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Isolation, Identification, and Pathogenicity of Pathogens from *Litopenaeus vannamei* With Acute Hepatopancreatic Necrosis Disease

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YZ analyzed the data and wrote the manuscript. JC, CH and HJ carried out the experiments. JH and LZ participated in data analysis and manuscript preparation. YG and GW conceived and designed the experiments. XQ revised the manuscript.

Key words
***Litopenaeus vannamei, Vibrio parahaemolyticus*, Biochemical characterization, Serotype, Pathogenicity**

ABSTRACT

Acute hepatopancreatic necrosis disease, which can cause 100% mortality, is one of the main threats to farming *Litopenaeus vannamei*. We identified seven strains of pathogenic bacteria from ponds with outbreaks of acute hepatopancreatic necrosis at a *L. vannamei* farm in Zhuhai, China. Among the seven bacteria strains, one strain was isolated from aquaculture water, two strains were isolated from hepatopancreas, and the other four strains were isolated from juvenile shrimp. The results of 16S rRNA sequencing and biochemical identification showed that all seven pathogenic bacteria were *Vibrio parahaemolyticus* and that they carried the *pirA* and *pirB* virulence genes. These bacteria showed β-hemolysis with translucent rings, and six serotypes were identified. The regression infection results showed that the lethal rates of the three *V. parahaemolyticus* strains of serotypes O1:KUT and OUT:KUT to *L. vannamei* were significantly higher than those of the other serotypes. The three *V. parahaemolyticus* strains had the same growth characteristics, and they all entered the growth plateau at 4 h after inoculation. In addition, the pathogenicity of the three *V. parahaemolyticus* strains were similar. In the present study, *V. parahaemolyticus* was identified as one of the pathogenic bacteria causing acute hepatopancreatic necrosis of *L. vannamei*, and the O1:KUT and OUT:KUT serotypes may be the main pathogenic strains in Zhuhai. These results provided a foundation for the isolation, identification, and pathogenicity study of acute hepatopancreatic necrosis of *L. vannamei*, providing a reference for the prevention and control direction of *L. vannamei* disease.

INTRODUCTION

Vibrio parahaemolyticus, a gram-negative bacterium belonging to the genus *Vibrio* of the Vibrio family, is a halophilic opportunistic pathogen (Thompson *et al*., 2009). In aquaculture, *V. parahaemolyticus* is a type of pathogenic bacteria that causes great harm by infecting various aquaculture species, such as *Litopenaeus vannamei* (Jiao *et al*., 2020; Muhammad *et al*., 2017), *Penaeus monodon* (Ahmmed et al., 2019; Zhao et al., 2020), *Marsupenaeus japonicus* (Elshopakey *et al*., 2018), flounder (*Paralichthys olivaceus*) (Kim *et al*., 2016), and oysters (*Ostrea gigasthunberg*) (Elmahdi *et al*., 2018). *V. parahaemolyticus* easily proliferates in high temperature, fluctuating water temperature, poor water quality, and poor physical conditions, causing disease in cultured animals (Zhao *et al*., 2020). *V. parahaemolyticus* not only causes immense harm to aquaculture varieties but also causes human food poisoning. After consuming food contaminated by *V. parahaemolyticus*, the bacteria develop a variety of toxins in the human body, leading to diarrhea, vomiting, and other food poisoning symptoms, thus endangering life in serious cases (Wang *et al*., 2021).

V. parahaemolyticus is one of the pathogens that cause acute hepatopancreatic necrosis of *L. vannamei*. Diseased shrimp have various symptoms, such as empty jejunum, empty stomach, white stool, and slow feeding (Tran *et al*.,

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2013), which endanger the health of cultured *L. vannamei*. Research has shown that *V. parahaemolyticus*, which causes acute hepatopancreatic necrosis disease of *L. vannamei*, contains a 70 kb plasmid (pVA1), encoding ORF50 and ORF51, which are homologs of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB (Han *et al*., 2015a). Natural or experimental deletion of PirA and PirB leads to elimination of virulence of *V. parahaemolyticus* (Lee *et al*., 2015; Tinwongger *et al*., 2016). In addition, when *L. vannamei* are fed with natural PirA and PirB proteins extracted from medium or recombinant PirA and PirB from *Escherichia coli*, severe exudation of hepatopancreas tubules occurs in shrimp, presenting typical symptoms of acute hepatopancreatic necrosis (Sirikharin *et al*., 2015; Zhang *et al*., 2021), suggesting that PirA and PirB are the key virulence factors causing acute hepatopancreatic necrosis of *L. vannamei*. Additional studies have shown that pVA1 is a transferable plasmid via the conjugation transfer pathway, which is followed by stable inheritance on the receptor. Importantly, the plasmid may transfer between strains of *V. parahaemolyticus* as well as between different *Vibrio* species as demonstrated in *Vibrio campbellii* (Xuan *et al*., 2017).

In the present study, pathogenic bacteria were isolated from *L. vannamei* farms with outbreaks of acute hepatopancreatic necrosis from 2020 to 2021 in Zhuhai, China. The species of pathogenic bacteria were identified by 16S rRNA sequencing, biochemical characterization, hemolysis tests, and serotype identification, and the virulence genes of the strains were detected. According to the standard of pathogen isolation and identification using Koch's rule, seven strains of *V. parahaemolyticus* were isolated and identified, and they were further tested by regression infection to determine the strains with strong pathogenicity. Subsequently, the growth characteristics of the strains with strong pathogenicity were analyzed. Finally, the virulence differences of pathogenic *V. parahaemolyticus* were compared by infecting healthy *L. vannamei* larvae. The present study demonstrated that *V. parahaemolyticus* is one of the pathogenic bacteria causing acute hepatopancreatic necrosis in *L. vannamei*.

MATERIALS AND METHODS

Experimental materials

The diseased shrimp samples and water samples were collected from *L. vannamei* farms in Zhuhai, China. There were many white strips of feces in the breeding water and feeding platform at ponds with outbreaks of acute hepatopancreatic necrosis. The diseased shrimp had the following symptoms: poor swimming and feeding ability; white and empty intestines; and atrophic and scattered stomachs and hepatopancreases. For the infection experiment, 1–2 cm healthy *L. vannamei* were purchased from Haixingnong Shrimp Seedling Farm in Zhongshan. A 2% proportion of the shrimps was randomly selected and sent to Guangdong Huashite Detection Technology Co., Ltd. for nucleic acid detection of shrimp acute hepatopancreatic necrosis syndrome. The experimental shrimps were cultured in a tank containing 45–55 L of water with 5‰ mariculture solution at a density of 5 shrimps/L. The shrimps were fed every day with three meals of a Brekk. Su microdiet, which is especially designed for post-larvae, and the sewage was aspirated prior to feeding. The water was changed once a day.

Tryptic soy broth, lysogeny broth (LB) agar, and thiosulfate-citrate-bile salts-sucrose (TCBS) agar were purchased from Guangdong Huankai Microbial Technology Co., Ltd. Biochemical identification tubes for *V. parahaemolyticus*, Wagatsuma agar base, rabbit red blood cells, Voges-Proskauer (V-P) kits, sodium chloride polymyxin B broth base (SCPB), polymyxin B, and Vibrio chromogenic medium were purchased from Qingdao Hope Bio-Technology Co., Ltd. *V. parahaemolyticus* diagnostic serum was purchased from Tianjin Biochip Technology Co., Ltd. 2xSuper PCR Mix (with dye) was purchased from BGI.

Isolation and purification of bacteria

For diseased adult shrimp, the body surface was disinfected with 75% alcohol, placed on a sterile disposable petri dish, and transferred to a clean bench. The hepatopancreas tissue was removed using sterilized scissors and forceps, placed in a 1.5-mL sterile centrifuge tube, and homogenized with 500 µL of sterilized phosphate-buffered saline (PBS) buffer using a grinding rod. For sick juvenile shrimp, the body surface was disinfected with 75% alcohol, washed three times with sterilized PBS buffer, and homogenized with 500 µL of sterilized PBS buffer. Then, 100 µL of hepatopancreas or shrimp larvae homogenate was aspirated and spread on TCBS agar plates, which were inverted and cultured at 36±1°C for 24 h. For the collected aquaculture water, 100 µL of sample was plated on TCBS agar plates, which were inverted and cultured at 36±1°C for 24 h. After the colonies grew, the dominant colonies were selected using an inoculation ring, streaked, and inoculated onto a new TCBS plate, which was inverted and cultured at 36±1°C for 24 h. The single colonies on the plates were picked and numbered, and the single colonies were inoculated into 5 mL of tryptic soy broth medium containing 1.5% NaCl and incubated at 36±1°C for 24 h at 180 rpm.

Chromogenic culture of bacteria

To detect the chromogenic characteristics of the

isolated bacteria, 1 mL of the above bacterial solution was added to 9 mL of SCPB broth and incubated for 24 h at 36±1°C for enrichment. One ring of the bacterial solution was taken with a 3-mm inoculation ring, streaked, and inoculated onto Vibrio chromogenic culture plates, which were incubated at 36±1°C for 24 h. The chromogenic plates identified the following strains: *V. parahaemolyticus* showed large blue/green colonies; *Vibrio cholerae* and *Vibrio vulnificus* showed purplish red colonies; *Vibrio alginolyticus* showed light yellow or beige colonies; and most of the other bacteria were inhibited.

Detection of the pirA *and* pirA *virulence genes and* 16S rRNA *gene sequencing analysis*

Single colonies on chromogenic plates were inoculated into 5 mL of tryptone soy broth medium containing 1.5% NaCl and incubated overnight at 36±1°C with shaking at 180 rpm. Then, 1 mL of the bacterial solution was placed into a 1.5-mL centrifuge tube and centrifuged at 12,000 rpm for 1 min. The supernatant was removed, and the precipitate was resuspended using 200 µL of sterilized deionized water. The bacterial suspension was lysed in a metal bath at 95°C for 10 min and centrifuged at 12,000 rpm for 2 min. The supernatant was collected, and the genome quality was determined using a Nanodrop 2000. The extracted genome was stored at –20°C.

For the detection of the *pirA* virulence gene, the following primers were used: *pirA*-F, 5'-ATGAGTAACAATATAAAA-3'; and *pirA*-R, 5'-GTGGTAATAGATTGTACA-3' (Nakamura *et al*., 2019). The polymerase chain reaction (PCR) system contained 12.5 µL of 2xSuper PCR Mix (with dye), 1.5 µL of forward primer, 1.5 µL of reverse primer, and 1.0 µL of DNA template, and the total volume was adjusted to 25 µL with PCR grade water. The PCR thermocycler program was as follows: 98°C for 5 min; 35 cycles of 98°C for 10 s, 48°C for 30 s, and 72°C for 40 s; and 72°C for 10 min. The primers for the detection of the *pirB* virulence gene were as follows: *pirB*-F, 5'-TGATGAAGTGATGGGTGCTC-3'; and *pirB*-R, 5'-TGTAAGCGCCGTTTAACTCA-3' (Han *et al*., 2015b). The PCR system contained 12.5 µL of 2xSuper PCR Mix (with dye), 1.5 µL of forward primer, 1.5 µL of reverse primer, and 1.0 µL of DNA template, and the total volume was adjusted to 25 µL with PCR grade water. The PCR thermocycler program was as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 40 s; and 72°C for 10 min.

The extracted genome was subjected to 16S rRNA gene sequencing analysis using the following primers: 27F, 5'-AGAGTTTGATCCTGGTCAGAACGAACGCT-3'; and 1492R, 5'-TACGGCTACCTTGTTACGACT-TCACCCC-3'. The PCR system contained 25 µL of 2xSuper PCR Mix (with dye), 1.5 µL of forward primer, 1.5 µL of reverse primer, and 2.0 µL of DNA template, and the total volume was adjusted to 50 µL with PCR grade water. The PCR thermocycler program was as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min; and 72°C for 10 min. The PCR products were sent to BGI for sequencing. The phylogenetic tree of the seven *Vibrio* strains was constructed based on 16S rRNA sequencing to analyze phylogeny.

Biochemical characterization

At least three single colonies were selected from each chromogenic culture plate, streaked, and inoculated onto tryptone soy agar plates, which were inverted and incubated for 24 h at 33°C. The purified colonies were inoculated into 3% sodium chloride mannitol, 3% sodium chloride lysine decarboxylase, 3% sodium chloride amino acid decarboxylase control, 3% sodium chloride V-P semisolid, 3% sodium chloride β-D-galactoside (ONPG), sucrose, hydrogen sulfide, and glucose decomposition gas production biochemical tubes for detection according to the manufacturer's instructions. The results were assessed according to the instructions of the biochemical tube. The identification was repeated three times.

Kanagawa test

The isolated *V. parahaemolyticus* strains were tested for the presence of specific hemolysin by the Kanagawa test. The tested strains grown for 18 h on 3% NaCl tryptic soy agar plates were seeded with an inoculation ring onto Wagatsuma agar base plates. The plates were divided into four zones, and three to four bacteria were seeded on each plate. The bacteria were incubated for 20 to 48 h at 36±1°C. A positive result was β-hemolysis with a translucent ring around the colony, and the circle of hemolysis was well demarcated and 2–4 mm in size.

Identification of serotypes

Colonies were streaked onto LB agar plates containing 3% NaCl and incubated for 24 h at 35°C for subsequent assays. For K-antigen detection, 15–20 µL of test serum was added dropwise onto a glass slide. Colonies on the LB agar plates were picked and added to the test serum, and agglutination was observed within 1 min. For O-antigen detection, colonies on LB agar plates were washed off with 3% NaCl solution containing 5% glycerol using a disposable coating rod. After washing three times with 3% NaCl solution containing 5% glycerol, the bacterial solution was sterilized at 121°C for 1 h. The precipitate was collected by centrifugation at 4500 rpm for 15 min, and the precipitate was dissolved with 3% NaCl solution containing 5% glycerol and washed three times. Finally,

the precipitate was resuspended using the same volume of 3% NaCl solution containing 5% glycerol before sterilization. The agglutination phenomenon was observed within 1 min by adding 15 µL of detection serum to the slide followed by adding 5 µL of the sample to be tested.

Regression test

The experimental shrimps were randomly divided into seven experimental groups and one control group, with three replicates in each group and 50 shrimps in each replicate. Two replicates were used to count the number of deaths and calculate the cumulative mortality rate, and the other replicate was used to isolate and identify pathogenic bacteria as well as observe the symptoms of diseased shrimp. Each group of 50 shrimps was kept in a small tank containing 10 L of water for 7 days before the experiment, and the water temperature, salinity, and water quality were continuously monitored.

Single colonies from TCBS plates were picked, inoculated into tryptic soy broth medium containing 1.5% NaCl, and incubated overnight at 33°C at 180 rpm. Overnight cultures were inoculated at a percentage of 2% into 200 mL of tryptic soy broth medium containing 1.5% NaCl, and they were incubated at 33°C for 4 h at 180 rpm. Three tubes containing 1 mL of bacterial solution were aspirated from each strain, and after 10-fold gradient dilution with sterile PBS, 100 µL of the dilution was used to smear TCBS plates for a viable count. The counting method referred to the total number of colonies as described in GB 4789.2-2016 (National Standard for Food Safety Microbiological Inspection of Food). The bacterial solution was centrifuged at 7000 rpm for 15 min, and the precipitate was washed three times with sterile PBS before being resuspended for use. The bacterial solution was diluted to 1.0×10^6 cfu/mL using aquaculture water, and 50 shrimps in each replicate group were immersed in 1 L of $1.0\text{x}10^6$ cfu/mL of bacterial solution for 2 h. After immersion, the total volume was adjusted to 10 L with water. The number of remaining shrimps was counted for 10 days, and the symptoms of infected shrimp were observed for 10 days. The diseased shrimp with obvious symptoms were collected, washed with sterile PBS, homogenized in 500 µL sterilized PBS buffer, and spread on TCBS plates. The dominant colonies were selected and separated. A single colony was selected, placed in a 1.5-mL centrifuge tube containing 50 µL of sterile water, and lysed using a metal bath at 95℃ for 10 min. The *16S rRNA* gene of each colony was amplified as described above, and the PCR products were sent to BGI for sequencing.

Growth curves of the strains

To compare the growth characteristics of V.p.25, V.p.27, and V.p.29, the growth curves were determined. Single colonies from TCBS plates were picked, inoculated into 5 mL of tryptic soy broth containing 1.5% NaCl, and incubated overnight at 33°C at 180 rpm. The bacterial solution was then inoculated into 250 mL of tryptic soy broth containing 1.5% NaCl with 2% inoculum. A 3-mL bacterial solution was taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, and 10 h after inoculation. The optical density value at 600 nm (OD600) of the sample was measured, and each sample was assayed in triplicate. The OD600 values of the bacterial solution were maintained in the range of 0.2 to 0.8, and the samples were measured again after diluting with tryptic soy broth containing 1.5% NaCl when the values exceeded this range. Finally, growth curves were plotted based on the incubation time and OD600 values of the bacterial solution.

Pathogenicity analysis of V. parahaemolyticus

To compare the pathogenicity of V.p.25, V.p.27, and V.p.29, shrimps were challenged with different concentrations of each bacterium. In total, 250 shrimps were used for each strain, which were randomly divided into 5 groups with 50 shrimp in each group, and another 50 shrimps served as the control group, resulting in a total of 800 shrimps. Each group of 50 shrimps was kept in a tank containing 10 L of water for 7 days, and the water temperature, salinity, and water quality were continuously monitored.

V.p.25, V.p.27, and V.p.29 were cultured and counted as above. The bacterial concentration was diluted to 1.0×10^8 cfu/mL, 1.0×10^7 cfu/mL, 1.0×10^6 cfu/mL, 1.0×10^5 cfu/mL, and 1.0×10^4 cfu/mL using 5‰ prepared mariculture solution, and each group of 50 shrimps was immersed in 1 L of bacterial solution with the above concentrations for 2 h. After immersing the shrimps, the total volume was adjusted to 10 L with water. The number of remaining shrimps was counted for 10 days. The median lethal concentration was calculated by Koch's method as described in GB15193.3-2003 (acute toxicity experiments) using the following formulas: $M=XK\text{-}I(\sum p\text{-}0.5)$ and $LD50=\log^{-1}M$. According to the median lethal concentration, the pathogenicity of V.p.25, V.p.27, and V.p.29 was compared.

RESULTS

Isolation and sequencing identification of pathogenic bacteria

From 2020 to 2021, a total of seven dominant *Vibrio* strains (numbered as V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29) were isolated from the aquaculture ponds of *L. vannamei* in Zhuhai, China. The information of seven *Vibrio* strains is shown in Table I. V.p.20 was isolated from the aquaculture water, while V.p.24 and V.p.25 were isolated from the hepatopancreas of adult shrimp. In

addition, V.p.26, V.p.27, V.p.28, and V.p.29 were isolated from homogenized whole juvenile shrimp. All seven *Vibrio* strains appeared green on TCBS plates. The following 16S rRNA gene sequencing results were obtained: V.p.20, V.p.25, and V.p.26 were identified as *V. parahaemolyticus*; V.p.24 was identified as *V. parahaemolyticus* or *Vibrio alginolyticus*; V.p.27 was identified as *Vibrio cyaneus* or *Vibrio alginolyticus*; and V.p.29 was identified as *V. parahaemolyticus* or *Vibrio alginolyticus* (Table I). The phylogenetic tree of the seven *Vibrio* strains based on 16S rRNA sequencing showed that the seven *Vibrio* strains have high homology (Fig. 1). PCR analysis showed that all seven *Vibrio* strains carried the *pirA* and *pirB* virulence genes, which showed a single target band at 283 bp (Fig. 2A) and 392 bp (Fig. 2B), respectively. According to the color on TCBS plates and *16S rRNA* gene sequencing alignment results, these seven strains were predominately identified as *V. parahaemolyticus*.

Chromogenic culture of bacteria

The seven suspected *V. parahaemolyticus* strains (V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29) were tested by Vibrio chromogenic plates. V.p.20, V.p.24, and V.p.28 appeared dark green (Fig. 3A, B, F), and V.p.26 appeared as blue-green (Fig. 3D). In addition, V.p.25, V.p.27, and V.p.29 appeared light green (Fig. 3C, E, G), and the inoculated *Vibrio alginolyticus* was distinctly off-white (Fig. 3H). All seven pathogenic bacteria were consistent with the chromogenic results of *V. parahaemolyticus*, and each plate showed a single colony without miscellaneous bacteria. The results of the three repeated tests were consistent.

Biochemical characterization

Biochemical indicators of V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29, including oxidase, kinetic test, halophilic, mannitol, lysine decarboxylase, V-P test, ONPG, sucrose, glucose, lactose, and hydrogen sulfide, were identified using biochemical identification tubes.

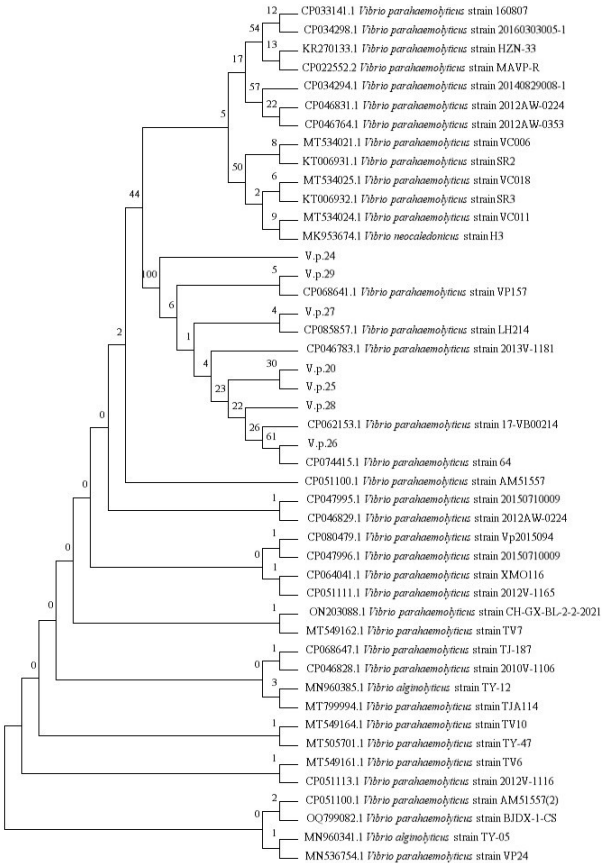

Fig. 1. The phylogenetic tree of the seven isolated *Vibrio* strains was constructed using MEGA 7. Multiple sequences use the muscle alignment algorithm with default parameters, the phylogenetic tree was built use the Neighbor joining method. The Bootstrap method was set to 1000, and other parameters were defaulted.

Table I. Information on pathogen isolation of *Litopenaeus vannamei* in Zhuhai.

Numbered	Virulence genes	Species name	Year	Site of separation	*16S rRNA* gene sequencing
V.p.20	*pirA/pirB*	*Litopenaeus vannamei*	2020	Aquaculture water	*Vibrio. parahaemolyticus*
V.p.24	*pirA/pirB*	*Litopenaeus vannamei*	2020	Hepatopancreas	*Vibrio. parahaemolyticus* or *Vibrio alginolyticus*
V.p.25	*pirA/pirB*	*Litopenaeus vannamei*	2020	Hepatopancreas	*Vibrio. parahaemolyticus*
V.p.26	*pirA/pirB*	*Litopenaeus vannamei*	2021	Juvenile shrimp	*Vibrio. parahaemolyticus*
V.p.27	*pirA/pirB*	*Litopenaeus vannamei*	2021	Juvenile shrimp	*Vibrio cyaneus* or *Vibrio alginolyticus*
V.p.28	*pirA/pirB*	*Litopenaeus vannamei*	2021	Juvenile shrimp	*Vibrio. parahaemolyticus*
V.p.29	*pirA/pirB*	*Litopenaeus vannamei*	2021	Juvenile shrimp	*Vibrio. Parahaemolyticus* or *Vibrio alginolyticus*

Table II. Biochemical characterization of seven *Vibrio parahaemolyticus.*

Index	Reference standard	V.p.20	V.p.24	V.p.25	V.p.26	V.p.27	V.p.28	V.p.29
Oxidase	+	+	+	+	+	+	+	+
Kinetic test	+	+	+	+	+	+	+	+
Halophilic	0% and 10% NaCl showed no or weak growth, 6% and 8% NaCl showed vigorous growth	0% and 10% NaCl showed no growth, 6% and 8% NaCl showed vigorous growth						
Sucrose	−	−	−	−	−	−	−	−
ONPG	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Glucose decomposition gas production	−	−	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	−	−
Hydrogen sulfide	−	−	−	−	−	−	−	−
Lysine decarboxylase	+	+	+	+	+	+	+	+
V-P test	−	−	−	−	−	−	−	−
β-D-galactoside	−	−	−	−	+	−	−	−

Notes: + represents positive, - represents negative. The reference basis is GB4789.7-2013 (National Standard for Food Microbiological Examination of *Vibrio. parahaemolyticus*).

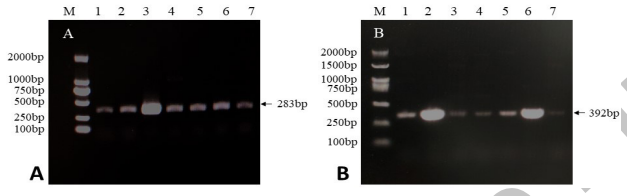

Fig. 2. PCR amplification results of the *pirA* and *pirB* virulence genes of the seven pathogenic bacteria. A, The amplification result of the *pirA* virulence gene. Lane M represents the DNA electrophoresis marker, and lanes 1-7 are V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29, respectively. B, The amplification result of *pirB* virulence gene. Lane M represents the DNA electrophoresis marker, and lanes 1-7 are V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29, respectively.

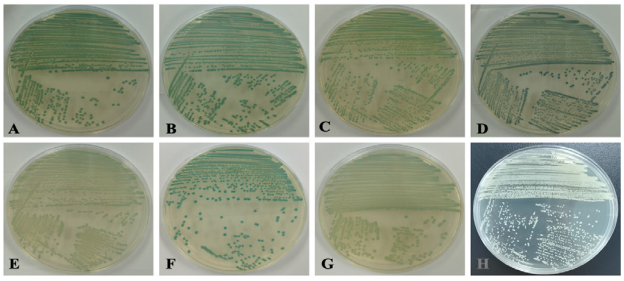

Fig. 3. Chromogenic culture of the strains. A-G represent V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29, respectively. The seven *Vibrio* strains presented green or blue-green color on the plates. H represents *Vibrio alginolyticus* and presented an off-white color on the plate.

The results of each index were compared with the indicators of *V. parahaemolyticus* described in GB4789.7-2013 (National Standard for Food Microbiological Examination of *V. parahaemolyticus*). Except for the abnormal and positive ONPG index of V.p.26, the other strains and indexes were consistent with the biochemical indexes of *V. parahaemolyticus* in the standard (Table II). The results of the two repeated tests of the above biochemical indicators were consistent.

Results of the Kanagawa test

The fresh V.p.20, V.p.24, V.p.25, V.p.26, V.p.27, V.p.28, and V.p.29 strains cultured overnight on TCBS plates were seeded on Wagatsuma agar base plates and cultured for 48 h at 35°C. After being cultured for 24 h, the peripheral colors of the colonies of the seven strains became lighter, and hemolytic rings gradually appeared (Fig. 4A, B). At 48 h of culture, obvious hemolytic rings were observed around all seven strains. The hemolytic rings were transparent and well-demarcated (Fig. 4C, D), consistent with typical β-hemolytic rings of *V. parahaemolyticus*. The size of β-hemolytic rings was 1–3 mm (Fig. 4E). These results suggested that all seven strains carrying the *pirA* and *pirB* virulence genes were hemolytic and pathogenic strains.

Serotypes of the seven V. parahaemolyticus *strains*

We next evaluated the serotypes of the seven *V. parahaemolyticus* strains. Compared to the control lacking a testing sample, the V.p.20, V.p.24, V.p.26, and V.p.28

Table III. Results of serotype detection of seven *Vibrio parahaemolyticus*.

Strains	K antigen multivalent antiserum	K antigen monovalent antiserum	O antigen multivalent antiserum	O antigen monovalent antiserum	Serotype
V.p.20	Multivalent antiserum 4	K25	Agglutination	O1	O1: K25
V.p.24	Multivalent antiserum 5	K33		O1	O1: K33
V.p.25	No agglutination	–		No agglutination	OUT: KUT
V.p.26	Multivalent antiserum 5	K36		O1	O1: K36
V.p.27	No agglutination	–		O1	O1: KUT
V.p.28	Multivalent antiserum 9	K68		O1	O1: K68
V.p.29	No agglutination	–		O1	O1: KUT

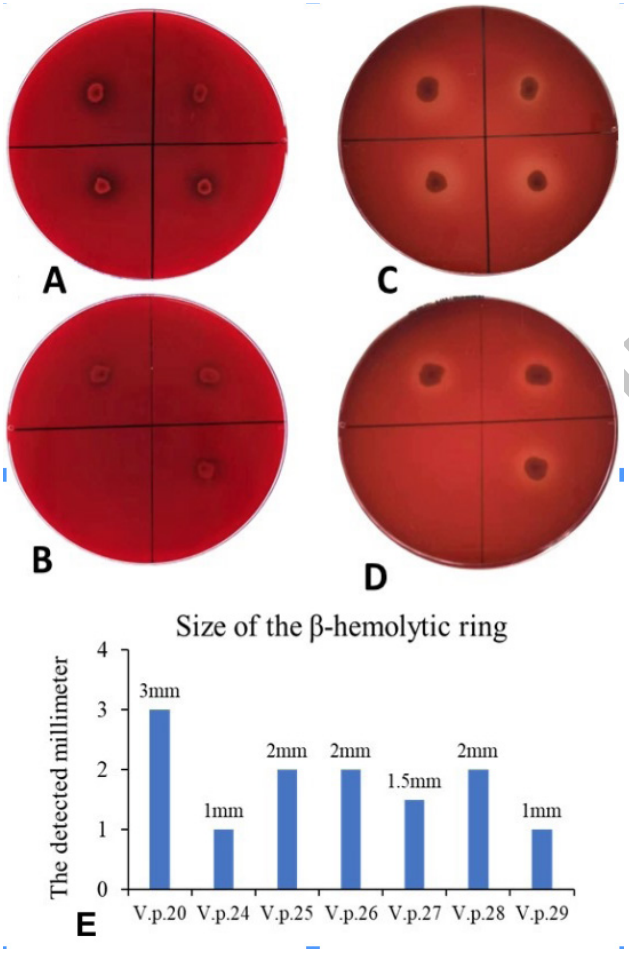

Fig. 4. Hemolytic detection of the strains. A and B, The seven *Vibrio* strains were inoculated on Wagatsuma agar base plates for 24 h. C and D, The seven *Vibrio* strains were inoculated on Wagatsuma agar base plates for 48 h, all seven strains were hemolytic as obvious β-hemolytic rings appeared on the Wagatsuma agar base plates. E: Size of the seven strains' β-hemolytic rings at 48h.

strains resulted in obvious agglutination particles after reaction with the test serum for 1 min. The following serotypes were identified: the serotype of V.p.20 was O1:K25; the serotype of V.p.24 was O1:K33; the serotype of V.p.26 was O1:K36; and the serotype of V.p.28 was O1:K68. The O antigen multivalent antiserum of V.p.25 had agglutination particles, but the monovalent antiserum did not show obvious agglutination within 1 min. Because the K antigen multivalent serum did not detect agglutination, the serotype of V.p.25 was OUT:KUT (OUT represents O untypeable and KUT represents K untypeable). V.p.27 and V.p.29 showed agglutination at approximately 1 min after reaction with monovalent O1 sera, and agglutination was not detected using the K antigen multivalent serum. Thus, the serotype of V.p.27 and V.p.29 was O1:KUT. The results of serotype testing are summarized in Table III, and the serotype identification was consistent after two repeated testing.

Regression test

A 1.0×10^6 cfu/mL bacterial solution was used to infect 1–2 cm healthy *L. vannamei*. During the regression infection test, the water temperature was 22–24°C, the salinity was 4–5‰, the ammonia nitrogen level was 0.2–0.6 mg/L, the nitrite level was 0.001–0.05 mg/L, and the pH was stable at 8. The water temperature, water quality, and salinity during the experiment were within the suitable growth range for shrimp seedlings. On day 2 postinfection, morbidity and death began to appear in each group. On day 3 postinfection, the death number increased significantly, while on day 5 postinfection, the death number increased continuously. Mortality on day 5 was 49% for V.p.20, 39% for V.p.24, 77% for V.p.25, 37% for V.p.26, 65% for V.p.27, 41% for V.p.28, 80% for V.p.29, and 7% for the control group. On day 8 postinfection, the mortality rates gradually stabilized in each group, and the cumulative mortality rates of V.p.25, V.p.27, and V.p.29 were significantly higher than that of the control and other

experimental groups (Fig. 5). The following symptoms were observed in the diseased shrimp: The intestinal tract of the diseased shrimp was transparent and free of contents; and the tails of some shrimp showed black carbonization; the stomach and liver were yellow; and the hepatopancreas was diffuse and red-brown with black spots on the body surface. The dominant bacteria isolated from the infected shrimp were identified as *V. parahaemolyticus*.

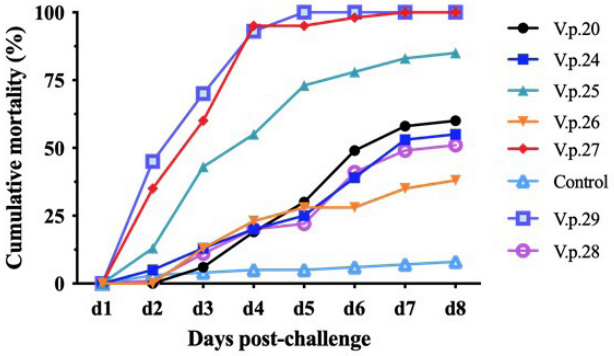

Fig. 5. Cumulative mortality curve of *V. parahaemolyticus* regression infection. The abscissa represents the days post immersion challenge, and the ordinate represents the cumulative mortality. The mortality rates of V.p.25, V.p.27, and V.p.29 were significantly higher than those of V.p.20, V.p.24, V.p.26, and V.p.28. The mortality rates of V.p.20, V.p.24, V.p.26, and V.p.28 were higher than those of the control group during the regression infection experiment.

Analysis of growth characteristics

The growth curves of V.p.25, V.p.27, and V.p.29 indicated that there was a lag period of approximately 1 h after inoculation followed by a rapid growth phase. According to the growth curves, the three strains of *V. parahaemolyticus* were in the logarithmic growth phase from 1 to 4 h after inoculation, and the growth rate slowed down and entered the plateau phase after 4 h (Fig. 6).

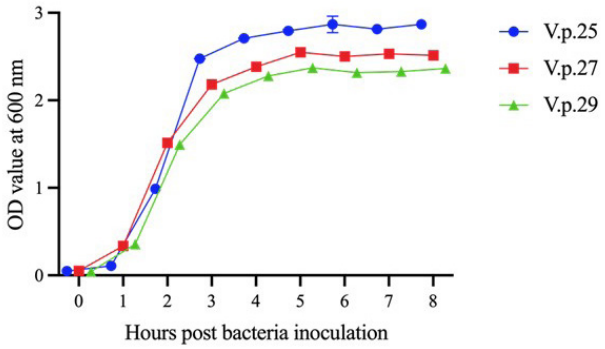

Fig. 6. Growth curves of V.p.25, V.p.27, and V.p.29. The three strains of *V. parahaemolyticus* reached the growth plateau at 4 h after inoculation. Error bars represent SD of three independent detected OD600 values of one sample.

Comparison of pathogenicity of strains

During the immersion infection experiment, the water temperature was 25–26°C, the salinity was 4%–5%, the ammonia nitrogen level was 0.2–0.4 mg/L, the nitrite level was 0.001–0.05 mg/L, the pH was stable at 8, and the water quality index was within the suitable growth range of shrimp seedlings. On day 2 postinfection, the shrimp in the high concentration immersion groups of 1.0×10^8 cfu/mL and 1.0×10^7 cfu/mL appeared sick and dead, and the diseased shrimp had empty stomachs, empty jejunums, and diffuse hepatopancreases, which were consistent with the typical symptoms of *V. parahaemolyticus* infection. Higher concentrations of immersion resulted in faster morbidity and mortality of shrimp. The cumulative mortality of the 1.0×10^8 cfu/mL and 1.0×10^7 cfu/mL immersion groups reached 100% and 80% on day 3 postinfection, respectively, and both reached 100% on day 5 postinfection. The mortality of each immersion group was stabilized after day 5 postinfection (Fig. 7). The median lethal concentrations of V.p.25, V.p.27, and V.p.29 were 1.10×10^4 cfu/mL, 1.15×10^4 cfu/mL, and 1.10×10^4 cfu/mL, respectively.

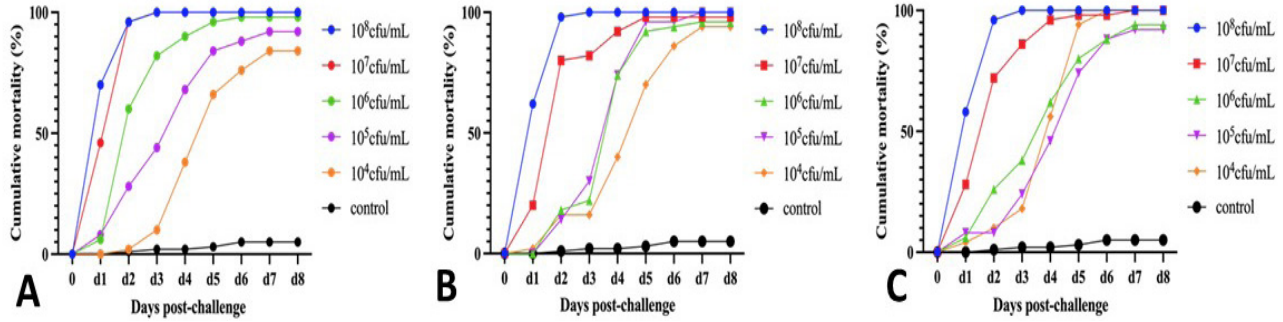

Fig. 7. Pathogenicity evaluation of V.p.25, V.p.27, and V.p.29. A, Cumulative mortality curve for V.p.25. B, Cumulative mortality curve for V.p.27. C, Cumulative mortality curve for V.p.29.

DISCUSSION

Pathogenic bacteria were isolated from the diseased ponds of *L. vannamei* farms from 2020 to 2021 in Zhuhai, China, and the dominant bacteria were all *V. parahaemolyticus*, indicating that *V. parahaemolyticus* may be one of the main pathogenic *Vibrio* species of shrimp in Zhuhai. Thus, it is necessary to continuously monitor the content changes of *V. parahaemolyticus* in water and shrimp during the culture of *L. vannamei*.

TCBS agar medium is often used for the isolation and identification of *Vibrio*. *Vibrio* generally shows blue-green, yellow, and black on this agar plate. The color of different *Vibrio* species is mainly determined by the pH of the plate (Pfeffer and Oliver, 2003). Some *Vibrio* species ferment sucrose in the medium to produce lactic acid, resulting in a decrease in pH, which makes the colony appear yellow. Some *Vibrio* species may not be able to ferment sucrose to produce acid, resulting in the colonies and their surroundings to appear the color of the plate itself. Some strains can produce hydrogen sulfide, which reacts with iron ions in the medium to produce iron sulfide, resulting in black colonies (Kobayashi *et al*., 1963; Nicholls *et al*., 1976). All seven *Vibrio* strains isolated in the present study showed a blue-green color on TCBS plates, and 16S rRNA sequencing showed that the strains were *V. parahaemolyticus*, *Vibrio cyaneus*, or *Vibrio alginolyticus*. Because *Vibrio alginolyticus* appears yellow on TCBS plates, the seven strains were either *V. parahaemolyticus* or *Vibrio cyaneus*. Furthermore, the seven strains appeared green on the chromogenic plate, which suggested that the isolated strains may be *V. parahaemolyticus*.

To further identify the species of the seven *Vibrio* strains, their biochemical characteristics were identified (Ciufecu *et al*., 1976; Xie *et al*., 2021). Except for the ONPG positive test for V.p.26, all other strains and indicators met the detection standards for *V. parahaemolyticus*. The principle of the biochemical detection of ONPG is that some bacteria have galactosidase, which hydrolyzes o-nitrophenol β-D-galactoside, and release yellow o-nitrophenol (Buelow, 1964; Ladero *et al*., 2001). The presence or absence of β-galactosidase is determined by a yellow reaction per unit time. In the present study, *16S rRNA* sequencing showed that V.p.26 was *V. parahaemolyticus*, suggesting that there was a mutation in the galactosidase gene in the V.p.26 strain, which led to the ONPG positive test result. However, this speculation needs to be confirmed by further gene sequencing and additional studies. Moreover, it is necessary to follow up with related studies on the abnormal ONPG index of *V. parahaemolyticus* and monitor the changes in pathogenicity, infectivity, and prevalence of V.p.26 in shrimp.

Hemolysin is the extracellular product of *V. parahaemolyticus*, and it is also an important virulence factor of *V. parahaemolyticus*. The direct hemolytic factors of *V. parahaemolyticus* are thermostable direct hemolysin and thermostable direct hemolysin-related hemolysin (Okuda *et al*., 1997; Robert-Pillot *et al*., 2004). In addition, there are thermolabile hemolysin (Vazquez-Morado *et al*., 2021). Thermostable direct hemolysin has cytotoxicity, enterotoxicity, and erythrocyte lytic activity, and it does not lose its biological activity even when heated at 100°C for 10 min. Thermostable direct hemolysin produces a specific β-hemolysis phenomenon on Wagatsuma agar base plates, namely the Kanagawa phenomenon (Miyamoto *et al*., 1969; Wagatsuma, 1968). Thermostable direct hemolysin-related hemolysin has intestinal toxicity and is sensitive to heat, and it loses its activity after 10 min above 60°C. Thermostable direct hemolysin-related hemolysin does not produce hemolysis circles on Wagatsuma agar base plates, but it does produce obvious hemolysis circles on ordinary blood plates (Kishishita, 1992). In the present study, all seven strains of *V. parahaemolyticus* had positive Kanagawa tests and formed obvious β-hemolytic rings on the Wagatsuma agar base plates, indicating that the seven strains of *V. parahaemolyticus* contained thermostable direct hemolysin and had certain pathogenicity to humans (Miyamoto *et al*., 1969).

The seven strains of *V. parahaemolyticus* were identified as six serotypes. Among these serotypes, O1:K25 and O1:KUT are the main serotypes that have caused multiple outbreaks of enteritis after 1996 (Li *et al*., 2018; Preeprem *et al*., 2019). O1:K36 was reported in foodborne *V. parahaemolyticus* isolates in Southeastern China from 2009 to 2017 (Chen *et al*., 2016, 2020; Li *et al*., 2015). O1:K68 is consistent with the serotype of the clinically isolated *V. parahaemolyticus* strain BAA-241 in the American Type Culture Collection (Ramamurthy *et al*., 2000). The pathogenicity to humans of the above *V. parahaemolyticus* serotypes isolated from *L. vannamei* was uncertain, but it is necessary to take protective measures in breeding production. In the present study, we found that the three *V. parahaemolyticus* strains with serotype O1:KUT or OUT:KUT were significantly more virulent to 1 cm *L. vannamei* than the other serotypes; 100% mortality was achieved after the immersion challenge after 2 to 3 days, and the median lethal concentrations of the two serotypes were almost the same, indicating that the pathogenicity of the three strains were similar. *V. parahaemolyticus* with serotype O1:KUT has been detected in *L. vannamei* and *Penaeus nipponense*, and it shows a strong ability to acutely infect animals (Jia, 2017). Moreover, the V.p.26 strain with the abnormal ONPG biochemical identification index (serotype O1:K36) did not show strong pathogenicity or

mortality. These results suggested that *V. parahaemolyticus* with serotype O1:KUT or OUT:KUT might be the main epidemic strains of *L. vannamei* in Zhuhai, China. Thus, it is necessary to continuously monitor the serotypes of *V. parahaemolyticus* in *L. vannamei* culture in Zhuhai.

CONCLUSION

In conclusion, we isolated seven bacterial strains from *L. vannamei* with acute hepatopancreatic necrosis disease in Zhuhai, China. The bacterial isolates were identified as *V. parahaemolyticus* based on morphological, biochemical characteristics, hemolysis, and molecular identification with 16S rRNA sequencing. The seven *V. parahaemolyticus* strains carried the *pirA* and *pirB* virulence genes, which cause acute hepatopancreatic necrosis disease, and they were identified as six serotypes. Three *V. parahaemolyticus* strains with serotype O1:KUT or OUT:KUT showed higher pathogenicity than the other serotypes, which had similar median lethal concentrations and showed the same growth characteristics. We suggested that *V. parahaemolyticus* strains with serotype O1:KUT or OUT:KUT might be the main epidemic strains that cause acute hepatopancreatic necrosis disease of cultured *L. vannamei* in Zhuhai, China. The present study laid a foundation for the isolation, identification, and pathogenicity study of *V. parahaemolyticus* in *L. vannamei*, thereby providing a reference for the prevention and control of *Vibrio* diseases.

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IRB approval

This study was approved by the IRB at Guangdong Haid Group. The operation and management of animal experiments carried out in the study are in line with the Articles of Association of Experimental Animal Ethics Committee of Guangdong Haid Group (permit number: HD-221110-1).

Ethical statement

All animial experiments were performed under anesthesia, and every effort was made to minimize suffering.

Statement of conflict of interest

The authors have declared no conflict of interest.

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