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Research article

Isolation, molecular identification, and pathogenicity of *Aeromonas hydrophila* disease in clown knife fish (*Chitala chitala*) in the Mekong Delta, Vietnam

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Abstract

Hemorrhagic septicemia disease caused by *Aeromonas hydrophila* bacteria appears to be more common and causes severe damage to many fish species worldwide. This research aimed to isolate, identify, and determine the pathogenicity and antibiotic susceptibility of A. *hydrophila* in septicemic clown knife (*Chitala chitala*) fish in the Mekong Delta, Vietnam. As a result, 116 bacterial strains were isolated from 176 septicemic fish samples. Two strains of bacteria, D2F71 and H1F39, were identified as A. *hydrophila* based on colony morphology, identification results by the API 20E kit, PCR technique, and aerolysin gene sequencing. In addition, these two strains of bacteria were experimentally infected on healthy clown knife fish (weight 15±4 g/fish) by injection method. The experimental results fulfilled Koch's postulates: infected fish showed signs of disease symptoms similar to those of naturally diseased fish. In particular, the two strains of bacteria D2F71 and H1F39 in the experiment had relatively high virulence, with LD50 values of 1.26×104 CFU/mL and 4.06×103 CFU/mL, respectively. The obtained findings demonstrated that the bacteria were susceptible to doxycycline, florfenicol, cefotaxime, flumequine, enrofloxacin, and sulfamethoxazole-trimethoprim at a rate of over 70%. On the contrary, the study found that bacterial isolates were resistant to ampicillin (100%), cefazolin (85%), streptomycin (80%), and colistin (80%). To our knowledge, this is the first report on hemorrhagic septicemic clown knife fish caused by A. *hydrophila* in Vietnam.

Keywords: *Aeromonas hydrophila*, Antibiotic susceptibility, Clown knife fish, LD_{50} value, Mekong Delta, Vietnam

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INTRODUCTION

Clown knife fish (*Chitala chitala*) is a valuable fish species in Vietnam. It is raised in many provinces in the Mekong Delta, such as Ben Tre, Dong Thap, A Giang, Long An, and Tien Giang, due to its beautiful shape and color, delicious meat of high quality, fast growth rate, and good adaptability to aquatic conditions (Gam et al., 2001). The region's most extensive farming area and productivity are found in the province of Hau Giang (So and Tuan, 2022). Clown knife fish production in Hau Giang province in 2017 was 50.8 ha and 2.775 thousand tons, according to information from the Hau Giang Fisheries Sub-Department (recited by So and Tuan, 2022). However, by 2020, the area and fish production increased to 86 ha and 6,880 tons, respectively. It has lately gained commercial significance as an aquaculture species, and its current annual production in the lower Mekong region alone is over 500 tons (Viet, 2015). In recent years, the farming of clown knifefish has not only been limited to raising ornamental fish but has also increasingly increased the demand for food. Because it is a new species of aquatic animal, the research mainly focuses on the nutritional needs of the fish development stages and artificial seed production to increase productivity and economic efficiency for farmers (Tien et al., 2012). However, the rapid development of aquaculture and the increasing density of intensive farming led to increased bacterial diseases (Noga, 2010). Many bacterial species causing fish diseases worldwide have been recorded, such as *Vibrio* spp. (Manchanayake et al., 2023), *Edwardsiella* spp. (Armwood et al., 2022), *Streptococcus* spp. (Van et al., 2022), *Aeromonas* spp. (Dubey et al., 2022), *Pseudomonas* spp. (Duman et al., 2021), *Mycobacterium* spp. (Delghandi et al., 2020), and *Flavobacterium* spp. (Lee et al., 2023).

A. *hydrophila* belongs to the genus *Aeromonas*, family Aeromonadaceae, order Aeromonadales, class Gamma proteobacteria, and phylum Proteobacteria (Janda and Abbott, 2010). To date, at least 26 species of this genus have been reported (Semwal et al., 2023), and most of them are pathogenic to many fish species worldwide, such as A. *hydrophila* (Samayanpaulraj et al., 2020), A. *veronii* (Zhu et al., 2022), A. *sobria* (Soliman, 2022), A. *caviae* (Xue et al., 2022), A. *salmonicida* (Gulla et al., 2019), and A. *dhakensis* (Soto-Rodriguez et al., 2018). In Vietnam, A. *hydrophila* has been reported as the causative agent of hemorrhagic disease in striped catfish (Crumlish et al., 2010), red tilapia (Nhinh et al., 2021), and most recently, snakehead fish (Duc et al., 2013). However, information about A. *hydrophila* bacteria causing disease in clown knife fish is still limited. Therefore, the study was carried out to isolate and identify the causative agent and assess the pathogenicity and antibiotic susceptibility of bacteria to serve as a basis for controlling and treating the disease in the future.

MATERIALS AND METHODS

Ethical approve

The study protocol was approved by the Regulations on Ethical Management in Animal Experiments (Decision No. 3965/QĐ-DHCT, October 15, 2021) of Can Tho University.

Collection of fish samples

Specimens of diseased clown knife fish were obtained from 79 intensive culture ponds, including Hau Giang (37 ponds), Dong Thap (29 ponds), Can Tho (11 ponds), and two ponds in Tien Giang provinces of the Mekong Delta, Vietnam (Figure 1) from January to September of the years 2022. Fish samples were collected when the fish showed unusual signs of disease. Each pond collects 6-10 moribund and 2-4 healthy fish. The diseased fish samples (liver, spleen, and kidney) were collected and checked for microbiological analysis.

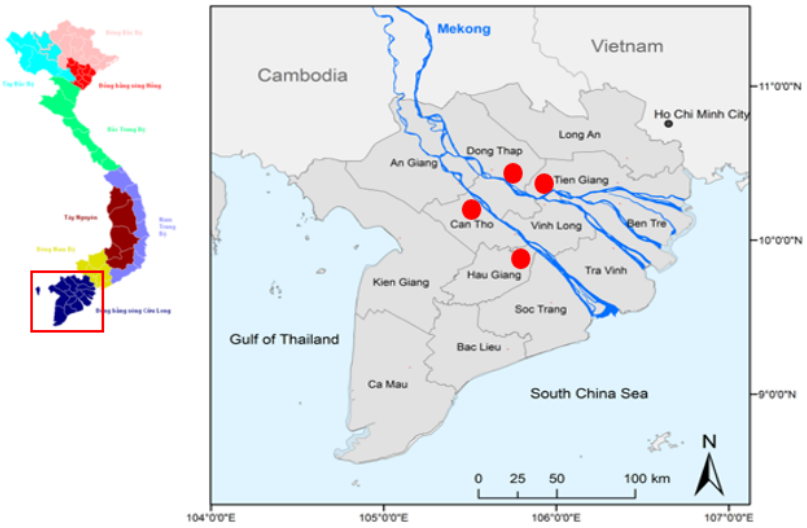

Figure 1 Location of diseased fish sample collection for *A. hydrophila* isolation (red circle)

Isolation of bacteria

The bacteria were recovered from the internal organs of diseased fish, such as the kidneys, liver, and spleen, according to the guidelines of Frerichs and Millar (1993). Briefly, the fish was wiped off with 70% alcohol and aseptically dissected with general anesthesia. Bacterial isolates were recovered from the kidney, liver, and spleen on tryptic soya agar (TSA, Merck, Germany) and glutamate starch phenol red agar (GSP, Merck, Germany) and incubated at 28°C for 24 hours. The presumptive colonies (yellow color) were subcultured many times for pure isolates. Finally, bacterial strains will be stored in brain heart infusion broth (BHIB, Merck, Germany) containing 20% glycerol (v/v) at -80°C.

Bacterial identification

The pure colonies were examined for essential morphological, physiological, and biochemical characteristics such as shape, color, colony size, cell shape, motility, Gram staining, oxidase, catalase, and oxidation-fermentation test. These characteristics were performed according to the manual of Cowan and Steel (Barrow and Feltham, 2004) and the guideline document of Buller (2014). In addition, other critical biochemical characteristics of the bacteria were tested using the API 20E commercial kit (Biomérieux, France)

Molecular identification

The bacterial DNA was extracted according to Sambrook et al. (1989) with some modifications. In brief, pure bacteria were cultured in a tryptic soya broth medium (TSB, Merck, Germany) and shaken at 120 rpm for 24 hours for DNA extraction. The obtained DNA was verified for purity, quantitated, and stored at -20°C until use and further analysis. The aerolysin gene of *A. hydrophila* was amplified using the primers AeroFd: 5'-CCAAGGGGTCTGTGGCGACA-3' and AeroRs: 5'-TTTCACCGGTAACAGGATTG-3' (Pollard et al., 1990). The composition of the PCR reaction used to identify *A. hydrophila* bacteria includes 1X PCR buffer, 2.0 mM $MgCl_2$, 20 mM dNTPs, 1U *Taq* DNA polymerase, 10 pmol of AeroFd primer, 10 pmol of AeroRs primers, DMSO (1%), and 40 ng of template DNA. The PCR cycle and conditions are as follows: initial denaturation at 95°C for 4 minutes, then 30 cycles of initial denaturation for 30 seconds at 95°C, annealing for 45 seconds at 60°C, extension for 30 seconds at 72°C, and final extension for 10 minutes at

72°C. PCR products (209 bp) were purified and sequenced at Macrogen Company, Korea (www.macrogen.com).

Experimental infection

Two strains of *A. hydrophila*, H1F39 and D2F71, from Hau Giang and Dong Thap provinces, were chosen for the challenge experiment. In brief, the bacteria were cultured in brain heart infusion broth medium (BHIB, Merck, Germany) on a shaker at 200 rpm for 24 hours. The culture was centrifuged at 4.000 rpm for 5 minutes and washed thrice with 0.85% NaCl physiological saline solution. Next, the bacteria were rinsed three times with 0.85% NaCl physiological saline, and the density was measured using a spectrophotometer at 610 nm (OD_{610} = 1±0.1 corresponds to the density of *A. hydrophila* bacteria of 10^8 CFU/mL) (Crumlish et al., 2010). Finally, the bacterial solution was diluted to 10^4, 10^5, and 10^6 CFU/mL densities for injection into fish.

The fingerlings weighing about 10±2 g/fish were domesticated for about 1-2 weeks, and before the experiment, the fish were checked for parasites and microbiology to select healthy and disease-free fish. The pathogenicity of bacterial strains *A. hydrophila* was determined by the experiment arranged in a completely randomized design with seven treatments, including 6 of them injected with two bacterial isolates (D2F71 isolate, and H1F39 isolate (each bacterial strain injected with three doses: 10^4, 10^5, and 10^6 CFU/fish) and the control injected with physiological saline (0.85% NaCl). Each treatment was repeated three times at a density of 10 fish per tank, and experimental fish were intraperitoneally injected with 0.1 mL of bacterial solution per fish. Thus, the total number of fish used for the experiment was 210. The lethal dose of 50% (LD_{50}) was determined according to the formula of Reed and Muench (1938). The lethargic or moribunds (including the control fish) were dissected to re-isolate and re-identify *A. hydrophila* from the moribunds.

Antibiotic sensitivity

Antibiotic sensitivity was evaluated using the Kirby-Bauer disc diffusion method (Bauer et al., 1966). Ten antibiotics (Oxoid, UK) were used to conduct the antibiogram: ampicillin (AMP/10µg), cefotaxime (CTX/10µg), cefazolin (KZ/10µg), cephalexin (CL/10µg), doxycycline (DO/30µg), enrofloxacin (ENR/5µg), flumequine (UB/30µg), florfenicol (FFC/30µg), streptomycin (S/10µg), colistin (CT/50µg), and sulfamethoxazole-trimethoprim (SXT/1,25/23,75µg).

A 0.85% saline solution was used to suspend bacterial colonies, and the turbidity was by the McFarland standard (bioMerieux, France). The Muller-Hinton agar (MHA, Merck, Germany) surface was then covered with the bacterial solution. The antibiotic discs were then positioned on the agar. The inhibition zone diameter was measured after 24-48 hours of incubation at 28°C. The determination of inhibitory zone diameters as resistant (R), intermediate (I), and susceptible (S) was based on a document from the CLSI (2020). As a quality control, *Escherichia coli* ATCC 25922 was utilized as the reference strain.

Data analysis

The rate of antibiotic resistance and cumulative mortality were calculated using descriptive statistics. Using the BLASTn (nucleotide-basic Local Alignment Search Tool) tool, the sequences of different bacterial strains were compared to sequences in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). The CLUSTALW program was then used to multiply the sequences (Thompson et al., 1997). The phylogenetic tree was created using the neighbor-joining approach with a bootstrap value of 1,000 replicates and MEGA6 (Molecular Evolutionary Genetics Analysis software) (Tamura et al., 2013)

RESULTS

Bacterial characterization

The study isolated 116 bacterial strains from 176 fish samples showing hemorrhagic lesions (Figure 2), of which 68/116 (58.62%) isolates originated from Hau Giang, 42/116 (35.34%) isolates from Dong Thap, and 6/116 (5.17% each) isolates from Can Tho and Tien Giang provinces (Table 1). In general, bacterial strains were derived from the liver (68/116 isolates, 58.62%), kidney (32/116 isolates, 27.59%), and spleen (26/116 isolates, 13.79%) of diseased fish on the TSA medium. Morphological examination results showed that the bacteria formed cream-colored, round colonies on TSA medium after 24 hours of incubation at 28°C (Figure 2C). On GSP medium, isolated bacterial strains with yellow colonies (Figure 2D). They are Gram-negative, have short rod (Figure 2E), have positive catalase and oxidase reactions, are resistant to O/129, capable of fermenting in both aerobic and anaerobic conditions, and can grow in media with 0-3% NaCl concentrations (Table 2).

The obtained results from the API 20E kit showed that two strains of D2F71 and H1F39 showed adverse reactions with the following criteria: ornithine, citrate, H_2S, urease, tryptophane deaminase, indole, inositol, sorbitol, rhamnose, melibiose, and arabinose; positive reactions for ortho-nitrophenyl galactosidase, arginine, lysine, Voges-Proskauer, gelatin, glucose, mannitol, saccharose, and amygdalin. Morphological, physiological, and biochemical characteristics of isolated *A. hydrophila* bacteria are presented in detail in Table 2.

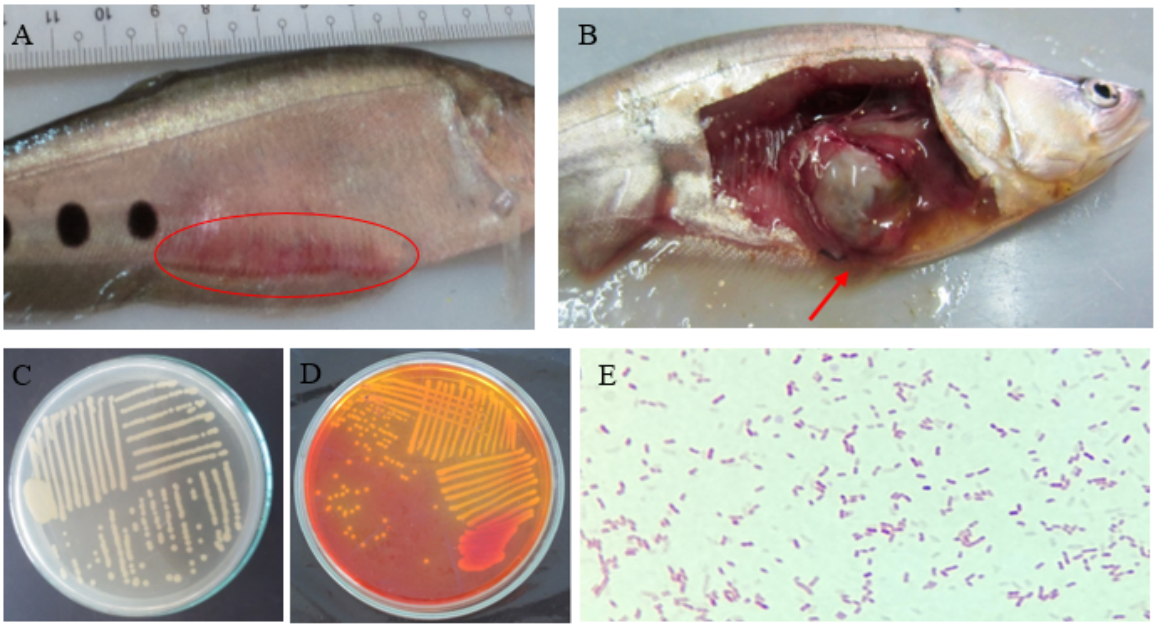

Figure 2 Signs of diseased fish and characteristics of isolated *A. hydrophila* bacteria. A. Lesion on fish (red circle); B. The congested internal organs and abdominal fluid (yellowish fluid, red arrow); C. Bacterial colonies isolated from diseased fish on TSA medium; D. Bacterial colonies isolated from diseased fish on GSP medium; E. Gram staining results (100X)

Table 1 Bacterial isolation results on clown knife fish

Sampling sites	Hemorrhagic septicemic disease fish samples	Number of bacterial isolates
Hau Giang	85	68
Dong Thap	63	42
Tien Giang	23	3
Can Tho	5	3
Total	176	116

Table 2 Morphological, physiological, and biochemical characteristics of isolated *A. hydrophila* isolates

Bacterial characteristics	Isolate D2F71	Isolate H1F39	*A. hydrophila**
Colony size	3-5 mm	3-5 mm	3-5 mm
Colony color	Yellow	Yellow	Yellow
Gram staining	Negative	Negative	Negative
Bacterial morphology	Rod-short	Rod-short	Rod-short
Motility	+	+	+
Oxidase test	+	+	+
Catalase test	+	+	+
Oxidation/Fermentation test	+/+	+/+	+/+
Growth on NaCl**			
1%	+++	+++	+++
2%	+++	+++	+++
3%	+++	+++	+++
β-galactosidase	+	+	+
Arginine dihydrolase	+	+	+
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	-	-	-
Citrate	+	-	-
Production of hydrogen sulphide	-	-	-
Urease	-	-	-
Tryptophane deaminase	-	-	-
Indole	+	+	+
Voges-Proskauer	+	+	+
Gelatin	+	+	+
Glucose	+	+	+
Mannitol	+	+	+
Inositol	-	-	-

Note: ONPG: ortho-nitrophenyl galactosidase +: positive -: negative; * Reference strain of *A. hydrophila*; ** The ability of bacteria to grow on media with different salt temperatures: +++: excellent growth, ++: good growth, +: weak and -: no growth.

Bacterial identification results by PCR

All tested bacterial strains could amplify the *A. hydrophila*-specific aerolysin gene with a DNA band appearing at 209 bp (Figure 3).

The BLASTn results demonstrated that representative isolate H1F39 showed a 100% similarity to *A. hydrophila* strain SWNV-3 (ON479619.1), a 99.32% homology to *A. hydrophila* strain CEMTC_8139 (OQ834572.1), a 97.48% homology to *A. hydrophila* strain Til013 (OQ625314.1), and *A. hydrophila* strain AHT23-2 (OQ613272.1) in the NCBI database. On the other hand, isolate D2F71 had a 100% similarity to *A. hydrophila* strain ZC1 (FJ608555.1), a 97.48% similarity to *A. hydrophila* strain wp3 (FJ608554.1), *A. hydrophila* strain NNG1 (JX512398.1), and *A. hydrophila* strain YBH090730L (GU169708.1) in the GenBank. Two isolates were placed into two separate clusters, according to the phylogenetic tree (Figure 4), in which isolate H1F39 and isolate D2F71 are genetically related to *A. hydrophila* strain SWNV-3 (ON479619.1), and *A. hydrophila* strain ZC1 (FJ608555.1), respectively.

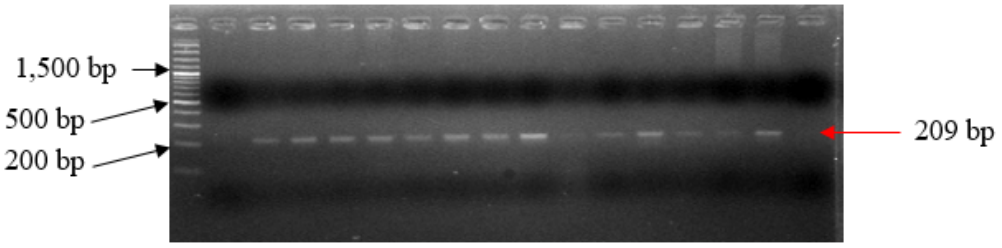

Figure 3 DNA electrophoresis of the isolated strain of *A. hydrophila* by PCR. Lane M. 100 bp plus DNA ladder; Lane 1. Negative control; Lane 2-15: isolates H1F1, H1F3, H1F5, H1F11, H1F39, H1F19, H1F39, D2F7, D2F8, D2F710, D2F712, D2F14, D2F41, and D2F71, respectively. Lane 16: negative sample

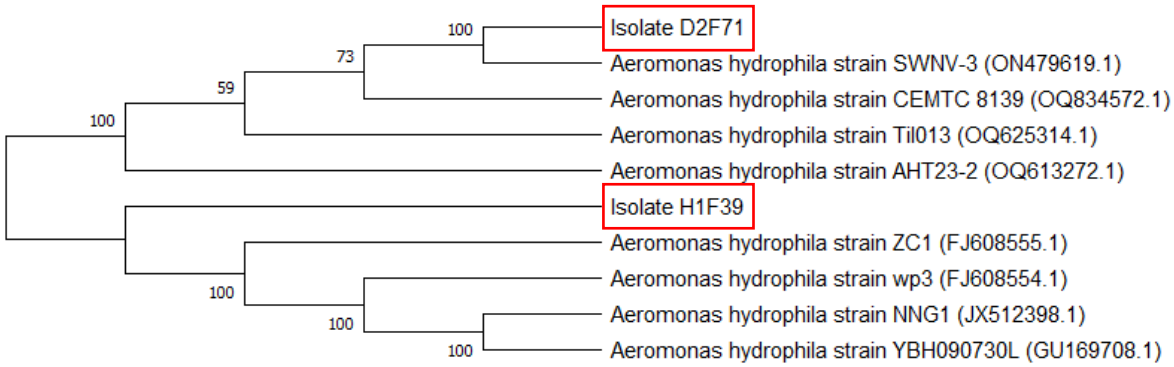

Figure 4 Phylogenetic tree illustrating the genetic relationship between isolates of *A. hydrophila* (the branching point numbers are bootstrap values).

Experimental infection

The results showed that the infected groups had different mortality rates (Figure 5), except the controls had no dead fish. For fish infected with the *A. hydrophila* strain, fish started to die after 12 hours in all groups. After 36 hours of infection, in the group of 10^6 CFU/mL, the fish died with the highest rates of 95% for isolate D2F71 and 80% for isolate H1F39, respectively. Besides, at 105 CFU/mL

group, the fish died at a relatively high rate of 90% in D2F71 and 70% in H1F39. Interestingly, for the bacterial injection group at a dose of 10^4 CFU/mL, the fish died at the lowest rate of 70% and 55% in isolates D2F71 and H1F39, respectively. The cumulative mortality of the groups (10^4, 10^5, and 10^6 CFU/mL) was not significantly different ($p > 0.05$). The obtained LD_{50} values of two strains of D2F71 and H1F39 in the experiment were 1.26×10^4 CFU/mL and 4.06×10^3 CFU/mL, respectively. After the challenge, two isolates, D2F71 and H1F39, were also re-isolated and re-identified from moribund fish. The morphological, physiological, and biochemical characteristics of two pathogenic bacterial strains recovered from the challenge fish had similar features to those obtained in the naturally diseased fish (Figure 5). Hence, the experimental results fulfilled Koch's postulates, and two strains of *A. hydrophila*, D2F71, and H1F39, are the causative agents of hemorrhagic septicemia disease in clown knife fish.

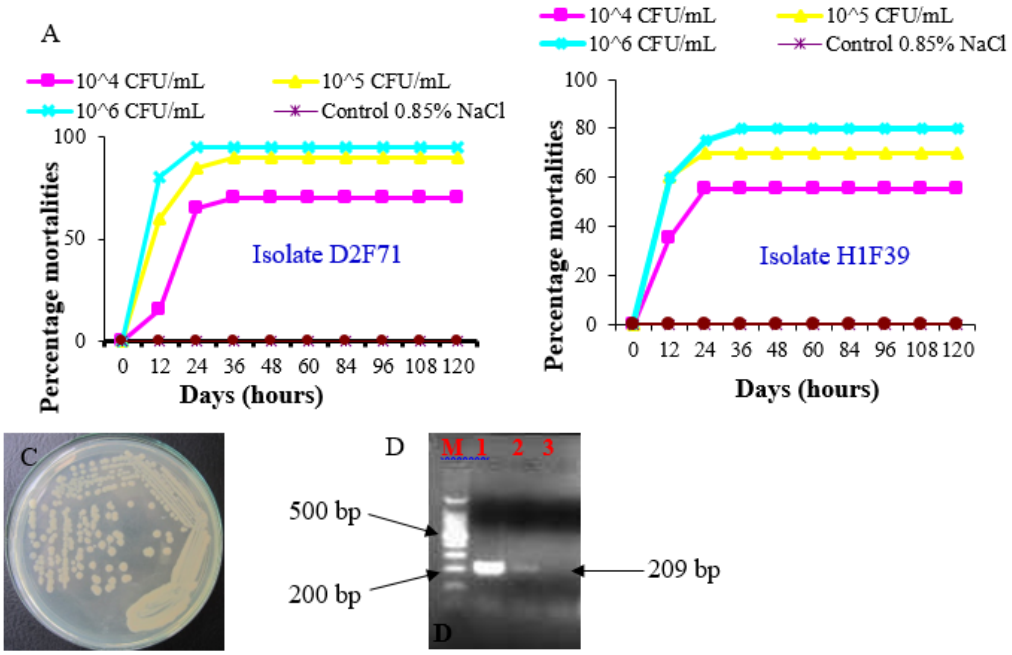

Figure 5 A&B. Cumulative mortality of clown knife fish infected with *A. hydrophila*; C&D. Results of re-isolation and identification of two D2F71 and H1F39 isolates after infection (Lane M. 100 bp DNA ladder; Lane 1-2: isolate D2F71 and H1F39, respectively; Lane 3: negative control).

Antibiotic sensitivity of isolates

The obtained results showed that *A. hydrophila* bacteria were sensitive to florfenicol (FFC) 100%, doxycycline (DO) 100%, cefotaxime (CTX) 100%, flumequine (UB) 100%, sulfamethoxazole-trimethoprim (SXT) 95%, and enrofloxacin (ENR) 75%. On the contrary, the present study found bacteria resistant to ampicillin (AMP) 100%, cefazolin (KZ) 85%, streptomycin (S) 80%, and colistin (CT) 80% (Figure 6).

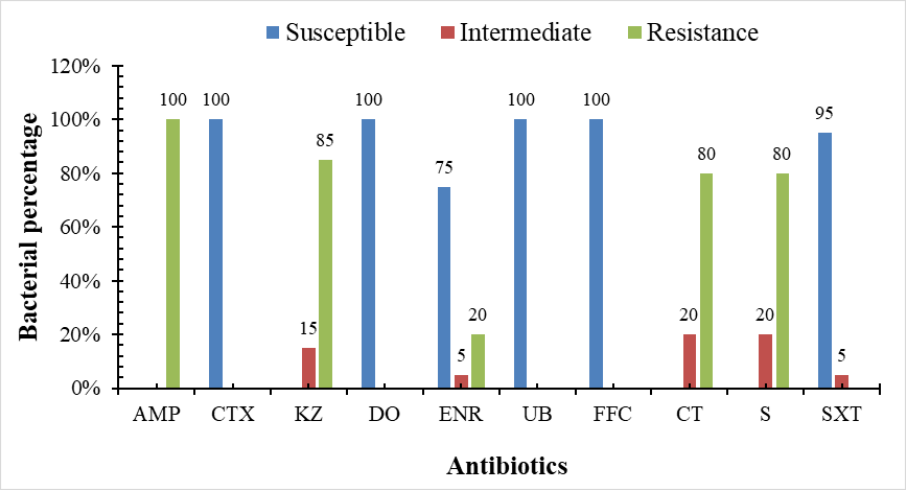

Figure 6 Antibiotic sensitivity results of *A. hydrophila* isolates
AMP: ampicillin, CTX: cefotaxime, KZ: cefazolin, CL: cephalexin, DO: doxycycline, ENR: enrofloxacin, UB: flumequine, FFC: florfenicol, S: streptomycin, CT: colistin, and SXT: sulfamethoxazole-trimethoprim.

DISCUSSION

A. hydrophila bacteria are considered to be the causative agent of hemorrhagic septicemia in many different fish species around the world (Newman, 1993). Outbreaks of disease caused by *A. hydrophila* in clown knife fish may be due to environmental changes causing shock to fish and unstable water environments, with high organic materials and pathogens from other nearby ponds. In this study, 116 bacterial strains were isolated from the diseased clown knife fish. According to Ali et al. (2016) and Pauzi et al. (2020), the morphological and biochemical traits in this study are generally compatible with those described by those researchers. Additionally, the biochemical characteristics of the two isolates, D2F71 and H1F39, were mostly in line with those of the *A. hydrophila* reference strain (Buller, 2014), with positive and negative reactions detailed in Table 2. Although it is valuable to determine the phenotypic characteristics of bacteria, doing so becomes unrealistic when solely using traditional phenotypic characteristics (Zhang et al., 2011). Thus, molecular identification using the aerolysin and 16S rRNA genes was used. One of the most influential and widely used techniques for identifying bacteria is 16S rRNA sequencing (Busse et al., 1996). However, due to the relatively low discrimination of *Aeromonas* spp. (Zhang et al., 2011), the 16S rRNA gene sequences are not the best for identifying species. According to BLAST alignments, the aerolysin gene sequences of the two isolates, D2F71 and H1F39, shared the most similarity with those of the known *A. hydrophila* strains. The two isolates, D2F71 and H1F39, were found to be associated with the *A. hydrophila* strains in the phylogenetic trees constructed using the sequences of the aerolysin gene, as predicted.

A. hydrophila is a significant bacterium that affects various aquatic animals and can manifest itself in various ways in cultivated species. Hemorrhagic septicemia, fin rot, abdominal destination, and skin ulcers are the symptoms of *A. hydrophila* infection in farmed and wild fish, according to Jacobs and Chenia (2007), Abolghait et al. (2010) and Dias et al. (2016). In the current study, the *A. hydrophila* bacteria causing hemorrhagic septicemic disease in clown knife fish exhibited pathological signs such as distention and hemorrhagic abdomen. The internal cavity contains yellowish fluid, and all internal organs are congested. This result is similar to that of *A. hydrophilic* hemorrhagic disease in fish species previously reported. Specifically, Pauzi et al. (2020) demonstrated that *A.*

hydrophila-infected red hybrid tilapia (*O. niloticus*×*Oreochromis mossambicus*) in Malaysia exhibited occasional erratic swimming patterns, localized hemorrhages, and depigmentation on the body and operculum areas, the fin destruction, enlargement of the gall bladder, and hemorrhage in internal organs. According to research by Nhinh et al. (2021), *A. hydrophila*-infected freshwater fish displayed severe symptoms such as abnormal swimming pale or darker skin with or without ulcer formation, hemorrhage around their mouth, operculum, fin bases, and fin destruction, as well as gross lesions such as an enlarged gall bladder and liver, an enlarged and darkened spleen, hemorrhage, and an empty stomach and intestines. Similar to this, a study by Sherif and Kassab (2023) found that experimental *O. niloticus* infected with *A. hydrophila* had clinical symptoms such as an opaque, slightly protruding eye, dented dorsal and tail fins, and surface hemorrhages. The post-mortem findings included an empty intestinal tract, an empty gall bladder, a dark brown liver, and splenomegaly.

Two strains of the present findings, D2F71 and H1F39, which had LD_{50} values of 1.26×10^4 CFU/mL and 4.06×10^3 CFU/mL, respectively, showed very high virulence, according to the infectious data. The findings are in line with the research of Pauzi et al. (2020), which showed that the LD_{50} value of *A. hydrophila* collected from red hybrid tilapia (*O. niloticus*×*O. mossambicus*) in Malaysia was determined at 1.1×10^4 CFU/mL. In the present study, however, the obtained LD_{50} value is lower than previously reported. The study by Pridgeon and Klesius (2009) demonstrated that three-quarters of *A. hydrophila* strains infecting American catfish had relatively high virulence, in which the virulence of strain AL09-71 was highest with an LD50 value of 1.1×10^5 CFU/mL, followed by strains AL09-73 (1.3×10^5 CFU/mL) and AL09-72 (1.9×10^5 CFU/mL). Sherif and Kassab (2011) showed that the median lethal doses of *A. hydrophila* recovered from Nile tilapia broodstock isolates in Egypt ranged from 2.62×10^4 to 3.02×10^6 CFU/mL. This result may be because bacteria from the genus *Aeromonas*, in general, and *A. hydrophila*, in particular, have various virulence genes. Numerous studies have discovered virulence genes in *Aeromonas*, including the *aerA*, *hlyA*, *ahpA*, *alt*, and *ast* genes, which code for toxic proteins such as aerolysin, hemolysin, a serine protease, and enterotoxins (Jin et al., 2020; Dubey et al., 2022), and *Aeromonas* strains that contain these genes are highly virulent (Zheng et al., 2012; Oliveira et al., 2012). In addition, the virulence of *Aeromonas* bacteria is related to virulence plasmids (Tanaka et al., 2017; Preena et al., 2021).

The antimicrobials test of the study revealed that *A. hydrophila* bacteria were 100% sensitive to florfenicol. This result is similar to that of Bannai et al. (2020), who demonstrated that 100% of *A. hydrophila*-infected *Cyprinus carpio* in Basrah governorate, Iraq, was sensitive to florfenicol. However, the percentage of *A. hydrophila* bacteria sensitive to florfenicol was higher than the study of Sherif and Kassab (2023), which reported that approximately 83.3% of *A. hydrophila* strains originating from Nile tilapia broodstock were sensitive to florfenicol. The high sensitivity of *A. hydrophila* to florfenicol may be because this antibiotic is rarely used in pond fish cultures. However, the future use of this antibiotic needs to be carefully managed, as many studies showing high bacterial resistance to florfenicol have been reported (Miranda and Rojas, 2007; Smith, 2008).

Antibiotics of the tetracycline class have been widely used in aquaculture in Vietnam (Phu et al., 2016). In the current findings, *A. hydrophila* was completely sensitive to doxycycline (100%). This result is in agreement with the study of Samal et al. (2014), who revealed that *A. hydrophila* isolates isolated from diseased fish, such as rohu, catla, mrigal, catfish, goldfish, and *Channa* spp., were 100% sensitive to oxytetracycline, and doxycycline. Many studies have shown that tetracycline resistance genes are located on R-plasmids and that they can transmit resistance genes to each other (Ramírez-Bayard et al., 2023). Therefore, it is necessary to detect R-plasmids in *A. hydrophila* isolates in this research to clarify this issue for strictly controlling the use of tetracycline antibiotics in aquaculture.

Sulfamethoxazole-trimethoprim is a broad-spectrum antibiotic inhibiting bacteria's folic acid synthesis (Masters et al., 2003). This investigation revealed that 95% of strains of *A. hydrophila* were sensitive to the antibiotic sulfamethoxazole-trimethoprim. Similarly, Revina et al. (2017) demonstrated that *A. hydrophila* bacteria originated from sea trout (*Salmo trutta*) in Latvia and were 100% sensitive to sulfamethoxazole-trimethoprim. However, according to research by Nguyen et al. (2014), up to 72% of *Aeromonas* spp. It was derived from the water of catfish culture ponds in Vietnam, which are resistant to this antibiotic. Recently, the research of Thi et al. (2023) also showed that 100% of *A. hydrophila* collected from striped catfish (*P. hypothalamus*) in the Mekong Delta, Vietnam, is resistant to sulfamethoxazole-trimethoprim.

As for the quinolone antibiotics, the study showed that *A. hydrophila* is still highly sensitive to the antibiotics of this class. Specifically, the percentage of bacteria susceptible to flumequine is 100%. The findings are in agreement with the work of Guz and Kozinska (2004), which revealed that all *Aeromonas* isolates recovered from diseased cultivated carp (*Cyprinus carpio* L.) in Poland were sensitive to flumequine. Another study by Syrova et al. (2018) showed that *Aeromonas* spp. from Czech carp are resistant to flumequine (14%). This finding is contrary to Chau et al. (2018), who indicated that *A. schubertii* causing white spot disease on snakehead fish (*Channa striata*) in intensive ponds in Tra Vinh province of the Mekong Delta, Vietnam, was 54,17% resistant to flumequine. According to Lee and Wendy (2017), *A. hydrophila* and *E. tarda* isolates derived from red hybrid tilapia (*Oreochromis* spp.) were highly sensitive to flumequine (73.3%). Meanwhile, bacteria have decreased susceptibility to enrofloxacin (75%), according to the results obtained from this study. Mulia et al. (2021) presented that all of the *Aeromonas* spp. from diseased walking catfish (*Clarias* sp.) in Indonesia were sensitive to enrofloxacin. However, the findings were obtained from the study of Didugu et al. (2016), who reported that *Aeromonas* spp. Isolates obtained from livestock products are found to be resistant to enrofloxacin. In a study, Guo et al. (2014) depicted that only 2.23% of the *A. hydrophila* isolates from clinical cases were resistant to enrofloxacin.

Several world regions have reported colistin resistance in *Aeromonas* and other bacterial species (Pungpian et al., 2021; Thaotumpitak et al., 2023). However, the present study found that bacterial isolates were still highly resistant to colistin (80%). This result is in line with Kaskhedikar and Chhabra (2010), who reported that all *A. hydrophila* isolates isolated from chicken samples in India were resistant to colistin. Similarly, a study by Qu et al. (2022) demonstrated that three *Aeromonas* species, consisting of *A. caviae*, *A. dhakensis*, and *A. hydrophila*, collected from food samples in China, were 100% resistant to colistin. On the other hand, the research of Pham et al. (2023) revealed that *A. schubertii* from diseased snakehead fish (*C. striata*) in the Mekong Delta, Vietnam, has resistance to colistin at a rate of 28%, much lower than this current study.

For β-lactam antibiotics, this investigation illustrated that 100% of *A. hydrophila* bacteria are sensitive to cefotaxime. This finding is similar to the research of Eid et al. (2022), who presented that the *A. hydrophila* complex (*A. hydrophila*, *A. sobria, A. caviae*, and *A. schubertii*) recovered from wild *Mugil cephalus* (striped mullet) and water samples in Egypt were utterly susceptible to cefotaxime. On the contrary, the present study found bacteria resistant to ampicillin and cefazolin at 100% and 85%, respectively. All *Aeromonas* strains recovered from asymptomatic *C. macropomum* cultured in Brazil were resistant to ampicillin (Sebastião, 2023). Similarly, *A. hydrophila* isolates from fresh, brackish, and marine fish were resistant to ampicillin, according to research by Dahdouh et al. (2016). Yang et al. (2018) found that 95.24% of *A. hydrophila* isolates from grass carp in south China were resistant to ampicillin. However, the research of Yang et al. (2018) also showed that *A. hydrophila* isolates resist cefazolin (6.35%), which is much lower than in our present study. *A. hydrophila* bacteria have a high resistance rate to β-lactam antibiotics because their cells secrete the enzyme β-lactamase, which

breaks the β-lactam ring, making the drug ineffective (Saavedra et al., 2004; Jalal et al., 2010). However, bacteria in this group are still susceptible to the antibiotic cefotaxime (100%). This result may be because cefotaxime is a 3rd generation cephalosporin antibiotic studied against the enzymatic degradation of β-lactamases by bacteria (Treves-Brown, 2000).

The present study found that bacteria were highly resistant to streptomycin (80%). The rate of streptomycin-resistant bacteria in this finding was lower than that reported by Hafez et al. (2018), who reported that 100% of *A. hydrophila* in frozen fish (mackerel, herrings, and fish fillets) in Egypt was recorded against streptomycin. According to Odeyemi and Ahmad (2017), *Aeromonas* species originated from different aquatic sources in Malaysia and were found to be 47.2% resistant to streptomycin. Similarly, Zdanowicz et al. (2020) revealed that all *Aeromonas* isolates from the water of the carp ponds in Poland were susceptible to streptomycin. The findings are comparable with the result of Zdanowicz et al. (2020), who emphasized that all *Aeromonas* strains from the water of three carp ponds in Ba Lan were susceptible to streptomycin. Dang et al. (2020) reported that 25% of *A. hydrophila* collected from tilapia and traditional freshwater fish in Northern Vietnam resisted streptomycin. On the contrary, none of the *A. hydrophila* isolates with resistance to streptomycin were found in the study of Sherif and Kassab (2023)., the results of the study show that florfenicol, doxycycline, cefotaxime, flumequine, enrofloxacin, and sulfamethoxazole-trimethoprim can be used to control *A. hydrophila*. However, strict management are needed to avoid drug-resistant bacteria. In the future, it is possible to use other methods to effectively prevent this bacterium in clown knife fish aquaculture in the Mekong Delta, such as herbs, probiotics, and immune stimulants, especially vaccine development.

CONCLUSIONS

The study demonstrated that two D2F71 and H1F39 isolates identified, *A. hydrophila*, were the etiological agents of hemorrhagic septicemia disease in clown knife fish. The experimental infection results showed signs of disease symptoms similar to those of natural hemorrhagic septicemia fish. Interestingly, the two strains of bacteria D2F71 and H1F39 in the experiment had relatively high virulence, with LD_{50} values of 1.26×10^4 and 4.06×10^3 CFU/mL, respectively. The obtained antibiogram results illustrated that over 70% of the bacteria were sensitive to florfenicol, doxycycline, cefotaxime, flumequine, enrofloxacin, and sulfamethoxazole-trimethoprim. Meanwhile, the study found bacteria highly resistant to ampicillin, cefazolin, streptomycin, and colistin. The results of this study show that farmers in the Mekong Delta should considerably use this antibiotic to treat hemorrhagic septicemia disease in clown knife fish.

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