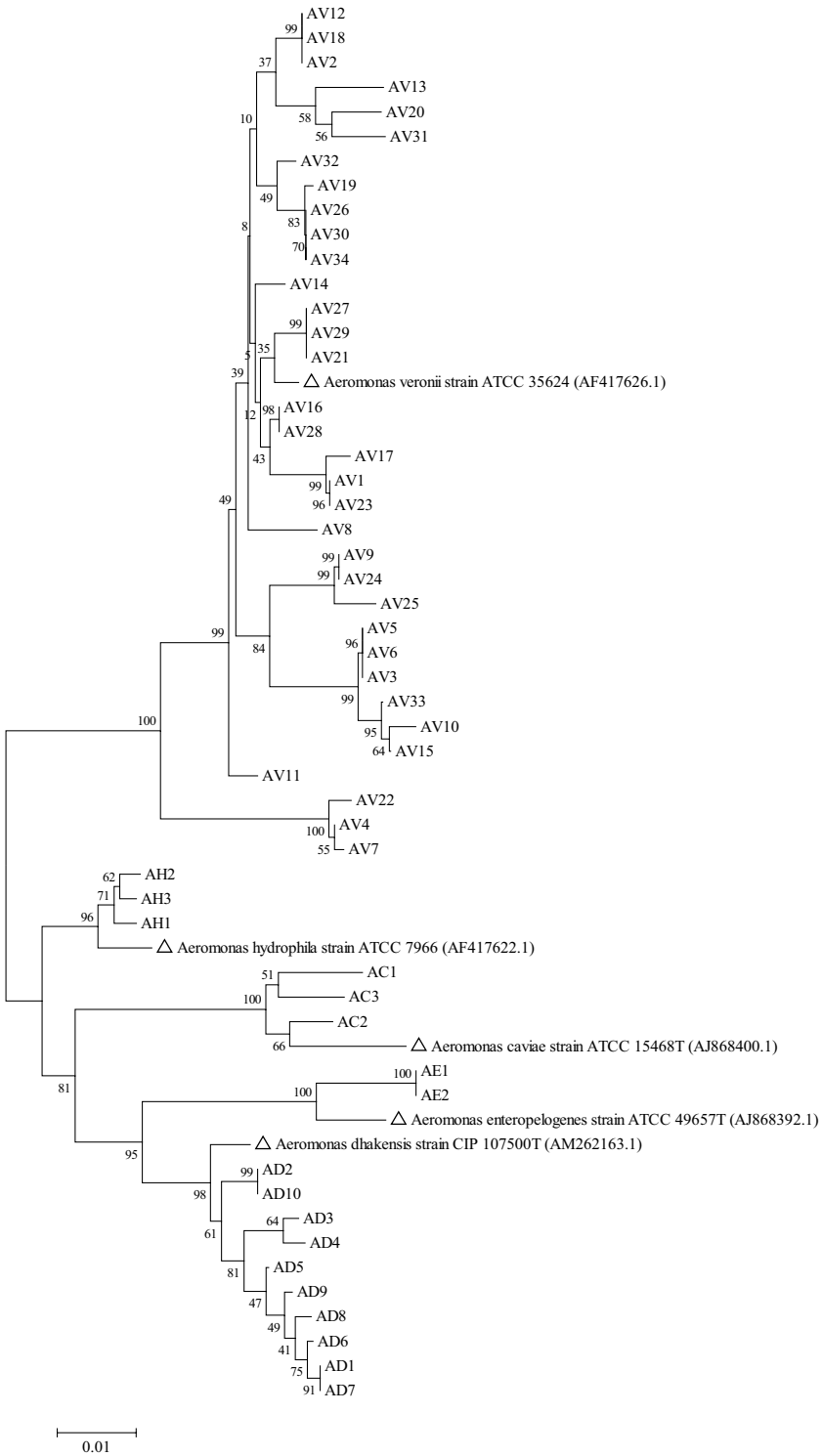

Fig. 1 Unrooted neighbor-joining phylogenetic tree based on *gyrB* sequences of *Aeromonas* spp. isolated from guppy (*Poecilia reticulata*). The GenBank sequences are labelled as (Δ) and the isolates including *A. hydrophila*, *A. veronii*, *A. caviae, A. dhakensis* and *A. enteropelogenes* are marked as AH, AV, AC, AD and AE, respectively

the other section included *A. hydrophila*, *A. enteropelogenes*, *A. dhakensis* and *A. caviae*. Every isolate grouped with their respective reference sequence.

Phenotypic characterization of virulence factors

Phenotypic virulence factors were analyzed among the tested species by several pathogenicity tests including the production of caseinase, DNase, gelatinase and lipase enzymes, β-hemolysis activity and slime production (Table 2).The β-hemolysis, slime, caseinase, DNase, gelatinase and lipase production were observed in 28 (53.9%), 33 (63.5%), 28 (53.9%), 42 (80.8%), 37 (71.2%) and 42 (80.8%) isolates, respectively.

Prevalence of virulence genes in *Aeromonas* species

The presence of 12 virulence genes was analyzed by PCR in this study (Table 2). Each strain contained at least 2 virulence genes. Two *A. dhakensis* and one *A. veronii* showed a compliment of 10 virulence genes which is the highest frequency among the isolates (data not shown). The virulence genes including *act* (84.6%), *hly* (80.8%), *aer* (73.1%), *lip* (73.1%), *gcaT* (73.1%), *ascV* (53.8%), *ahyB* (53.8%) *fla* (51.9%), *alt* (48.1%), *ast* (36.5%) and *ser* (34.6%) were detected among the isolates.

Antibiotic susceptibility patterns

The antibiotic susceptibility patterns of *Aeromonas* species are shown in Table 3. All the isolates were resistant to at least four antibiotics belonging to ≥4 structural classes.

All isolates showed resistance to amoxicillin, nalidixic acid and oxytetracycline. The resistance for ampicillin (92.3%), rifampicin (76.9%), imipenem (71.2%) was prevalent among the guppy-borne isolates. The resistance rates of cephalothin, tetracycline, trimethoprim/sulfamethoxazole, gentamicin, kanamycin, chloramphenicol, ciprofloxacin, ofloxacin, cefotaxime and ceftriaxone were 51.9, 51.9, 50.0, 21.2, 13.5, 5.8, 5.8, 3.9, 3.9 and 1.9%, respectively.

Discussion

The taxonomy of genus *Aeromonas* is complex due to the phenotypic and genotypic discrepancies at the species level. The 16S rRNA sequencing which is the most common molecular tool for taxonomic studies of the bacteria has proven ineffective for distinguishing different *Aeromonas* species because of low taxonomic resolution (Nagar et al. 2013). On the other hand, some housekeeping genes were reported with considerably higher discriminatory power than 16S rRNA, showing 92% and 89% average sequence similarity for *gyrB* and *rpoD*, respectively (Soler et al. 2004; Nagar et al. 2013). In this study, nucleotide sequencing of

Table 2 Distribution of virulence factors in guppy-borne *Aeromonas* species

Virulence factors	*A. veronii* (*n* = 34)	*A. dhakensis* (*n* = 10)	*A. caviae* (*n* = 3)	*A. hydrophila* (*n* = 3)	*A. enteropelogenes* (*n* = 2)	Total (*n* = 52)
Phenotypic virulence factors						
β-hemolysis	18 (52.9%)	8 (80.0%)	–	2 (66.7%)	–	28 (53.9%)
Biofilm formation	23 (67.7%)	5 (50.0%)	2 (66.7%)	2 (66.7%)	1 (50.0%)	33 (63.5%)
Caesinase production	11 (32.4%)	9 (90.0%)	3 (100.0%)	3 (100.0%)	2 (100.0%)	28 (53.9%)
DNase activity	24 (70.6%)	10 (100.0%)	3 (100.0%)	3 (100.0%)	2 (100.0%)	42 (80.8%)
Gelatinase production	26 (76.5%)	9 (90.0%)	1 (33.3%)	1 (33.3%)	–	37 (71.2%)
Lipase production	25 (73.5%)	9 (90.0%)	3 (100.0%)	3 (100.0%)	2 (100.0%)	42 (80.8%)
Virulence genes						
act	30 (88.2%)	9 (90.0%)	2 (66.7%)	3 (100.0%)	–	44 (84.6%)
alt	14 (41.2%)	9 (90.0%)	1 (33.3%)	1 (33.3%)	–	25 (48.1%)
ast	11 (32.4%)	5 (50.0%)	1 (33.3%)	2 (66.7%)	–	19 (36.5%)
aer	27 (79.4%)	6 (60.0%)	–	3 (100.0%)	2 (100.0)	38 (73.1%)
hly	29 (85.3%)	10 (100.0%)	–	3 (100.0%)	–	42 (80.8%)
ser	7 (20.6%)	7 (70.0%)	2 (66.7%)	–	2 (100.0)	18 (34.6%)
lip	24 (70.6%)	10 (100.0%)	2 (66.7%)	2 (66.7%)	–	38 (73.1%)
gcaT	22 (64.7%)	10 (100.0%)	3 (100.0%)	3 (100.0%)	–	38 (73.1%)
fla	18 (52.9%)	5 (50.0%)	3 (100.0%)	1 (33.3)	–	27 (51.9%)
ascV	18 (52.9%)	6 (60.0)	1 (33.3%)	3 (100.0%)	–	28 (53.8%)
ahyB	17 (50.0)	7 (70.0)	2 (66.7%)	1 (33.3%)	1 (50.0%)	28 (53.8%)

Table 3 Antibiotic susceptibility patterns of different *Aeromonas* spp. isolated from guppy

Antibiotics	Number of resistance isolates (%)					
	A. veronii (*n* = 34)	*A. dhakensis* (*n* = 10)	*A. caviae* (*n* = 3)	*A. hydrophila* (*n* = 3)	*A. entero-pelogenes* (*n* = 2)	Total (%)
Penicillin						
Ampicillin (10 µg)	33 (97.1%)	10 (100.0%)	2 (66.7%)	2 (66.7%)	1 (50.0%)	48 (92.3%)
Amoxicillin (30 µg)	34 (100.0%)	10 (100.0%)	3 (100.0%)	3 (100.0%)	2 (100.0%)	52 (100.0%)
Cephalosporin						
Cephalothin (30 µg)	11 (32.4%)	9 (90.0%)	3 (100.0%)	2 (66.7%)	2 (100.0%)	27 (51.9%)
Cefotaxime (30 µg)	2 (5.9%)	–	–	–	–	2 (3.9%)
Ceftriaxone (30 µg)	1 (2.9%)	–	–	–	–	1 (1.9%)
Carbapenem						
Imipenem (10 µg)	27 (79.4%)	8 (80.0%)	–	2 (66.7%)	–	37 (71.2%)
Quinolone						
Nalidixic acid (30 µg)	34 (100.0%)	10 (100.0%)	3 (100.0%)	3 (100.0%)	2 (100.0%)	52 (100.0%)
Ciprofloxacin (5 µg)	2 (5.9%)	–	1 (33.3%)	–	–	3 (5.8%)
Ofloxacin (5 µg)	1 (2.9%)	–	1 (33.3%)	–	–	2 (3.9%)
Aminoglycoside						
Gentamicin (10 µg)	4 (11.8%)	5 (50.0%)	1 (33.3%)	1 (33.3%)	–	11 (21.2%)
Kanamycin (30 µg)	1 (2.9%)	5 (50.0%)	–	1 (33.3%)	–	7 (13.5%)
Tetracycline						
Oxytetracycline (30 µg)	34 (100.0%)	10 (100.0%)	3 (100.0%)	3 (100.0%)	2 (100.0%)	52 (100.0%)
Tetracycline (30 µg)	22 (64.7%)	2 (20.0%)	1 (33.3%)	1 (33.3%)	1 (50.0%)	27 (51.9%)
Folate pathway inhibitor						
Trimethoprim/sulfamethoxazole (1.25/23.75 µg)	14 (41.1%)	8 (80.0%)	2 (66.7%)	2 (66.7%)	–	26 (50.0%)
Phenicol						
Chloramphenicol (30 µg)	3 (8.8%)	–	–	–	–	3 (5.8%)
Ansamycin						
Rifampicin (5 µg)	26 (76.5%)	9 (90.0%)	2 (66.7%)	2 (66.7%)	2 (100.0%)	40 (76.9%)

the *gyrB* gene has proved to be a suitable tool for taxonomic and phylogenetic studies in *Aeromonas* spp. (Figueras et al. 2011; Hoel et al. 2019). The intraspecies nucleotide substitution rates of *gyrB* gene sequences were found as < 3%. Lower nucleotide substitution rates (< 3%) were observed in a few strains in interspecies level and the only exception was *A. hydrophila* with nucleotide substitution of 2.7%. Nucleotide substitution rates generally range from 0 to 2% in the intraspecies level while the substitution rate > 3% is considered as different species (Soler et al. 2004).

All *Aeromonas* spp. isolates were assayed for six phenotypic pathogenicity tests (Table 2). The Extracellular lipase plays an important role in the establishment of infections in humans by disturbing immune system functions and DNase allows the propagation of the bacteria (Tomas 2012). According to our results, 42 (80.8%) of the isolates were positive for lipase and DNase enzyme production and 37 (71.2%) isolates were positive for gelatinase activity. The majority of ornamental fish-borne *Aeromonas* spp. were also found to produce lipase, DNase and gelatinase in a previous study (John and Hatha 2013).

The slime production reflects the bacterial capacity to adhere in the specific host tissues and produce invasive micro-colonies. The slime production was found in 33 (63.5%) of the guppy-borne aeromonads. The slime production is responsible for acting against phagocytosis, chemotaxis and the action of antibiotics (Tomas 2012). Hemolysis activity is strongly related to enterotoxin production (Burke et al. 1983). The production of serine protease (caseinase) is responsible for the proteolysis activity and aid to the maturation of exotoxins (e.g. aerolysin). The current study could detect 28 (53.9%) β-hemolysis and caseinase positive isolates from guppies. Similarly, about 44.2% of β-hemolytic *Aeromonas* spp. were isolated from fish and beef (Arslan and Kucuksari 2015). In contrast to our results, 95.9% of caseinase producing isolates were reported by the same study.

The cytotoxic enterotoxin related *act* gene (84.6%) was highly distributed among the isolates (Table 2). The *act* gene

encoding a cytotoxin (Act) was highly found with a high frequency in the *Aeromonas* isolates from different sources such as seafood (75.0%) and drinking water (70.0%) (Sen and Rodgers 2004). The enterotoxin genes including *alt* (48.1%) and *ast* (36.5%) were less prevalent in the isolates which is in agreement with a previous study (Yano et al. 2015). Among the enterotoxins, *act* gene is one of the most important virulence determinants linked to hemolytic, cytotoxic and enterotoxic actions. The enterotoxin genes, *alt* and *ast* were also observed which are mainly related to diarrhea that influence fluid secretion in the intestine of animals (Nawaz et al. 2010). The high occurrence of *aer* and *hly* genes was identified in 73.1% and 80.8% isolates, respectively. Wong et al. (1998) proposed a two-toxin model for *aerA* and *hlyA* genes in which both genes equally contributed to *Aeromonas* virulence and they might be knocked out to reduce the virulence. We observed a heterogeneous distribution of all toxin genes in *A. veronii*, *A. dhakensis* and *A. hydrophila.* None of the *A. enteropelogenes* harbored *act*, *alt*, *ast* and *hlyA* genes and no hemolysin genes (*aer* and *hlyA*) were found in *A. caviae* isolates. The lack of specific genes might be attributed to certain species while considering the diversity in the distribution of various virulence genes among the isolates from different sources and the possibility of horizontal gene transfer of virulence determinants among the species (Hoel et al. 2019).

The extracellular enzymes of *Aeromonas* spp. are generally secreted by means of well-categorized type II or general secretory pathway in the establishment of the pathogenicity (Pemberton et al. 1997). The majority of *Aeromonas* strains exhibited extracellular lipase and glycerophospholipid-cholesterol acyltransferase related *lip* (73.1%) and *gcaT* (73.1%) genes. The presence of other extracellular enzyme encoded genes such as elastase (*ahyB*) (53.8%), T3SS (*ascV*) (53.8%) and serine-protease (*ser*) (34.6%) genes were present. These enzymes have been increasingly linked to microbial virulence since they are involved in the activation of aerolysin (*aerA*), degradation of host cell components, enhancing adhesion, contributing to cell nutrition and evade host cell (Nawaz et al. 2010).

In the present study, polar flagella related *fla* (51.9%) gene was observed among the strains. Bacterial flagellar motility enabled by a lateral or polar flagellum, have several biological roles in pathogens such as chemotaxis, adhesion and invasion (Haiko and Westerlund-Wikström 2013).

A misalliance between genotypic and phenotypic virulence factors was noticed in the aeromonads isolated from guppies. All *A. enteropelogenes* produce lipase in the phenotypic pathogenicity tests but failed to amplify *lip* gene in PCR assay. Also, every *A. hydrophila* lacked *ser* gene but could produce caseinase. In contrast, phenotypically negative *A. enteropelogenes* for β-hemolysis in sheep-blood agar conferred *aer* gene in PCR amplification. This issue has been reported previously in *Aeromonas* spp. in which the mismatch was observed with different primer sets (Yano et al. 2015). This discrepancy might be attributed to the genetic variability of virulence determinants in *Aeromonas* as previously suggested (De Silva et al. 2018). However, further studies are needed for the assessment of the specific mechanisms of genetic variability.

The antibiotic susceptibility patterns of *Aeromonas* species are shown in Table 3. All isolates were resistant to at least four antibiotics belonging to ≥ 4 structural classes. The high occurrence of β-lactam (penicillin, cephalosporin and carbapenem) antibiotics was observed among the isolates. The higher percentage of β-lactam resistant isolates was found due to the natural production of chromosomal β-lactamases by aeromonads (Tayler et al. 2010). Amoxicillin resistance was displayed in all guppy-borne isolates (Table 3). Resistance to ampicillin was shown by 48 (92.3%) isolates. This result is similar to a recent report where the majority of goldfish-borne *Aeromonas* spp. showed resistance against amoxicillin and ampicillin (Hossain et al. 2018a). High resistance to first-generation cephalosporin (cephalothin) and a decreased susceptibility to third-generation cephalosporins (cefotaxime and ceftriaxone) were previously reported in fish-borne aeromonads (Rall et al. 1998; Imziln 2001). In our study, the resistance cephalothin has been observed in 27 (51.9%) isolates and *A. enteropelogenes* (100.0%), while 2 (5.9%) and 1 (2.9%) of *A. veronii* isolates showed cefotaxime and ceftriaxone, respectively. In addition, imipenem resistance was observed in 37 (71.2%) isolates including *A. dhakensis* (80.0%), *A. veronii* (79.4%) and *A. hydrophila* (66.7%) whereas all *A. caviae* and *A. enteropelogenes* isolates were susceptible to imipenem. Castro-Escarpulli et al. (2003) have also reported the occurrence of imipenem resistance among the *Aeromonas* spp.

In our study, quinolone, folate pathway inhibitor and tetracycline-resistant *Aeromonas* species were also observed with a high percentage. In the present study, every isolate showed resistance to nalidixic acid. Resistance to ciprofloxacin was identified in *A. caviae* (33.3%) and *A. veronii* (5.9%) isolates and ofloxacin resistance was observed in only *A. caviae* (33.3%) and *A. veronii* (2.9%). In the case of the tetracycline group, all isolates showed resistance against oxytetracycline, and 52.9% of the isolates were resistant to tetracycline. Also, all *A. enteropelogenes* showed susceptibility to trimethoprim/sulfamethoxazole while 50.0% of the aeromonads displayed resistance to this antibiotic. Indeed, quinolone, tetracycline and folate pathway inhibitor antibiotic groups had been extensively used in clinical medicine, veterinary and agriculture sectors for decades contributing to a higher level of bacterial resistance. Therefore, the resistance phenotype against these important antibiotic group among the guppy-borne *Aeromonas* spp. should not be underestimated.

The results for the aminoglycoside group (gentamicin, kanamycin and amikacin) were also observed. None of the isolates were resistance to amikacin. Besides, gentamicin was resistant to 11 (21.2%) isolates except for *A. enteropelogenes*. Kanamycin resistance was displayed in 7 (13.5%) isolates including *A. veronii* (2.9%), *A. dhakensis* (50.0%) and *A. hydrophila* (33.3%). Similarly, the high incidence of gentamicin and kanamycin resistance was observed in goldfish-borne and zebrafish-borne aeromonads (Hossain et al. 2018a, b). Finally, rifampicin resistance was exhibited by 40.0% of the isolates. However, only 3 *A. veronii* (8.8%) isolates were resistant to chloramphenicol. In the previous study, 86.2% and 4.6% of the isolates were resistant to rifampicin and chloramphenicol, respectively (Hossain et al. 2018a).

Our results revealed the high prevalence of *Aeromonas* spp. among the ornamental guppy. All isolates displayed a significant proportion of phenotypic virulence factors and harbored several virulence genes which reveal the virulence potential of guppy-borne *Aeromonas* spp. Majority of the isolates showed resistance against different structural classes of antibiotics which revealed the indiscriminate use of those antibiotics in the ornamental fish industry. The occurrence of virulent and drug-resistant guppy-borne *Aeromonas* spp. can cause a serious threat to the ornamental fish industry. Therefore, further studies should be focused on the characterization of virulence and antimicrobial resistance profiles of *Aeromonas* spp. isolated from other ornamental fish. Considering the rapid growth of the ornamental fish industry, the antibiotic usage for prophylactic purposes must be substituted by better husbandry especially well-maintenance of water quality and good transportation practice.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The American Veterinary Medical Association (AVMA) guidelines were followed to euthanize the fish.

References

Abd-El-Malek AM (2017) Incidence and virulence characteristics of *Aeromonas* spp. in fish. Vet World 10:34–37

Arslan S, Kucuksari R (2015) Phenotypic and genotypic virulence factors and antimicrobial resistance of motile *Aeromonas* spp. from fish and ground beef. J Food Saf 35:551–559

Burke V, Gracey M, Robinson J et al (1983) The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents. J Infect Dis 148:68–74

Carvalho-Castro GA, Lopes CO, Leal CA et al (2010) Detection of type III secretion system genes in *Aeromonas hydrophila* and their relationship with virulence in Nile tilapia. Vet Microbiol 144:371–376

Castro-Escarpulli G, Figueras MJ, Aguilera-Arreola G et al (2003) Characterisation of *Aeromonas* spp. isolated from frozen fsh intended for human consumption in Mexico. Int J Food Microbiol 84:41–49

CLSI (2014) Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals; Second informational supplement. CLSI document VET03/VET04-S2. Wayne, PA: Clinical and Laboratory Standards Institute

De Silva BCJ, Hossain S, Wimalasena SHMP et al (2018) Putative virulence traits and antibiogram profile of *Aeromonas* spp. isolated from frozen white-leg shrimp (*Litopenaeus vannamei*) marketed in Korea. J Food Saf 38:e12470

Figueras MJ, Alperi A, Beaz-Hidalgo R et al (2011) *Aeromonas rivuli* sp. nov., isolated from the upstream region of a karst water rivulet. Int J Syst Evol Microbiol 61:242–248

Freeman DJ, Falkiner FR, Keane CT (1989) New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol 42:872–884

Haiko J, Westerlund-Wikström B (2013) The role of the bacterial flagellum in adhesion and virulence. Biology 2:1242–1267

Harley JP, Prescott LM (2002) Laboratory exercises in microbiology. The McGraw-Hill Companies, New York

Hoel S, Vadstein O, Jakobsen AN (2019) The significance of mesophilic *Aeromonas* spp. in minimally processed ready-to-eat seafood. Microorganisms. 7:91

Hossain S, De Silva BCJ, Wimalasena SHMP et al (2018a) Distribution of antimicrobial resistance genes and class 1 integron gene cassette arrays in motile *Aeromonas* spp. isolated from goldfish (*Carassius auratus*). Microb Drug Res 24:1217–1225

Hossain S, De Silva BCJ, Dahanayake PS et al (2018b) Characterization of virulence properties and multidrug resistance profiles in motile *Aeromonas* spp. isolated from zebrafish (*Danio rerio*). Lett Appl Microbiol 67:598–605

Imziln B (2001) Occurrence and antibiotic resistance of mesophilic Aeromonas in three riverine freshwaters of Marrakech, Morocco. Sci World J 1:796–807

Janda JM, Abbott SL (2010) The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23:35–73

John N, Hatha AAM (2013) Distribution, extracellular virulence factors and drug resistance of motile aeromonads in freshwater ornamental fishes and associated carriage water. Int J Fish Aquac 3:92–100

Jun JW, Kim JH, Gomez DK et al (2010) Occurrence of tetracycline-resistant *Aeromonas hydrophila* infection in Korean cyprinid loach (*Misgurnus anguillicaudatus*). Afr J Microbiol Res 4:849–855

Kigigha LT, Oku IY, Ojesanmi AS (2012) Enumeration and characterization of bacteria associated with backwater fish species in Wilberforce island Bayelsa state Nigeria. Cont J Biol Sci 5:32–37

Krishnakumar K, Raghavan R, Prasad G (2009) When pets become pests—exotic aquarium fishes and biological invasions in Kerala, India. Curr Sci 97:474–476

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874

Lewbart GA (2001) Bacteria and ornamental fish. Semin Avian Exot Pet Med 10:48–56

Nagar V, Shashidhar R, Bandekar JR (2013) Characterization of *Aeromonas* strains isolated from Indian foods using *rpoD* gene sequencing and whole cell protein analysis. World J Microbiol Biotechnol 29:745–752

Navarro A, Martínez-Murcia A (2018) Phylogenetic analyses of the genus based on housekeeping gene sequencing and its influence on systematics. J Appl Microbiol 125:622–631

Nawaz M, Khan SA, Khan AA et al (2010) Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiol 27:327–331

Pemberton JM, Kidd SP, Schmidt R (1997) Secreted enzymes of *Aeromonas*. FEMS Microbiol Lett 152:1–10

Rall VLM, Iaria ST, Heidtmann S, Pimenta FC et al (1998) *Aeromonas* species isolated from Pintado fish (*Pseudoplatystoma* sp): virulence factors and drug susceptibility. Rev Microbiol 29

Rowe-Magnus DA, Guerout AM, Mazel D (2002) Bacterial resistance evolution by recruitment of super-integron gene cassettes. Mol Microbiol 43:1657–1669

Sen K, Rodgers M (2004) Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. J Appl Microbiol 97:1077–1086

Soler L, Yáñez MA, Chacón MR (2004) Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. Int J Syst Evol Microbiol 54:1511–1519

Sreedharan K, Philip R, Singh IS (2013) Characterization and virulence potential of phenotypically diverse *Aeromonas veronii* isolates recovered from moribund freshwater ornamental fishes of Kerala, India. Antonie Leeuwenhoek 103:53–67

Tayler AE, Ayala JA, Niumsup P et al (2010) Induction of beta-lactamase production in *Aeromonas hydrophila* is responsive to beta-lactam-mediated changes in peptidoglycan composition. Microbiology 156:2327–2335

Teunis P, Figueras MJ (2016) Reassessment of the enteropathogenicity of mesophilic *Aeromonas* species. Front Microbiol 7:1395

Tomas JM (2012) The main *Aeromonas* pathogenic factors. ISRN Microbiol 2012:256–261

Toranzo AE, Magariños B, Romalde JL (2005) A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246:37–61

Trust TJ, Whitby JL (1976) Antibiotic resistance of bacteria in water containing ornamental fishes. Antimicrob Agent Chemother 10:598–603

Wong CYF, Heuzenroeder MW, Flower RLP (1998) Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. Microbiology 144:291–298

Yáñez MA, Catalán V, Apráiz D et al (2003) Phylogenetic analysis of members of the genus *Aeromonas* based on *gyrB* gene sequences. Int J Syst Evol Microbiol 53:875–883

Yano Y, Hamano K, Tsutsui I et al (2015) Occurrence, molecular characterization, and antimicrobial susceptibility of *Aeromonas* spp. in marine species of shrimps cultured at inland low salinity ponds. Food Microb 47:21–27

Yu HB, Zhang YL, Lau YL et al (2005) Identification and characterization of putative virulence genes and gene clusters in *Aeromonas hydrophila* PPD134/91. Appl Environ Microbiol 71:4469–4477

Yu JH, Han JJ, Kim HJ, Kang SG et al (2010) First report of *Aeromonas veronii* infection in farmed Israeli carp *Cyprinus carpio* in Korea. J Fish Pathol 23:165–176

Yu J, Koo BH, Kim DH et al (2015) *Aeromonas sobria* infection in farmed mud loach (*Misgurnus mizolepis*) in Korea, a bacteriological survey. Iran J Vet Res 16:194–201