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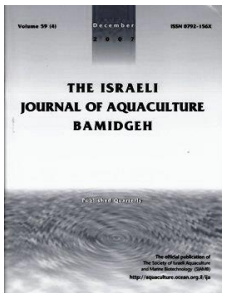

Isolation, Identification, and Detection, of the Virulence Factor of *Vibrio cholerae* in Grass Carp

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Key words: *Grass carp*; *Vibrio cholera*; isolation; identification; detection of virulence factor

Abstract

In this study, we examined the mass mortality of grass carp (*Ctenopharyngodon idellus*) caused by *Vibrio cholerae* in Jiangsu Province. We evaluated the phenotypic, morphological, physiological, biochemical, and virulence factor characteristics as well as the drug sensitivity of the CY-1 strain isolated from the liver, spleen, and kidney, of dying grass carp. The 16S rRNA gene (GenBank accession number: KF386612) was amplified and compared with sequences deposited in GenBank. A phylogenetic tree analysis showed that the isolated pathogen was *V. cholerae* and that it was most closely related to *V. cholerae* (GenBank accession number: JN555611). Artificially infected grass carp that died exhibited the same disease symptoms as those of naturally infected fish. An amplified band in the corresponding 564-bp position was detected as the virulence factor, and it was the same as that of the *V. cholerae* virulence factor ctxA. Drug sensitivity tests showed that *V. cholerae* was resistant to penicillin, tobramycin, cotrimoxazole, and amoxicillin, and was highly sensitive to cefuroxime, amikacin, tetracycline, and nitrofurantoin. These could possibly be used in the treatment of this disease. The findings of this study can be used as a reference for disease control and health management in the cultivation of *C. Idellus*.

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Introduction

Grass carp (*Ctenopharyngodon idellus*) are herbivorous freshwater fish of the family *Cyprinidae* and the only species in the genus *Ctenopharyngodon*. Grass carp are an important aquaculture species and are popular as a 'large freshwater fish'. They are widely cultivated and appeal to people of all ages because of their delicious meat. The low-cost of production makes this a popular fish for aquaculture in China. However frequent occurrence of various carp diseases has seriously affected intensive farming. Many studies have described bacterial diseases of grass carp, such as a histological study on bacterial gill rot disease (Huang et al., 1983), detection of bacterial septicemic pathogens (Fan et al., 2009), and a phylogenetic study of *Aeromonas hydrophila* isolated from grass carp enteritis (Zhang et al., 2006).

An outbreak of 'bacterial sepsis' which lasted a month, occurred in August 2014 at a grass carp farm in Dafeng City, Jiangsu Province, during which approximately 20,000 fish were infected and died. We isolated the CY-1 strain of *V. cholerae* from naturally infected grass carp and using regression tests, confirmed that the isolated strain had strong pathogenicity, and the virulence factor was detected. In this paper we report the incidence of the disease in grass carp, clinical manifestations of diseased fish, phenotypic characteristics of the isolated bacteria, the main biological characteristics, the main virulence factors, drug resistance, and the 16S rRNA gene sequence, to provide a reference for further research on effective inspection and prevention of *V. cholerae* disease infections in grass carp.

Materials and Methods

Grass carp source, and disease symptoms. Grass carp were obtained from a farm in Dafeng City, Jiangsu Province, in August 2014. The fins and gill of moribund fish showed slight bleeding, and postmortem observations revealed that the intestine contained no food and was red from hyperemia; there were many yellowish effusions in the abdomen. Pathogenic tissue samples (liver, spleen) were taken from 10 dying grass carp (weight, 150 $\pm$ 10 g) to determine and record pathological changes and symptoms. 48 fish confirmed healthy by PCR were sampled and challenged with pure isolated bacteria to test bacterial pathogenicity.

Isolation and purification. Parasite and fungal infections were excluded by microscopic observations. Grass carp with typical symptoms such as gill cover and fin hemorrhaging were selected to obtain the mucus from the ulcers or red areas with a vaccination bar used to isolate the bacterial markings on TSA agar plates. At the same time, the liver, spleen, and kidney tissue samples were obtained, streaked on TSA agar plates, and incubated at 28°C to observe the colony characteristics. Five colonies were selected and were used for pathogenicity testing. Then single colonies were selected for further purification and crossed until the CY-1 strain was obtained as a pure culture.

Pathogen morphology. The isolated strain was Gram stained, and its cell morphology observed under an optical microscope.

Artificial infection experiment. A pure isolated CY-1 culture strain was used to test bacterial pathogenicity. The bacteria were inoculated aseptically in nutrient broth at concentrations of 1×10^8, 10^7, 10^6, 10^5 and 10^4 CFU/mL and incubated for 24 h at 160 rpm on a shaker. They were then injected intraperitoneally (150 µL) into the smaller, healthy grass carp divided into five groups of eight fish each. The control group included eight fish inoculated with the same volume of sterile nutrient broth. After inoculation, the fish were fed ordinary commercial feed in an isolation tank, and the water was changed once daily, for a period of three weeks. The incidence of symptoms and mortality was recorded. At the onset of disease symptoms, bacteria were isolated under sterile conditions. The isolated bacteria were then compared with the original colonies of bacteria used for the inoculation.

Biochemical identification. Purified bacteria were transferred to a corresponding biochemical reaction tube for identification. After 24 h of incubation at 28°C, the bacteria were identified by gross morphology, colony morphology, and cultural characteristics while referring to Bergey's Manual of Systematic Bacteriology, second edition (Garrity et al., 2004; Austin B and Austin DA, 1999), the Common Bacterial System Identification Manual (Dong and Cai, 2001) to identify the isolates.

Serological identification. The strain CY-1 was used to identify serotype by *Vibrio cholera* O antisera (Tianjin Biochip Corporation, Tianjin, China). The kit includes *Vibrio cholera* Ogwan serogroup O1, *Vibrio cholera* Inaba serogroup O1, *Vibrio cholera* O1 serogroup, and *Vibrio cholera* O139 serogroup.

Drug sensitivity test. A 100-μL aliquot of the bacterial broth from liquid culture was pipetted and added evenly to a flat plate. A sterile cotton swab was dipped in the bacterial suspension and swabbed evenly on TSA agar plates and antimicrobial discs (Hangzhou Tianhe Microbial Co. Ltd., Hangzhou, China) were placed over them with sterile tweezers. The plates were then incubated at 28°C for 18 h. The diameter (mm) of the inhibition zones was recorded, and the results were interpreted according to the antibacterial diameter ranges recommended by the American Clinical and Laboratory Standards Institute (NCCLS).

16S rRNA gene sequence analysis. Preparation of PCR template DNA: Pure bacterial cultures were inoculated in nutrient broth and incubated at 28°C for 16 h. DNA was extracted and used as a PCR template using the UNIQ-10 column bacterial genome extraction kit (Shenggong Co., Ltd., Shanghai, China), according to the manufacturer's instructions.

The following two 16S rRNA gene PCR amplification primers were used:
27F (forward), 5′-AGA GTT TGA TC (C/A) TGG CTC AG-3′; 1492R (reverse), 5′-GGT TAC CTT GTT ACG ACT T-3′ (Polz and Cavanaugh, 1998). The PCR (20 μL) reaction system included 2 μL of 1× PCR buffer, 0.5 μL of 2-mM dNTPs, 0.5 μL of Taq DNA polymerase, 0.5 μL of the upper and lower primers each, 2 μL of template, 2 μL of Mg^{2+} solution and 12 mL of sterile distilled water. The PCR reaction conditions were as follows: denaturation for 3 min at 95°C, denaturation for 1 min at 94°C, refolding for 1 min at 55°C, extension for 1 min at 72°C and a 6-min incubation at 72°C, for a total of 30 cycles. A 6-μL aliquot (3 μL of loading buffer and 3 μL of PCR product) of the reaction product was electrophoresed on 1% agarose gel containing Goldview stain and observed with a gel imaging system. Gene sequencing of the PCR products was performed by Shanghai Biological Engineering Technology Co. (Shanghai, China)

Detection of virulence factors in the isolated strains. The DNA templates for the CY-1 strain, *V. alginolyticus*, *Listonella anguillarum* and *V. vulnificus* were extracted and separated according to the method described above. The isolated genomic DNA was treated as a PCR template to amplify the bacterial cells using the following specific primers: ctxA-F, 5′-CGG GCA GAT TCT AGA CCT CCT G-3′; ctxA-R, 5′-CGA TGA TCT TGG AGC ATT CCC AC-3′. The PCR reaction conditions and agarose gel electrophoresis were as mentioned above.

Results

Symptoms of diseased fish. The body length of the adult grass carp was 21 ± 1 cm. The main symptoms of the moribund fish were mainly manifested in the hyperemia of mouth, head, eye, gill cover edge, fin, and caudal peduncle, and anal swelling. The postmortem observations showed that the intestine contained no food and was red from hyperemia, there were many yellowish effusions in the abdomen, the liver was purple, and the gallbladder swollen.

Isolation and identification of the strains. Physical characteristics of isolates: they were Gram-negative rods with rounded ends, slightly curved, occurring singly, approximately 0.5-1.0 nm wide and 1.2-2.0 nm in length, and without endospores. The results of the physiological and biochemical tests are shown in Table 1.

Table 1. Physiological and biochemical characteristics of the isolated CY-1 strain

Characteristic	*Strain*		*Characteristic*	*Strain*	
	CY-1	*V.cholerae**		*CY-1*	*V.cholerae**
Glucose	+	+	Acetate		
Lactose	+	-	Tartaric acid salt	+	+
Maltose	+	+	Mucic acid	+	-
Mannitol	+	+	Phenylalanine	-	-
Mannose	+	d	Trehalose	+	+
Sucrose	+	+	Raffinose	+	-
L- Arabia sugar	+	-	Fructose	+	•
Arabitol	-	-	Melibiose	+	+
Xylose	+	-	Cellobiose	+	+
Xylitol	+	•	SM peptone water	+	•
Galactose	+	+	Glucose ammonium	+	•
Melezitose	+	•	Ornithine decarboxylase	+	+
Sorbitol	-	-	Lysine decarboxylase	+	+
Dulcitol	+	+	Arginine decarboxylase	+	+
Erythritol	-	-	Arginine hydrolase	+	•
Amygdalin	+	•	Gelatin	+	+
Rhamnose	-	-	Nitrate (reduction)	+	+
Dextrin	+	•	Nitrate (gas)	-	•
Inositol	+	-	OF tube	F	•
D(-)-Salicin	+	-	Potassium cyanide	+	•
Urea	-	•	α- methyl -D-glucoside	+	-
Bile esculin	-	-	Motility	+	+
Beta galactosidase	-	+	Gluconate	-	+
Malonate	+	-	Pyruvate	+	•
NaCl 0%	+	+	Citrate	-	d
NaCl 6%	+	+	Oxidase	+	+
NaCl 10%	-	-			

Note: "+": positive result; "-":negative result ; "·": not recorded; "*": The data in the table are from Bergey's Manual of systematic bacteriology, Second edition (George M. Garrity et al, 2004) and Common bacterial System Identification Manual (Dong Xiuzhu, Cai Miaoying, 2001)

The serologic results. The strain CY-1 agglutinated with *Vibrio Cholera* Inaba serogroup O1, *Vibrio Cholera* O1 serogroup and did not agglutinate with *Vibrio Cholera* Ogwan serogroup O1, *Vibrio Cholera* O139 serogroup.

*The analysis results of 16S rDNA gene sequence analysis***.** The length of the PCR product was 1441 bp (GenBank accession number: KF386612). The results retrieved were 16S rRNA gene sequences, including *V. cholerae* and *V. eTSAe*, and the similarity was 98–99%. Phylogenetic tree analysis showed that the strains were most closely related to *V. cholerae* (GenBank accession number: JN555611) (Fig. 1).

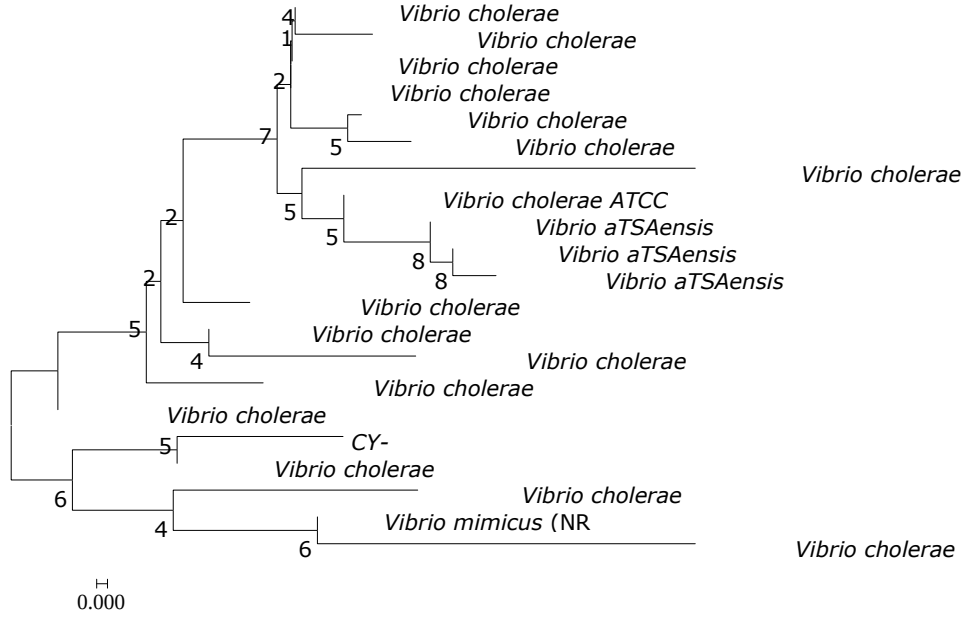

Fig. 1. Phylogenetic tree of the CY-1 strain based on 16S rRNA gene sequence

The drug sensitivity test. The diameter of the inhibition zones were calculated from the drug sensitivity tests using 45 kinds of drugs, according to NCCLS criteria (Huang et al., 2012). Antibiotic resistance was measured and the results are shown in Table 2. The results showed that the strains were sensitive to fosfomycin, amikacin, aztreonam, tetracycline, and 20 other antibiotics. They were moderately sensitive to rifampicin, kanamycin, vancomycin, and seven other antibiotics but were resistant to penicillin, tobramycin, cotrimoxazole, amoxicillin, and 18 other antibiotics.

Table 2. Drug sensitivity test results of the isolated CY-1 strain

Drug name	*Drug*	*Bacteriostatic*	*Result*	*Drug name*	*Drug*	*Bacteriostat*	*Result*
Penicillin	10	7	R	Cephalothin	30	22	S
Ampicillin	10	7	R	Doxycycline	30	17	I
Carbenicillin	100	7	R	Norfloxacin	10	18	I
Oxacillin	7	7	R	Nalidixic acid	30	13	R
Cefuroxime	30	30	S	Acetyl	30	12	R
Cefazolin	30	20	S	Maddie mycin	30	13	R
Cefoperazone	75	25	S	Enrofloxacin	5	21	S
Ceftazidime	30	30	S	Cephalexin	30	25	S
Ceftriaxone	30	35	S	Azlocillin	30	21	S
Rifampicin	5	16	I	Lomefloxacin	10	23	S
Streptomycin	10	8	R	Enoxacin	10	18	I
Kanamycin	30	17.5	I	Levofloxacin	5	23	S
Amikacin	30	20	S	Aztreonam	30	30	S
Gentamicin	10	21	S	Minocycline	30	22	S
Tobramycin	10	14	R	Amoxicillin	20	7	R
Novobiocin	30	9	R	Cefradine	30	18	I
Tetracycline	30	22	S	Spectinomycin	100	9	R
Chloramphenic	30	14	R	Ofloxacin	5	22	S
Erythromycin	15	7	R	O/129	10	7	R
Lin Ke mycin	2	12	R	Neomycin	30	23	S
Cotrimoxazole	23.7/1.25	7	R	Teicoplanin	30	7	R
Nitrofurantoin	300	20	S	Fosfomycin	200	37	S
Vancomycin	30	15	I				

Note: S: The diameter of inhibition zone including drug diameter 7mm; Denotes high sensitivity (d≥20mm); I: Denotes moderate sensitivity (15 mm≤d≤19 mm); R: Denotes low or no sensitivity (7 mm≤d≤14 mm)

The detection of virulence factors. PCR results for the isolates are shown in Fig. 2. The gel electrophoresis results revealed amplified bands in the corresponding 564-bp position. No other corresponding virulence factors were detected for the other three aquatic animal pathogens.

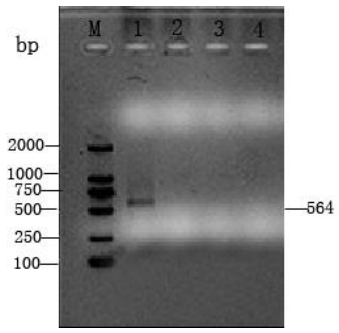

Fig. 2. Polymerase chain reaction sequence results of the isolated CY-1 strain
M. DL2000; 1. CY-1; 2. *V. alginolyticus*; 3. *L. anguillarum*; 4. *V. vulnificus*

Artificial infection experiment. Grass carp infected by artificially injecting different doses of the isolated bacterium showed different mortality rates, whereas all fish in the control group survived (Table 3). The mortality rate was 100% in fish injected with 1×10^7 CFU/mL, whereas only 50% of the fish injected with 1×10^6 CFU/mL died.

Table 3. Results of the artificial infection experiment with the isolate

Group	*CY-1/cfu·ml^{-1}*	*Deaths*	*Trials*	*Mortality*
test groups	1×10^8	8	8	100%
	1×10^7	8	8	100%
	1×10^6	4	8	50%
	1×10^5	0	8	0
	1×10^4	0	8	0
Cont	Sterile broth	0	8	0

Discussion

V. cholerae belongs to the family *Vibrionaceae* and is a typical *Vibrio* species. It is a short Gram-negative, comma-shaped bacterium, with a single flagellum and fimbriae, which are part of the capsule (Wu and Pan, 2001). *V. cholerae* is very prevalent in aquatic environments and products, and the food-borne cholera outbreaks that have occurred in China in recent years were caused mainly by water contaminated with *V. cholerae* (Cao et al., 2013; Zhang et al., 2010). *V. parahaemolyticus* and *V. fluvialis* are other important food-borne pathogens (Wu et al., 2007).

In this study, the bacterium that was isolated from diseased organs of grass carp was confirmed to be the same pathogen injected at the earlier stage of the experiment, and the onset of symptoms were the same as the primary symptoms. According to the Common Bacterial System Identification Manual, the bacterium isolated in this study metabolises D-glucose, other carbohydrates, and acids, but not gas; it can reduce nitrate to nitrite; ferment maltose, mannose, and trehalose, and is sensitive to the *Vibrio* inhibitor O/129 (Dong and Cai, 2001). The biochemical test results showed that there may be differences between strains: the isolated CY-1 strain and *V. cholerae* had similar physical and chemical properties. Moreover, the 16S rRNA gene sequencing and phylogenetic tree analyses further increased the accuracy of the identification.

V. cholerae strains carrying a toxin are an important reference index for judging an epidemic. The virulence factors in pathogenic *V. cholerae* are not potent, as they only cause diarrhoea and other mild clinical symptoms but do not lead to a pandemic (Jin et al., 2011). Therefore, strains that can cause a cholera epidemic or food poisoning have a stable genetic structure and particular molecular genetics. The main O1 CVC strain group of *V. cholerae* has major ctx virulence genes and includes the epidemic strains EVC and the O139 group, which have conserved ctx genes in the core region (virulence component) above the consensus sequence strains (Hou et al., 2006; Xu et al., 2004).

One of the main pathogenic factors in *V. cholerae* is the cholera enterotoxin (ctxA) (Mekalanos et al., 1983), which is a heat-sensitive protein that is denatured by heating at 56°C for 30 min (Li et al., 2010). *V. cholerae* multiply rapidly in the digestive tract by adhering to the intestinal mucosal surface, where they produce the cholera toxin, which causes excess secretions by the intestinal mucosa, intestinal inflammation, and other symptoms (Zhang et al., 2003; Luo and Hu, 2008).

The test strip showed that only the isolated CY-1 strain had amplified bands at the position corresponding to 564 bp, consistent with the ctxA gene, and no bands were detected for *L. anguillarum* or *V. vulnificus*, which contains the ctxA pathogenic factor. A previous study selected ctxA as a probe to detect the *V. cholerae* cholera toxin (CT) gene and used *in situ* hybridisation and blot hybridisation to identify various virulence genes but chromosome replication and amplification were not necessarily consistent (Yang et al., 1999). Dazhi et al. designed primers and probes to more accurately and rapidly detect the *V. cholerae* ctxA, ace and zot 3 toxin genes (Jin et al., 2011).

Isolating and identifying pathogenic bacterial diseases of grass carp (Li et al., 2011) and mandarin fish (Cao et al., 2013), particularly the source of the recent *V. cholera* outbreak, can aid in the discovery of methods for treating human diseases. The results of the present study provide a reliable basis for further disease prevention and control measures in cultured aquaculture species, in order to detect virulence factors of bacteria carrying toxigenic strains or to judge the probability of an outbreak and evaluate disease severity.

Acknowledgements

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