Identification of *Vibrio alginolyticus* as a causative pathogen associated with mass summer mortality of the Pacific Oyster (*Crassostrea gigas*) in China

Ben Yang [a], Shangyu Zhai [a], Xin Li [a], Jing Tian [a], Qi Li [a], Hongwei Shan [a], Shikai Liu [a,b,*]

[a] *Key Laboratory of Mariculture (Ocean University of China), Ministry of Education, and College of Fisheries, Ocean University of China, Qingdao 266003, China*
[b] *Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China*

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ABSTRACT

In August 2019, an outbreak of mass mortality occurred in the Pacific oysters farmed in Shandong, China, resulting in over 60% mortality. Bacterial pathogens were isolated from moribund oysters, among which one strain identified as gram-negative rod-shaped bacterium, named as Cg5, was further characterized in this study. Based on 16S rRNA phylogenetic analysis and biochemical characterization, Cg5 was determined to be *Vibrio alginolyticus*. Injection of *V. alginolyticus* isolate into healthy oysters revealed its pathogenicity, which showed same symptoms and histopathological changes as the naturally infected oysters. Analysis of growth capability of the isolate at different temperatures showed that high temperature was conducive to pathogen proliferation. These results implied that high temperature could facilitate the growth and pathogenicity of *V. alginolyticus*, suggesting the inducement of disease outbreak by high temperature in summer. Additionally, the important virulence genes, including *AspA, Collagenase, flaB, fur, ompW, tlh* and *toxR,* were all detected from the *V. alginolyticus*, further supporting its strong pathogenicity. To the best of our knowledge, this is the first report of *V. alginolyticus* as a causative pathogen associated with mass summer mortality of the Pacific oysters cultured in China, which provides valuable information for disease surveillance and genetic breeding program to produce oyster strains with high disease resistance.

1. Introduction

The Pacific oyster (*Crassostrea gigas*) originated in the Northeast Asia and has been introduced to many countries for aquaculture (Troost 2010), for its wide geographical distribution, fast growth and high yield (Zhang et al., 2019). The *C. gigas* has become one of the major aquaculture species around the world with global aquaculture production reaching over 643 kt in 2018 (FAO 2020). In recent years, the aquaculture industry of *C. gigas* has been seriously affected by mass summer mortality around the world (Patrick et al., 2005). Summer is the main season for mass mortality of *C. gigas* due to viral and bacterial infections, coupled with environmental stresses (Friedman et al. 2005; Burge et al. 2007; Garnier et al. 2007). Although the virus, *Ostreid herpesvirus* 1 (OsHV-1), has been reported as a main cause of mass mortality (Segarra et al. 2010), recent investigations suggested that *Vibrios* could also play a critical role in causing mortality of *C. gigas* (de Lorgeril et al. 2018).

As a common pathogen in seawater, *Vibrios* has been reported to infect a variety of aquatic animals (Pass et al. 1987; Nicolas et al. 2002;

Cardinaud et al. 2014; Zhang et al., 2014). The Gram-negative bacterium *V. alginolyticus* is a ubiquitous bacterium in the ocean (Chart 2012). It was previously considered to be a normal component of the marine fauna, which can be isolated from various aquatic animals (Yan et al. 2007). However, in recent years, many mortality events of marine animals have been associated with *V. alginolyticus* (Liu et al. 2001; Rameshkumar et al. 2017; Xie et al. 2020). In addition, the *V. alginolyticus* is reported to be involved in emerging human infections, leading to otitis externa, food poisoning and bacteremia (Dronda et al. 1991; Jacobs Slifka et al. 2017; Fu et al. 2016).

China is one of the major countries for oyster aquaculture, accounting for over 85% of the global aquaculture production (FAO 2020). In August 2019, an outbreak of mass mortality occurred in *C. gigas* farmed in Shandong, China, resulting in the mortality of over 60% of oysters. On-site investigation showed that the mortality started in July and lasted until October. The deaths were mainly adult oysters (over one year old). The water quality indicators (dissolved oxygen, ammonia nitrogen, nitrite, salinity) of the farm were normal and no other animals

were observed to be affected. Bacterial pathogens were isolated from moribund oysters, among which one strain identified as gram-negative rod-shaped bacterium, named as Cg5, was further characterized in this study. We determined its identity through phylogenetic analysis and characterized its pathogenicity to the healthy oysters by injection. Effects of temperature on pathogen proliferation and pathogenicity were also investigated. These results should provide valuable information for disease surveillance and genetic breeding program to generate disease resistant oyster strains.

2. Materials and methods

2.1. Sample collection

Eighty moribund oysters (14 months old, mean shell height 58.35 mm, mean wet weight 16.18 g) were collected from a farm in Rongcheng, Shandong Province in August 2019 (Supplementary Table 1). The oysters cultured in this farm were experiencing a high mortality rate of over 60% during summer. Eighteen moribund oysters were randomly selected and used for virus detection and bacterial screening.

2.2. Detection of viruses

About 50 mg of mantle was dissected from the soft tissue of oysters, and DNA was extracted using TIANamap Marine Animal DNA Kit (Tiangen Biotech, Beijing) according to the manufacturer's manual. The polymerase chain reaction (PCR) with specific primers C2/C6 (Table 1) was carried out to detect OsHV-1 DNA according to Arzul et al. (2001). PCR reactions were performed in 50 μL system, containing 25 μL Premix Taq™ (Takara, Kyoto, Japan), 22 μL distilled water, 1 μL primers (10 pMol), and 2 μL template DNA (200 ng). The PCR procedure was set as: first heating at 94 °C for 2 min, followed by 35 cycles consisted of DNA melting at 94 °C for 1 min, primer annealing at 50 °C for 1 min, and primer extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. A total of 3 μL PCR products was used to run the 1.5% Agarose gel electrophoresis for the OsHV-1 detection.

Presence of other possible viruses was determined by observing mortality after injection. Ten moribund oysters were used to prepare tissue homogenate using the approach as described previously (Schikorski et al. 2011). The homogenate was filtered by the bacterial filter and was inoculated on TSA agar to ensure its sterility. Healthy oysters (*C. gigas*, 14 months old, mean shell height 54.75 mm, mean wet weight 15.12 g) were collected from a farm in Yantai, Shandong Province (Supplementary Table 1). Eighteen oysters were randomly selected for

pathogen detection, and the results showed that no OsHV-1 was detected in these oysters and the *Vibrio* load in hemolymph was less than 50 CFU/mL. Then, sixty healthy oysters were divided into two groups ($n = 30$) and were anesthetized by magnesium chloride (MgCl$_2$; 50 g L^{-1}) for 4 h as described by Gay et al. (2004) before injection. In the treatment group, 100 μL fresh suspension was injected via the adductor muscle of each oyster, while in the control group, the same amount of ASW were injected. The dead oysters were counted every 12 h for one week.

2.3. Isolation of bacteria

According to Bruto et al. (2017), the bacteria were isolated from the hemolymph of oysters. A small opening near the oyster adductor muscle was made using a scalpel, then 200 μL blood was collected from adductor muscle lymph of each oyster using an 18-gauge needle. After 10-time dilution with ASW, 100 μL blood were plastered on Zobell Marine Agar 2216E and *Vibrio* selective agar (thiosulfate-citrate-bile salts-sucrose agar, TCBS), and plates were incubated at 28 °C for 48 h. Three bacterial colonies were randomly selected from each plate. The pure cultures of bacteria were stored in 2216E broth containing 25% glycerol (*v*/v) at −80 °C.

2.4. Molecular characterization of bacterial isolate

The TIANamp Bacteria DNA Kit (Tiangen Biotech, China) was used to extract bacterial genomic DNA according to the manufacturer's instructions. The 16S rRNA was amplified using bacterial universal primers (Table 1) as described in Weisburg et al. (1991). Briefly, PCR amplifications were performed in a total volume of 25 μL reaction mixture containing 12.5 μL Premix Taq™ (Takara, Kyoto, Japan), 11 μL distilled water, 0.5 μL primers (5 pMol), and 1 μL template DNA (100 ng). The reaction mixture was pre-heated at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and final extension for 7 min. PCR products were detected by running 1% Agarose gel electrophoresis, and were outsourced for sequencing by the Sangon Biotech Co., Ltd. (Shanghai, China). The Basic Local Alignment Search Tool (BLAST) was used for molecular identification and homology comparison of 16S rRNA sequences. The phylogenetic tree was constructed using the neighborjoining algorithm in the program MEGA 7.0.26 (Kumar et al. 2016) and bootstrapped 1000 times.

2.5. Biochemical characterization

The pure bacterial isolate was cultured on 2216E agar at 28 °C for 24 h for biochemical test. The biochemical characterization of the isolated strain was carried out by *Vibrio* biochemical identification Kit (Huankai microbial Co., Ltd., China) following the manufacturer's instructions. The Gram staining kit was used to carry out Gram staining and observe the morphology of bacteria. All biochemical tests were carried out in triplicate, including growth test at different conditions (Temperatures at 4 °C, 20 °C, 30 °C, 35 °C, 42 °C, and Salinities at 3%, 6%, 8% 10% of NaCl), gram's staining, arginine dihydrolase, lysin decarboxylase, indol, vp test, glucose, inositol, sucrose, arabinose, mannose, O/129. The biochemical characterization tube was cultured at 28 °C for 24 h, and the results were determined according to the manufacturer's instructions.

2.6. Growth test

The growth test of isolate was determined based on the growth curve. The isolate was inoculated on 2216E agar plate for 12 h, followed by selection of a bacterial colony with inoculating needle and cultured into a conical flask containing 50 mL 2216E broth. When the OD$_{600}$ value reached 0.5, 100 μL of the bacterial solution was inoculated into the conical flask containing 100 mL 2216E broth, and cultured at different

Table 1
Sequences of primer pairs used in this study.

Genes	Primer sequences (from 5′ to 3′)	Annealing Tm	Amplicon (bp)
16S rRNA	F-AGAGTT TGATCMTGGCTCAG R-CGGTTACCTTGTTACGACTT	58	1500
OsHV-1	C2-CTCTTTACCATGAAGATACCCACC C6-GTGCACGGCTTACCATTTTT	50	709
AspA	F-GCATGGTACTCACGTAGCGG R-CTTTCACAAGACCAGAAGAGTAACC	59	146
Clg	F-GTACTACGACATTGGCGAAGG R-CCCGACCATACATTTCATACTG	59	591
flaB	F-AACGTATCAGCGATGACC R-TTGAAACGGTTCTGGAAT	54	928
fur	F-ATTAACCCTTTGAAGTTCGTGG R-TGACATATACTTTCCCGTTGGATC	60	111
ompW	F-TCGTGTCACCAAGTGTTTTCG R-CGTGGCTGAATGGTGTTGC	59	213
toxR	F-GGATTCAACCAAATCTCCAGAGT R-GCTCAATAGAAGGCAACCAGTT	56	434
tlh	F-CGAACGAGAACGCAGACATT R-CTTTGTTGATTTGATCTGGCTG	60	108

temperatures ($15 \pm 0.5\,°C$, $20 \pm 0.5\,°C$, $25 \pm 0.5\,°C$) for 24 h. OD_{600} was used as an indicator of bacterial growth and was measured by UV Spectrophotometer. The 2216E agar was inoculated with 1 μL bacterial suspension, then cultured at different temperatures ($15 \pm 0.5\,°C$, $20 \pm 0.5\,°C$, $25 \pm 0.5\,°C$) for 24 h, and the colony diameter was measured with vernier caliper. All the tests were carried out in triplicate.

2.7. Experimental infection

Two hundred healthy oysters (*C. gigas*, about 15 g in wet weight) were collected from a farm in Yantai, Shandong Province and cultured in a 400 L glass tank (containing 300 L of UV treated seawater) for two weeks. Care of animals was conducted in compliance with the guidelines of the Animal Experiment Committee, Ocean University of China.

The experimental animals were divided into 6 groups (3 treatment groups and 3 control groups, *n* = 30). The water temperature was changed by increasing or decreasing 0.5 °C every day to reach the experimental temperature ($15 \pm 0.5\,°C$, $20 \pm 0.5\,°C$, $25 \pm 0.5\,°C$). Oysters were experimentally infected after being acclimated at different water temperatures for one week. The water quality control was carried out as follows: pH at 8.1–8.2, dissolved oxygen at 8.0–9.0 ppm, salinity at 30–32, and nitrite at 0.2–0.4 ppm. The isolate was sub-cultured in 200 mL 2216E broth at 28 °C for 12 h. After centrifugation (8000 *g*, 5 min, 4 °C), the supernatant was discarded, bacterial cells were washed three times with ASW. The concentration of bacteria was measured by optical density value and gradient dilution culture and adjusted to 5×10^7 CFU/mL. The challenged group was injected with 100 μl of bacterial suspension in adductor muscle, and the control group was injected with ASW. Then put the oyster back into the water tank with different temperatures. The dead oysters were counted every 12 h for one week. Statistical analyses were performed using Log-rank (Mantel-Cox) test to compare different conditions.

2.8. Histological assay

The mantle, gill, adductor muscle, labial palp and digestive gland of the oysters (*n* = 6) were dissected and fixed by Bouin solution, dehydrated by graded alcohol, and embedded in paraffin, and were sectioned at 5 μm thickness for HE (hematoxylin-eosin) staining. The observations were performed using 400 × optical microscope.

2.9. Antibiotic sensitivity test

The agar plate diffusion technique (Bauer et al. 1966) was used to test the drug sensitivity of 30 antibiotics. After incubation at 28 °C for 24 h, the antimicrobial sensitivity (S), moderate sensitivity (M) and drug resistance (R) were determined by measuring the diameter of inhibition zone.

2.10. PCR detection of virulence genes

Potential virulence genes of *V. alginolyticus*, including alkaline serine protein (*AspA*), collagenase (*Clg*), flagellin B (*flaB*), ferric uptake regulatory protein (*fur*), outer membrane protein (*ompW*), thermolable thermolysin (*tlh*) and transmembrane transcription regulator (*toxR*), were detected by PCR with specific primers (Table 1). PCR were performed in a total volume of 25 μL reaction mixture containing 12.5 μL Premix Taq™(Takara, Kyoto, Japan), 11 μL distilled water, 0.5 μL primers (5 pMol), and 1 μL template DNA (100 ng). The PCR procedure was set as: first heating at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, annealing at specific temperature of each gene for 1 min, extension at 72 °C for 1 min and final extension for 7 min. A total of 3 μL PCR products was used to run the 1.5% Agarose gel electrophoresis for the gene detection.

2.11. Temperature data collection

The data of water temperature in Shandong coastal area comes from National Marine Data Center, National Science & Technology Resource Sharing Service Platform of China (http://mds.nmdis.org.cn/).

3. Results

3.1. Symptoms of diseased oysters and pathogen screening

For the naturally diseased oysters, the shell and soft tissues showed no visible lesions but emitted pungent odor. The contraction of adductor muscle becomes slow and could not fully close the shell. The *Vibrio* bacteria were detected with TCBS medium, no parasites were found with light microscopy, and no OsHV-1 DNA was amplified in the diseased oysters (Supplementary Table 1). Healthy oysters injected with sterile tissue suspension of diseased oysters resulted in no mortality, suggesting that no other virus was present.

3.2. Biochemical characterization of Vibrio isolate Cg5

The *Vibrio* isolate Cg5 was further analyzed for its biochemical characterization. When being incubated on TCBS agar for 12 h, Cg5 was pale-yellow with a round and moist, central uplift and a neat edge. Gram staining showed that Cg5 was rod-shaped, morphologically homogeneous, gram-negative bacteria. Because it can utilize sucrose, the yellow colony on TCBS plate was formed (Fig. 1), which is different from the green colony formed by *V. parahaemolyticus*.

Phenotypic characteristics of Cg5 were provided in Table 2. As revealed by motility test, Cg5 is a motile bacterium. It's positive for tests of voges-proskauer (vp), indole, glucose, mannose, lysine decarboxylase, O/129(10 μg), and O/129(150 μg). It can grow at salinity of 3% NaCl, 6% NaCl, 8% NaCl, and 10% NaCl, and at temperatures of 20 °C, 30 °C, 35 °C. The 3% NaCl and 35 °C was the optimum salinity and temperature for Cg5 growth (Table 2).

3.3. Phylogenetic analysis based on 16S rRNA

The 16S rRNA sequence of *Vibrio alginolyticus* Cg5 has been deposited to NCBI database with accession number of MW295458. The16S rRNA sequencing and BLAST based analysis showed that the identity was more than 99% between Cg5 in this study and *V. alginolyticus* from public

Fig. 1. Colony morphology of *Vibrio alginolyticus* Cg5 on TCBS agar plate.

Table 2
Phenotypic characteristics of the identified *Vibrio alginolyticus* Cg5.

Characteristics	The present study	Type strain[a]
Gram stain	−	−
TCBS	+	+
Motility	+	+
Glucose (gas)	−	−
Voges-Proskauer	+	+
Indole	+	+
Glucose	+	+
Sucrose	+	+
Mannose	+	+
Arabinose	−	−
Inositol	−	−
Lysine decarboxylase	+	+
Arginine dihydrolase	−	−
O/129(10 μg)	+	+
O/129(150 μg)	+	+
Growth at 0% NaCl	−	NA
Growth at 3% NaCl	+	+
Growth at 6% NaCl	+	+
Growth at 8% NaCl	+	+
Growth at 10% NaCl	+	+
Growth at 4 °C	−	NA
Growth at 20 °C	+	+
Growth at 30 °C	+	+
Growth at 35 °C	+	+
Growth at 42 °C	−	−

Notes: +, positive; −, negative; NA, Not Available.
[a] Data from Xie et al. (2020).

database. The phylogenetic tree constructed based on the 16S rRNA gene sequences of *Vibrio* spp. further verified that the isolated strain Cg5 in the current study was *Vibrio alginolyticus* (Fig. 2).

3.4. Growth test of Cg5 at different temperature conditions

As shown in Fig. 3, the Cg5 showed a significant growth advantage at high temperature (20 °C and 25 °C versus 15 °C). The colony diameter was measured as 1.32 mm, 2.20 mm and 2.83 mm at 15 °C, 20 °C and 25 °C, respectively (Fig. 3A). The growth of Cg5 at 25 °C was significantly faster than that at 20 °C and 15 °C. Growth curve showed that the growth of Cg5 was significantly inhibited by low temperature at 15 °C (Fig. 3B).
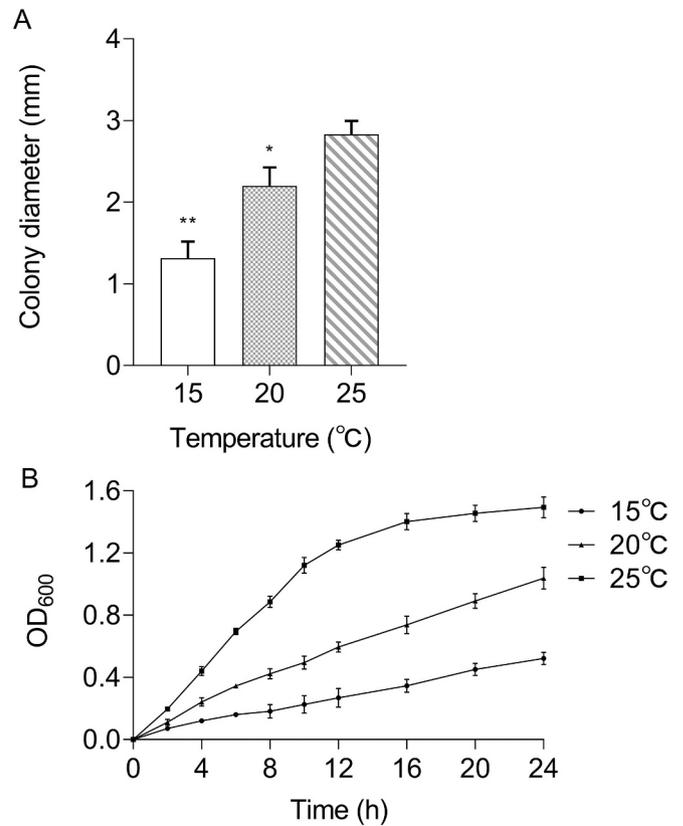
Fig. 3. The growth of *V. alginolyticus* Cg5 strain was dependent on temperature. A. Growth test of Cg5 at different temperatures (**$P < .01$; *$P < .05$). B. Growth curves of Cg5 at different temperatures.

3.5. Survival analysis

The survival curve of oysters after *V. alginolyticus* Cg5 infection is presented as shown in Fig. 4. Oysters began to die within one day after injection, and no mortality was found in all the control oysters. Daily observation last for a week, while no oyster died after day 3. The survival rates at 25 ± 0.5 °C, 20 ± 0.5 °C and 15 ± 0.5 °C were 30%, 63.33% and 80%, respectively. The cumulative mortality of challenged oysters
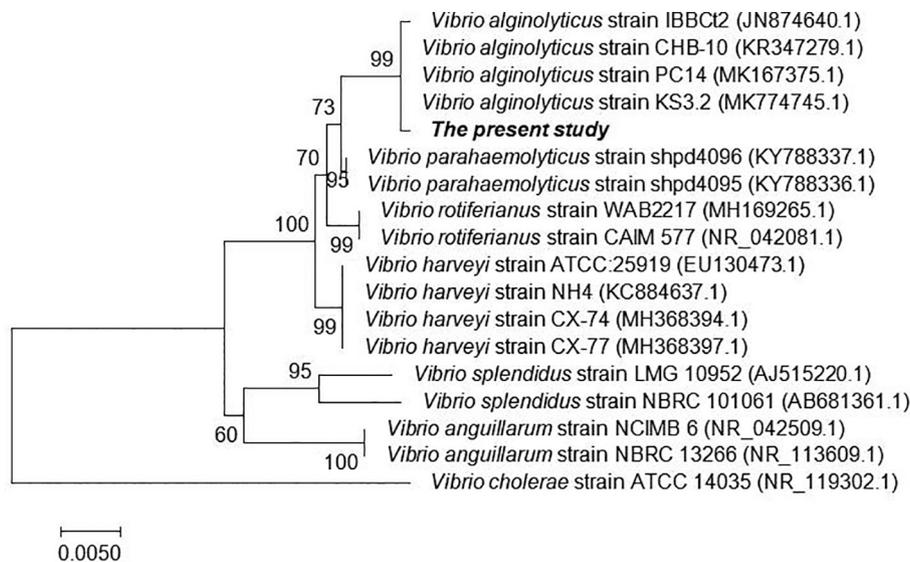
Fig. 2. Phylogenetic analysis of *Vibrio* spp. based on 16S rRNA. The phylogenetic tree was constructed using the neighbor-joining method by the MEGA 7.0.26 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. Bootstrap values are shown at the nodes.
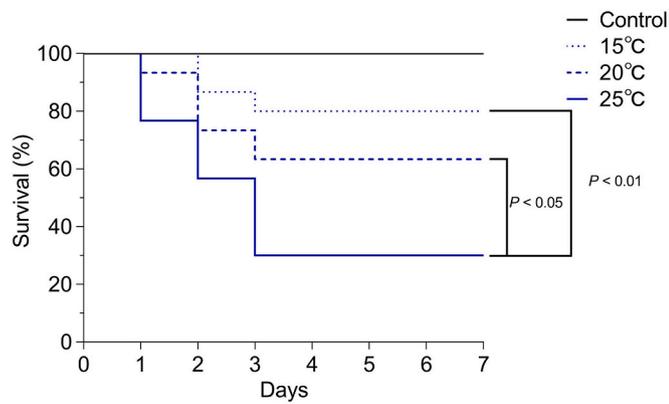
Fig. 4. Survival curve of *C. gigas* challenged with *V. alginolyticus* at different temperature (15 ± 0.5 °C, 20 ± 0.5 °C and 25 ± 0.5 °C) via intramuscular injection.

at 25 °C was significantly higher than that of oysters at 15 and 20 °C, but there was no significant difference between 15 and 20 °C (Fig. 4). The strain was re-isolated from the hemolymph, gill, digestive gland and adductor muscle of the infected oyster, and was identified to be *V. alginolyticus* based on 16S rRNA sequencing.

3.6. Histological changes

Same symptoms were observed in the oysters injected with the isolated *V. alginolyticus* Cg5 as the naturally diseased oysters. Histologically, the digestive glands of oysters (6/6) were obviously damaged after injection of Cg5 (Fig. 5). The digestive gland tubules were enlarged, absorption cells were atrophied, accompanying lipofuscin accumulation and connective tissue necrosis (Fig. 5B). Same histological changes were observed in all (6/6) naturally diseased oysters (Fig. 5C), and no similar damage (0/6) was observed in healthy oysters (Fig. 5A).

3.7. Antibiotic sensitivity test

The antimicrobial susceptibility tests were conducted and illustrated in Table 3. Cg5 strain was resistant (R) to penicillin, oxacillina, ampicilin, carbenicillin, piperacillin, cephalexin, ceftriaxone, norfloxacin, ofloxacin, enrofloxacin, vancomycin, midecamycinum, erythromycin, oxytetracycline, tetracycline, novobiocin, rifampicin; moderately susceptible (M) to cefamezin, cefradine, cefuroxim, cefoperazone, ciprofloxacin, polymyxin B; and susceptible (S) to ceftazidime, compound sulfamethoxazole, trimethoprim, furazolidone, streptomycin, gentamicin, chloramphenicol.

Table 3
Antibiotic Sensitivity pattern of *Vibrio alginolyticus* isolate.

Antibiotics	Concentration (disc^{-1})	Inhibition zone (mm)	Result
Penicillin	10 U	0	R
Oxacillin	1 µg	0	R
Ampicilin	10 µg	0	R
Carbenicillin	100 µg	0	R
Piperacillin	100 µg	15	R
Cephalexin	30 µg	13	R
Cefamezin	30 µg	15	M
Cefradine	30 µg	16	M
Cefuroxim	30 µg	16	M
Ceftazidime	30 µg	20	S
Ceftriaxone	30 µg	11	R
Cefoperazone	75 µg	20	M
Norfloxacin	10 µg	12	R
Ofloxacin	5 µg	12	R
Ciprofloxacin	5 µg	16	M
Enrofloxacin	5 µg	13	R
Vancomycin	30 µg	0	R
Compound Sulfamethoxazole	23.5 µg	22	S
Trimethoprim	5 µg	16	S
Furazolidone	300 µg	22	S
Midecamycinum	30 µg	0	R
Erythromycin	15 µg	10	R
Streptomycin	300 µg	22	S
Gentamicin	10 µg	16	S
Oxytetracycline	30 µg	0	R
Tetracycline	30 µg	14	R
Chloramphenicol	30 µg	22	S
Novobiocin	5 µg	0	R
Polymyxin B	300 IU	10	M
Rifampicin	5 µg	14	R

Abbreviations: S, susceptible; M, moderately susceptible; R, resistant.

3.8. PCR detection of virulence genes

The detection of virulence gene is shown in Fig. 6. Target PCR products with sizes of 146 bp, 591 bp, 928 bp, 111 bp, 213 bp, 108 bp and 434 bp were all amplified with specific primers of the seven virulence genes, including *AspA, Clg, flaB, fur, ompW, tlh*, and *toxR*, respectively. The results revealed that all the seven tested virulence genes were detected from the Cg5.

3.9. Effect of water temperature on disease outbreak

The annual water temperature records in the coastal area near Shandong Province were retrieved from the public data platform (http://mds.nmdis.org.cn/). According to Fig. 7, the average water temperature was higher than 25 °C in the months of disease outbreak, while the
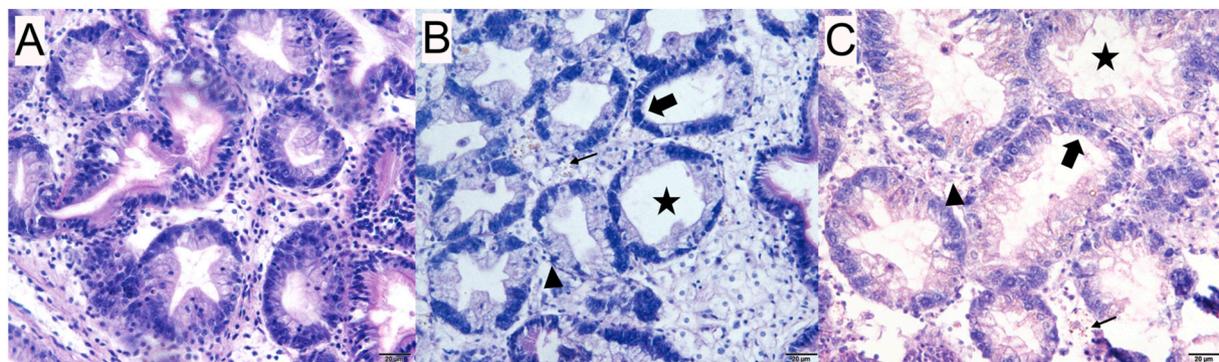

Fig. 5. Pathological observation of *C. gigas* under different conditions. A. HE staining of digestive glands of healthy oysters. B. HE staining of digestive glands of *C. gigas* injected with Cg5. C. HE staining of digestive glands of *C. gigas* collected during the outbreak of mass mortality. →, lipofuscin accumulation; ☆, digestive gland tubules were enlarged; △, connective tissue necrosis; ⇨, absorption cells were atrophied.
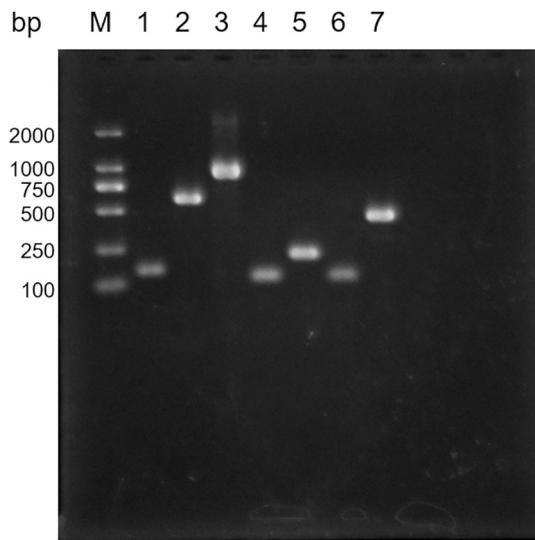
Fig. 6. Specific PCR amplification of *V. alginolyticus* virulence gene from Cg5. M: DL2000 DNA Markers. Lane 1: *AspA* detection of Cg5; Lane 2: *Clg* detection of Cg5; Lane 3: *flaB* detection of Cg5; Lane 4: *fur* detection of Cg5; Lane 5: *ompW* detection of Cg5; Lane 6: *tlh* detection of Cg5; Lane 7: *toxR* detection of Cg5.
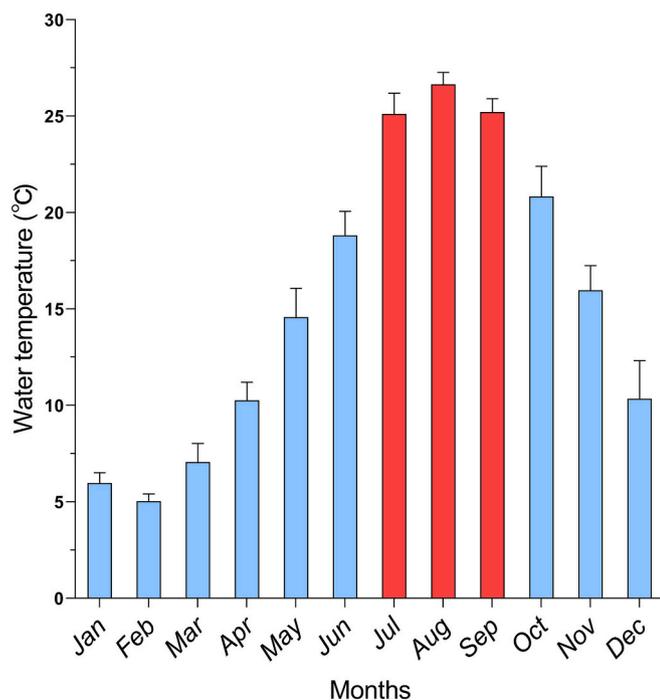

Fig. 7. Annual water temperature in coastal areas of Shandong Province in 2019, China. The water temperature is above 25 °C (red bars) in July, August and September during the year (Data retrieved from http://mds.nmdis.org.cn/). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water temperature in other months was lower than 20 °C. The 15 °C, 20 °C and 25 °C designed in this study are based on the data of water temperature, which represent the water temperature in different seasons, in order to illustrate the effect of temperature on the outbreak of disease.

4. Discussion

Over the past two decades, the global industry of Pacific oysters has

been threatened by disease outbreaks in summer. It is generally recognized that *Ostreid herpesvirus* 1 (OsHV-1) and its variants is one of the major causes of mass mortality events around the world (Schikorski et al. 2011; Martenot et al. 2012), but recent evidence suggests that *Vibrios* play a major role in mass mortality events (Petton et al. 2015). There is currently no report of mass mortality associated with OsHV-1 in the oysters in China, though mass mortality caused by OsHV-1 has been reported in clams (Bai et al. 2015; Xia et al. 2015). In the present study, no OsHV-1 was detected in the moribund oysters. However, we showed that *V. alginolyticus* isolated from diseased oysters was one of the critical pathogenic factors causing mass mortality in summer. Although there have been reports on the mass mortality of oysters caused by *Vibrios* (Garnier et al. 2007; Saulnier et al. 2010; Lacoste et al. 2001), to the best of our knowledge, this is the first report on *V. alginolyticus* as a causative pathogen for oyster mass mortality in China.

As an opportunistic pathogen widely existing in seawater, *V. alginolyticus* can cause diseases in many marine animals including cobia (Liu et al., 2004b), seabass (Kahla-Nakbi et al. 2006), seahorse (Martins et al. 2010), shrimp (Liu et al., 2004a) and shellfish (Gómez-León et al. 2005), leading to serious economic losses. *V. alginolyticus*, which is euryhaline bacterium, may also subsist in fresh water and is reported to infect Nile tilapia cultured in freshwater (Al-Sunaiher et al. 2010). With the continuous increase of intensive aquaculture, frequent diseases may become a major obstacle to the development of the industry, and the widespread pathogen *V. alginolyticus* should be taken as a key pathogen to surveillance, so as to ensure the sustainable development of the industry.

Mass mortality of oysters often occurs in summer, which suggests that environmental factors, especially the high temperature in summer, may play a vital role in mass mortality outbreaks (Leung and Bates, 2013). The rising of water temperature in ocean caused by global warming will continue to intensify (Frölicher et al. 2018). In the coastal area of Shandong Province in northern China, the water temperature will keep above 25 °C in summer, which could greatly improve the propagation of opportunistic pathogens. This study also confirmed the importance of temperature effect on the growth and propagation of *Vibrio*. In addition, high temperature significantly affected the pathogenicity of *Vibrio* and inhibited the immunity of oysters (Green et al. 2019; Kimes et al. 2012). Previous studies have shown that the virus OsHV-1 suppressed the immune system of oysters and caused opportunistic bacterial invasion, resulting in bacteremia and death of oysters (de Lorgeril et al. 2018). Although the virus was not detected in this study, high temperature in summer could be an environmental stressor to suppress immune system followed by bacterial invasion. The mortality of oysters under high temperature was significantly higher than that of low temperature group, which was in line with the speculation of high temperature suppressing oyster immune system (Kimes et al. 2012; Delisle et al. 2018; Rahman et al. 2019). With the continuous occurrence of high temperature during summer, future genetic breeding for oysters with high survival rate may need to consider both high temperature tolerance and pathogen resistance.

Previous studies have shown that OsHV-1 mainly infects the early life stage (larvae and spat) of oysters and has no lethal effect on adult oysters (Dégremont, 2013; Dégremont et al., 2015), while *Vibrio* plays a major role in the mortality of adult oysters (Green et al. 2016), which is similar to the results we have observed in the oysters infected with *V. alginolyticus*. Field observation on oyster farm showed that all oysters with outbreaks of disease were adult, and the *Vibrio* load in diseased oysters were far higher than healthy oysters, which further confirmed the role of *Vibrio* in the outbreak of adult oyster mortality events. Previous studies have shown that oysters have high plasticity to OsHV-1, after several generations of breeding, high resistance strains for OsHV-1 can be generated (Dégremont et al. 2015), while the genetic gain of resistance breeding to *Vibrio* is slow (Azéma et al. 2015; Azéma et al. 2017; Dégremont et al. 2020). This work showed that bacteria including *V. alginolyticus* were the vital pathogens affecting oyster industry, and

V. alginolyticus may become a long-term problem in oyster industry in China.

The potential pathogenicity of bacteria is related to the presence of virulence genes (Iraola et al. 2012; Bruto et al. 2018; Rønneseth et al. 2017). Seven virulence genes of *V. alginolyticus* were amplified by specific primers in order to characterize the potential virulence of Cg5, and the results were all positive. Extracellular proteases, motility and iron uptake system have been identified as the main virulence factors of *V. alginolyticus*, which determine the pathogenicity of *V. alginolyticus*. (Gu et al. 2016). Among these genes, serine protease and collagenase belong to extracellular proteases (Lafisca et al. 2008; Rui et al. 2009; Supuran et al. 2002)., flagellum is the key element bacterial motility (Alcaide et al. 2005; Luo et al. 2016), outer membrane protein and ferric uptake regulator are involved in iron uptake (Balebona et al. 1998; Mey et al. 2005; Sperandio et al. 1995; Thode et al. 2018), hemolysin and toxR are widely distributed virulence factors, participate in the virulence system of *Vibrios* (Jia et al. 2010; Ruwandeepika et al. 2010). These virulence genes may have significant roles in the pathogenesis of *Vibrio*. The results showed that the isolate Cg5 possessed these genes, which further supported its pathogenicity to the oysters.

The frequent use of antibiotics in aquaculture has led to a sharp increase in the number of drug-resistant bacteria in water (Reverter et al. 2020). In this study, Cg5 showed resistance to 17 of 30 antibiotics examined and was highly sensitive to 7 of the tested antibiotics. Oysters farmers do not use antibiotics in the process of aquaculture, however, oyster farms are located in the coastal area, so it is easy to be affected by human activity. The horizontal transfer of drug-resistant genes will also lead to the drug-resistant bacteria (Luczkiewicz et al. 2015; von Wintersdorff et al. 2016). The antibiotic susceptibility test revealed the type of antibiotic resistance of *Vibrio* in aquaculture area. The emergence of drug-resistant bacteria may be caused by the horizontal gene transfer and the sewage containing antibiotics discharged by human activity. In addition, antibiotics that can significantly inhibit bacteria may be of great significance in oyster larval production (Matsubara et al. 2002).

In conclusion, we investigated the causative pathogenic factors of mass mortality of *C. gigas* in China. Our work showed that *V. alginolyticus* Cg5 isolated from diseased oysters had strong pathogenicity to oysters, and the oysters infected with Cg5 showed the same symptoms and histopathological changes as naturally infected oysters. Temperature significantly affected the ability of *V. alginolyticus* to cause oyster death, which suggested that temperature was a critical environmental factor involved in disease outbreak. Additionally, seven virulence genes were all detected in the isolated *V. alginolyticus*, which further demonstrated its potential pathogenicity. To our knowledge, this is the first report of *V. alginolyticus* as a causative pathogen of massive summer mortality of oysters cultured in China. These results will provide valuable information for disease surveillance and genetic breeding program to generate oyster strains with high disease resistance.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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