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Pathogenicity and antibiotic resistance analysis of *Vibrio* species found in coastal water at mainly beach of Shenzhen, China

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Offshore industries and resources are the links between humans and the marine ecosystem. Several risk factors are associated with beach activities. The risk of *Vibrio* infection at bathing beaches is often overlooked as there are virtually no regulations regarding them. In this study, we investigated the current prevalence of pathogenic *Vibrio* spp. on the main beaches of Shenzhen. The risk indicator levels of pathogenic *Vibrio* species obtained were analyzed using 17 virulence genes mainly associated with *Vibrio* species and are responsible for several ailments and infections. A total of 60 *Vibrio* strains were isolated and identified by morphological observation, evolutionary tree alignment, and biochemical testing. There was a high abundance of *Vibrio* in the seawater. Also, a positive correlation was observed between the presence of virulence genes and the exhibition of high pathogenicity after artificially infecting fish with some of the virulent *Vibrio* species. In the infection experiment, it was observed that all the zebrafish infected with MEDF7 (*Vibrio alginolyticus*) and JSW-YELLOW (*Vibrio harveyi*) died a day after injection, with varying degrees of abdominal enlargement and skin ulceration. The mortality rates for strains with medium risk and low risk were 65% and 45%, respectively. Lethal dose 50 (LD₅₀) values were 5.67 × 10⁴ CFU/fish, 3.72 × 10⁵ CFU/fish, and 4.31 × 10⁵ CFU/fish for the high-risk, medium-risk, and low-risk strains of zebrafish, respectively. The results of the antibiotic sensitivity test showed that all the six experimental strains, except JSW-YELLOW, were resistant to doxycycline and neopenicillin. In summary, our study first identified and evaluated the pathogenicity of *Vibrio* in the Shenzhen beach baths, serving as a scientific benchmark for *Vibrio* risk prevention and control as well as guidance for *Vibrio* diagnosis through virulence factor detection and risk classification.

KEYWORDS

pathogenicity, virulence gene, antibiotic resistance, risk classification, Shenzhen

Introduction

Bathing beaches are of economic, social, and cultural importance. They provide many jobs for coastal residents and the agriculture industry. China's coastal tourism market is rapidly expanding, with revenues predicted to exceed RMB 180.86 billion by 2019 (Yu et al., 2020). According to a study conducted by Choe in 2021, 56% of Chinese travelers plan to travel within China in 2020, with beaches being the most popular destination (Choe, 2021). Shenzhen is accelerating its development as a global center of the ocean, with millions of tourists expected every year.

Human infection with vibriosis occurs frequently (Vugia et al., 2009). Pathogenic marine microorganisms come into close contact with human beings through bathing beaches, water sports bases, mariculture animals, marine aquatic products, and estuaries, which cause major public health incidents (Zulkifli et al., 2009). A new study shows that even frequently monitored beaches with very low fecal index concentrations are at risk of swimming-related illness (Tomenchok et al., 2020). Several species of Vibrionaceae, including *Vibrio harveyi*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus*, are mostly associated with health problems in marine animals (Fahmy et al., 2021). Groups at higher risk of infection include young children, people over the age of 64, and people with chronic illnesses or weak immune systems (Tomenchok et al., 2020). According to the United States Centers for Disease Control and Prevention, the incidence of *Vibrio* infections has increased dramatically since 2010 (Vugia et al., 2009). Common symptoms of vibriosis include chills, nausea, diarrhea, and fever. In severe cases of infection, it can cause conjunctivitis, otitis media, etc. (Eiler et al., 2006; Li et al., 2005; Hsieh et al., 2007).

The pathogenesis of *Vibrio* has been a hot topic of research, and virulence factors encoded by virulence genes are the main factors in the pathogenicity of *Vibrio* (Schroeder et al., 2017). Five major virulence factors have been identified: capsular polysaccharide, adhesion factor, cytotoxin, lipopolysaccharide, and flagellum (Wu et al., 2008; Daniela et al., 2013). *Vibrio* infects and damages the host through these virulence factors, allowing pathogens to attach, enter, establish and multiply while avoiding the host's immune defense system (Darshane Ruwandeepika et al., 2012). In addition, *Vibrio* can acquire atypical virulence genes, which increase pathogenicity (Deng et al., 2020). It has been found that these atypical virulence genes can be acquired by horizontal gene transfer (HGT) from the environment and/or other bacteria (Soucy et al., 2015). Global climate change, antibiotics, heavy metals, and environmental eutrophication can increase the pathogenicity and resistance of pathogens by affecting horizontal gene transfer (HGT) (Sun et al., 2015; Hu et al., 2016). Shenzhen belongs to the subtropical monsoon climate, with a long summer and short winter, a mild climate, sufficient sunshine, and abundant rainfall. The average

annual temperature is 23.3°C, which is very favorable for the growth of *Vibrio* (Ferchichi et al., 2020). According to the investigation, vibriosis occurs worldwide, usually in summer and autumn, with a high incidence at 25–32°C and a rapid epidemic over 28°C (Jin et al., 2004). Multiple regional outbreaks of vibriosis in Alaska (McLaughlin et al., 2005), Denmark (Dalsgaard et al., 1999), and Spain (Martinez-Urtaza et al., 2005) support the hypothesis that vibriosis epidemics are influenced by rising global temperatures. Furthermore, some studies have shown that Shenzhen's coastal waters have a certain degree of heavy metal pollution (Huang et al., 2017). Therefore, investigating and studying *Vibrio* virulence genes (especially atypical virulence genes) is very meaningful for *Vibrio* control.

Antibiotics are widely used to combat bacterial infections in aquaculture. It was estimated that in 2013, 54,000 tons of antibiotics were excreted into the environment by humans in China, an amount that has gradually increased over the following years (Q.-Q. Zhang et al., 2015). Antibiotics can be transmitted directly to humans through the consumption of contaminated food or water (Duarte et al., 2022). In addition, repeated exposure to antibiotics by pathogenic microorganisms has led to a decrease or even loss of susceptibility to the drugs, resulting in reduced efficacy or even failure of the drugs against drug-resistant bacteria (Letchumanan et al., 2015). Antibiotic resistance has become one of the most serious threats to global aquaculture and public health, and the level of resistance of *Vibrio* to antibiotics is on the increase (Larsson & Flach, 2021). As a result, determining the antibiotic resistance of pathogens in a specific region is essential for developing local antibiotic use policies and programs.

For the first time, we investigated the abundance of *Vibrio* in Shenzhen sea bathing beaches as well as the distribution of virulence genes in 60 strains of *Vibrio*. This study is important for preventing and controlling vibriosis in aquaculture and protecting public health.

Materials and methods

Ethics statement

All animal studies were carried out according to the Guangdong Ocean University's Animal Ethics Committee Guide for the Care and Use of Laboratory Animals. Guangdong Ocean University's Animal Ethics Committee approved the animal research (Zhanjiang, China).

Study area

The study area was the main bathing beach in Shenzhen, China. Shenzhen is located at longitude 113 degrees 46' to 114 degrees 37' east and latitude of 22 degrees 27' to 22 degrees 52'

north, south of Guangdong, and on the east bank of the Pearl River Estuary, with Daya Bay and Dapeng Bay in the east, the Pearl River Estuary and Lingdingyang in the west (Figure 1).

Bacterial isolates

The experimental samples were collected from Shenzhen's main 8 bathing beaches (Table 3). The samples collected are coastal water. Three replicates were set for each sampling site, and a total of 24 samples were collected and obtained. In this experiment, 200 μ L of seawater (each sample) was inoculated on a TCBS solid plate for isolation and cultured at 28°C for 18 h. The dominant colonies were selected with repeated scribing separation to obtain the pure cultured strains. The same volume of 30% glycerol was added for mixed preservation and stored at -80°C.

DNA extraction

Bacterial DNA was extracted using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver. 3.0 from Dalian Bao Biological Co. and stored at -20°C.

16S rRNA and gram stain

The 16S rRNA primers used are shown in Table 1. The PCR was performed in 25 μ L reactions containing 12.0 μ L of Premix Taq (TaKaRa Taq Version 2.0 plus dye) (Takara, Japan), 1.0 μ L

of each primer (10 μ M), 1.0 μ L of template DNA (20 ng/L), and 10.0 μ L of sterilized water. The amplification was performed in an automatic thermal cycler (Bio-Rad, USA) as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s/kb; and final extension at 72°C for 10 min. The gram stain was used using the HB8278 Gram Stain Kit (Hopebiol Co., Ltd., Qingdao, China).

Biochemical tests

Biochemical tests were performed using a GYZ-9V (070070) biochemical identification kit for *Vibrio* bacteria (Huankai Microorganism Reagent Co., Ltd., China). Presumptive tests included glucose, arabinose, mannose, sucrose, lysine, arginine dihydrolase, peptone water, and the Voges-Proskauer test. Growth conditions with incubation at 25°C for 24–48 hours with 0%, 3%, 6%, 8%, and 10% NaCl (Table 2). The pathogens were analyzed and identified by referring to the “Manual of Common Bacterial System Identification” and the “Manual of Berger's Bacterial Identification” (ninth edition) (Floyd et al., 2020).

Detection of virulence genes

To evaluate the distribution of virulence genes in *Vibrio* species, 8 typical *V. harveyi* virulence genes (*vhpA*, *vhhA*, *vhhB*, *luxR*, *chiA*, *vhml*, *vhs*, and *toxRVh*), the *V. vulnificus* hemolysin gene (*vvh*), the *V. parahaemolyticus* virulence genes (*trh*), the *V. anguillarum* virulence genes (*flaB*, *flaC*), the *V. alginolyticus*

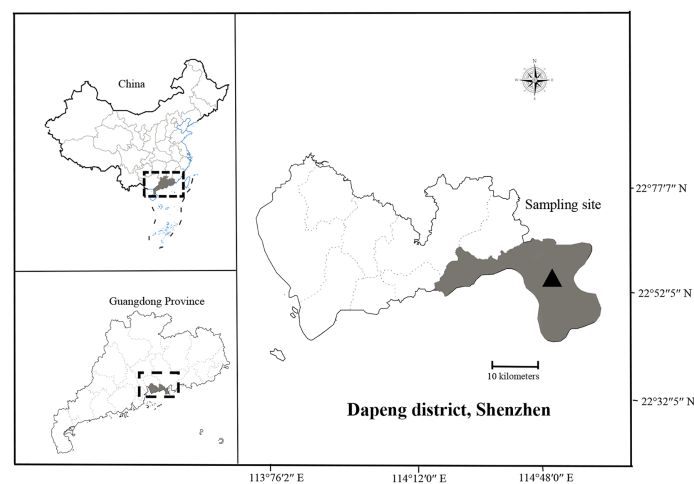


FIGURE 1
Map showing sampling location at Shenzhen, China.

TABLE 1 List of primers used for amplification of 16S rRNA gene and virulent genes.

| Gene | Primer Sequence(5'-3') | Product Size(bp) | Reference |
|-------------------|-----------------------------|------------------|-------------------------------------------|
| 16S rRNA-F: | AGAGTTTGATCCTGGCTCAG | 1600 | (Ruwandeeepika and Defoirdt et al., 2010) |
| 16S rRNA-R: | GGTTACCTTGTACGACTT | 1600 | |
| luxR-F | ATGGACTCAATTGCAAAGAG | 618 | (Ruwandeeepika and Defoirdt et al., 2010) |
| luxR-R | TTAGTGATGTTCACGGTTGT | 618 | |
| toxRVh-F | CGACAACCAAAATACGGAA | 131 | (Ruwandeeepika and Defoirdt et al., 2010) |
| toxRVh-R | AGAGCAATTTGCTGAAGCTA | 131 | |
| serine protease-F | TGCACGACCAGTTGCTTTAG | 232 | (Ruwandeeepika and Defoirdt et al., 2010) |
| serine protease-R | AAGTGGTCGTCAGCAAATCC | 232 | |
| vhml-F | TGATCATGCCGATGGTCTTA | 180 | (Ruwandeeepika and Defoirdt et al., 2010) |
| vhml-R | GGTCAAAATCCACACATCC | 180 | |
| chiA-F | GGAAGATGGCGTGATTGACT | 232 | (Ruwandeeepika and Defoirdt et al., 2010) |
| chiA-R | GGCATCAATTTCCCAAGAGA | 232 | |
| vhpA-F | CTGAACGACGCCATTATTT | 201 | (Ruwandeeepika and Defoirdt et al., 2010) |
| vhpA-R | CGCTGACACATCAAGGCTAA | 201 | |
| vhs-F | CCGGAAGGTTACAGCATTGT | 185 | (Ruwandeeepika and Defoirdt et al., 2010) |
| vhs-R | GCGTCGGTCTTCTCAAGTTC | 185 | |
| vhpA-F | CTGAACGACGCCATTATTT | 201 | (Ruwandeeepika and Defoirdt et al., 2010) |
| vhpA-R | CGCTGACACATCAAGGCTAA | 201 | |
| flaB -R | AACGTATCAGCGATGACC | 928 | (Bai and Pang et al., 2008) |
| flaB -F | TTGAAACGGTTCTGGAAT | 928 | |
| toxR-F | GAAGCAGCACTCACCGAT | 390 | (Conejero and Hedreyda, 2003) |
| toxR-R | GAAGCAGCACTCACCGAT | 390 | |
| vhhA-F | ATCATGAATAAAAATTATTACGTTACT | 1324 | (Conejero and Hedreyda, 2004) |
| vhhA-R | GAAAGGATGGTTTGACAAT | 1324 | |
| vhhB-F | TCAGTGCCTCTCAAGTAAGA | 216 | (Conejero and Hedreyda, 2004) |
| vhhB-R | GCTTGATAACACTTTGCGCT | 216 | |
| zot-R | CACTGGGCGAGAAAGGAC | 737 | (Rivera and Chun et al., 2001) |
| zot-F | CACTGGGCGAGAAAGGAC | 737 | |
| tcpA-R | ACCGTGGTCTAGGTAATT | 617 | Genbank AF315787 |
| tcpA -F | CAACGCCGAATGGAGCAG | 617 | |
| flaC-F | AAATCATTCCAAATCGGTGC | 580 | (Bai and Pang et al., 2008) |
| flaC-R | TCTTTGATTCCGGCTCTTA | 580 | |
| vvh-F | GCTATTTACCCGCCCTCAC | 222 | (Lee et al., 2002) |
| vvh-R | CCGCAGAGCCGTAACCGAA | 222 | |
| toxS -R | CCACTGGCGGACAAAATAACC | 640 | (Sechi and Duprè et al., 2000) |
| toxS -F | AACAGTACCGTAGAACCGTGA | 640 | |
| trh-R | CATTTCCGCTCATATGC | 250 | (Tada et al., 1992) |
| trh-F | GGCTCAAAATGGTTAAGCG | 250 | |

hemolysin gene *serine protease*, and 5 *V. cholera* typical virulence genes (*toxR*, *toxS*, *tcpA*, *ctxA*, and *zot*) were analyzed with PCR. The PCR was performed in 25 μ L reactions containing 12.0 μ L of Premix Taq (TaKaRa Taq Version 2.0 plus dye) (Takara, Japan), 1.0 μ L of each primer (10 μ M), 1.0 μ L of template DNA (20 ng/L), and 10.0 μ L of sterilized water. The amplification was performed in an automatic thermal cycler (Bio-Rad, USA) as follows: initial denaturation at 95 $^{\circ}$ C for 5 min; 33 cycles of denaturation at 95 $^{\circ}$ C for 30 s, annealing at the annealing temperature for 30 s, and extension at 72 $^{\circ}$ C for 60 s/kb; and

final extension at 72 $^{\circ}$ C for 10 min. The PCR amplification products were assessed by 1.0% agarose gel electrophoresis. Following electrophoresis, gels were photographed under UV illumination.

Pathogenicity test

A pathogenicity test was performed according to Saleh and Hela (Saleh et al., 2021) for a single randomly selected isolate

TABLE 2 The biochemical profile of six strain of *Vibrio*.

| Bacteria | Reaction | Oxidase Test | V-P Experiment | Mannitol | β -galactoside | Lysine | Amino Acid Decarboxylation Control | Saccharose | Growth in NaCl(%) |
|---------------------------|----------|--------------|----------------|----------|----------------------|--------|------------------------------------|------------|-------------------|
| | | | | | | | | | 0 6 8 10 |
| <i>MEDF7</i> | | + | + | + | - | + | - | + | - + + + |
| <i>V. alginolyticus</i> | | | | | | | | | |
| <i>JSW-YELLOW</i> | | - | + | - | - | - | - | - | - + - - |
| <i>V. harveyi</i> | | | | | | | | | |
| <i>DY3</i> | | + | + | + | + | - | - | - | - + + - |
| <i>V. coralliilyticus</i> | | | | | | | | | |
| <i>DY1</i> | | + | + | + | - | + | - | + | - + + + |
| <i>V. alginolyticus</i> | | | | | | | | | |
| <i>XY11</i> | | + | + | + | + | + | - | - | - + + + |
| <i>V. campbellii</i> | | | | | | | | | |
| <i>JSW-GREEN</i> | | + | + | + | + | + | - | - | - + + + |
| <i>V. campbellii</i> | | | | | | | | | |

+: positive reaction; -: negative reaction.

from each recovered bacterial species to ensure the virulence of *Danio rerio*. Three hundred and twenty-two fish were used for pathogenicity testing. Groups were randomly divided into 10 fish in each group. The experimental group was intramuscularly injected with 10 μ L bacterial solution per tail (2×10^8 CFU/fish). The control group was injected with 1 \times PBS in the same way. The mortality of fish within 7 days was recorded until the death was stable. The median lethal dose was calculated using the Finney method (Finney, 1985). Feeding was restricted for 24 hours before infection and resumed at 12 hours post-infection. All fish groups were kept under observation for 7 days for abnormal clinical signs, postmortem lesions, and mortality rates.

Clinical examination

Infected fish were examined to determine any external abnormalities. The challenged fish were observed twice daily throughout the experiment period according to the method described by Austin and Austin (Austin & Austin, 2016).

Histopathological investigation

Infected fish were fixed in tissue fixative fluid. Samples were sent to Seville Biotechnology Co., Ltd. for case section observation. The histopathological examination was performed as described by Suvarna and Layton (Suvarna et al., 2019).

Antimicrobial resistance testing

Two strains were selected from three groups of high, medium, and low-risk of virulence gene distribution for antibiotic resistance testing, respectively. Strains *MEDF7*, *JCW-*

YELLOW, *DY1*, *DY3*, *JCW-Green*, and *XY11* were investigated for doxycycline (DOX, 30 μ g/disk), chloramphenicol (CHL, 30 μ g/disk), ciprofloxacin (CIP, 5 μ g/disk), trimethoprim-sulfamethoxazole (T/S, 23.75/1.25 μ g/disk), neopenicillin (NEO, 30 μ g/disk), macrodantin (MAC, 300 μ g/disk), gentamicin (GEN, 10 μ g/disk), rifampicin (RIF, 5 μ g/disk), meropenem (MER, 10 μ g/disk), florfenicol (FLO, 10 μ g/disk), ampicillin (AMP, 10 μ g/disk), streptomycin (STR, 10 μ g/disk), and erythromycin (ERY, 15 μ g/disk). All the bacterial isolates were tested for resistance or sensitivity to different antibiotics using the standard disc diffusion method (Kirby Bauer test). For the disc diffusion assay, bacteria were grown between 18 and 24 h on Mueller-Hinton agar and then suspended in a 0.85% sterile physiological saline solution adjusted to a 0.5 McFarland turbidity standard, corresponding to 10^8 CFU/ml. Antimicrobial discs were gently fixed into the agar surface by fine forceps. The agar plates were incubated at 35°C for 24 h; *Escherichia coli* ATCC 25922 was used as a control strain. The inhibition zone was measured to the nearest mm using a digital caliper and interpreted according to breakpoints mentioned by the Clinical and Laboratory Standards Institute (CLSI) (2016) and shown in Table 6.

Results

Physiochemical water parameters

Water parameters were in the acceptable range. The average value of dissolved oxygen was higher than the optimum concentration (5 mg L⁻¹); the temperature was about 31.3°C the concentration of heterotrophic bacteria was 1.209×10^4 CFU/ml; the concentration of *Vibrio* was about 2.407×10^3 CFU/ml as presented in Table 3.

Isolation of *Vibrio* species from coastal water

A total of 60 *Vibrio* strains were collected from coastal water in Shenzhen Beach in September 2021. These bacterial species accounted for approximately 70% of the dominant clones. Among them, 18 *V. harveyi*, 14 *V. campbellii*, 5 *V. owenii*, 3 *V. coralliilyticus*, 3 *V. sinaloensis*, 3 *V. mediterranei*, 2 *V. ponticus*, 2 *V. orientalis*, 2 *V. alginolyticus*, 2 *V. aestivus*, 2 *V. tubiashii*, 1 *V. alfarensis*, 1 *V. parahaemolyticus*, 1 *V. hangzhouensis*, and 1 *V. rotiferianus* were identified, accounting for 30%, 23.33%, 8.33%, 5%, 5%, 5%, 3.33%, 3.33%, 3.33%, 3.33%, 1.67%, 1.67%, 1.67% and 1.67% of the total strains, respectively (Figure 2). The 16S rRNA phylogenetic tree clustered with the corresponding reference strain and was separate from other reference strains (Figure 3); the biochemical reactions of 6 *Vibrio* strains were detected (Table 3); the gram stain of *V. alginolyticus* (*MEDF7*) showed a negative, short rod-shaped, and slightly curved (Figure 4).

Distribution of virulence genes in *Vibrio* isolates

The isolated *Vibrio* strains were identified by the risk classification of virulence genes for 17 common *Vibrio* virulence genes. Isolates carrying virulence genes over 10 ($\geq 60\%$) were considered high-risk strains, and isolates carrying virulence genes less than 7 ($\leq 40\%$) were considered low-risk strains. Those carrying 8-9 virulence genes were classified as medium-risk strains. PCR analysis showed that *MEDF7* and *JSW-YELLOW* were high-risk strains with 11 and 10 virulence genes, respectively. Strains *DY1*, *DY3*, *X1* and *X3* were medium-risk strains with 8 virulence genes, respectively. The remaining strains contained 1-7 virulence genes and were low-risk strains. In 60 *Vibrio* strains, the detection rate of pathogenic genes was 5.00%–71.67% (Figure 5), and the one-time detection rate was: *serine protease*, 71.67%; *chiA*, 56.67%; *luxR*, 53.33%; *flaC*, 31.67%; *toxR_{Vh}*, 26.67%; *flaB*, 18.33%; *flaA*, 16.67%; *vhhB*, 16.67%; *toxR*, 15%; *toxS*, 15% and *vvh*, 15%. Six atypical virulence genes, *luxR*, *toxR*, *vhhA*, *flaA*, *chiA*, and *trh* were detected in the high-risk strain *MEDF7*, accounting for 54.54%. Four atypical virulence genes *vvh*, *flaA*, *flaC*, and *tcpA* were detected in strain *JSW-YELLOW*, accounting for 40% (Supplementary Tables).

Pathogenicity test

Strains *MEDF7*, *JCW-YELLOW*, *DY1*, *DY3*, *JCW-GREEN*, and *XY11* were selected for the zebrafish infection experiment. Zebrafish began to die 10 hours after infection with *MEDF7* and *JSW-YELLOW* strains (2×10^8 CFU/fish). All the zebrafish infected

with the *MEDF7* strain died within 24 hours (2×10^8 CFU/fish), and the mortality rate was about 70% within 7 days in the medium-risk strain group and 50% in the low-risk strain group (Table 4). *MEDF7*, *DY1*, and *JSW-GREEN* strains had a lethal dose of 50 (LD₅₀) to zebrafish of 5.67×10^4 CFU/fish, 3.72×10^5 CFU/fish, and 4.31×10^6 CFU/fish, respectively (Table 5).

Clinical and pathological examination

The first clinical sign observed in naturally and experimentally infected fish was a decrease in feed intake. The infected zebrafish got darker and accumulated at the water surface, gasping for air, particularly in the afternoon. Diseased fish showed hemorrhagic ulcerations on the external body surface and eroded hemorrhagic pectoral and pelvic fins (Figure 6). Internally, the diseased fish showed congested and hemorrhagic internal organs mainly, while the gill filaments and intestinal tract were damaged, and inflammation increased (Figure 7).

Antimicrobial resistance

The antibiotic resistance results showed that the strain *MEDF7* was resistant to ampicillin, neopenicillin G, doxycycline, rifampicin, meropenem, streptomycin, and flubenicol; *JSW-YELLOW* was resistant to ampicillin, trimethoprim-sulfamethoxazole, and doxycycline; *DY1* was resistant to streptomycin, erythromycin, and neopenicillin; *DY3* was resistant to ampicillin, erythromycin, and trimethoprim-sulfamethoxazole; *JSW-GREEN* was resistant to gentamicin, and doxycycline; *XY11* was resistant to erythromycin, neopenicillin, gentamicin, and doxycycline. The 6 *Vibrio* strains except *JSW-YELLOW* were all resistant to doxycycline and neopenicillin (Table 6).

Discussion

With the rise of global temperature and the aggravation of offshore pollution, the pathogenicity and antibiotic resistance of *Vibrio* bacteria are increasing, and the existence of pathogenic *Vibrio* bacteria in the ocean is attracting more and more attention because of their ability to cause systemic infection, resulting in fish death and even human disease (Md Yasin et al., 2019).

Vibriosis outbreaks have been widely reported in the summer (Baker-Austin et al., 2016). In this study, the average temperature of the seawater was suitable for the growth of *Vibrio*. The abundance of *Vibrio* was high and accounted for 19.91% of the heterotrophic bacteria in the seawater. Several 15 *Vibrio* species and 60 *Vibrio* strains were discovered. The most

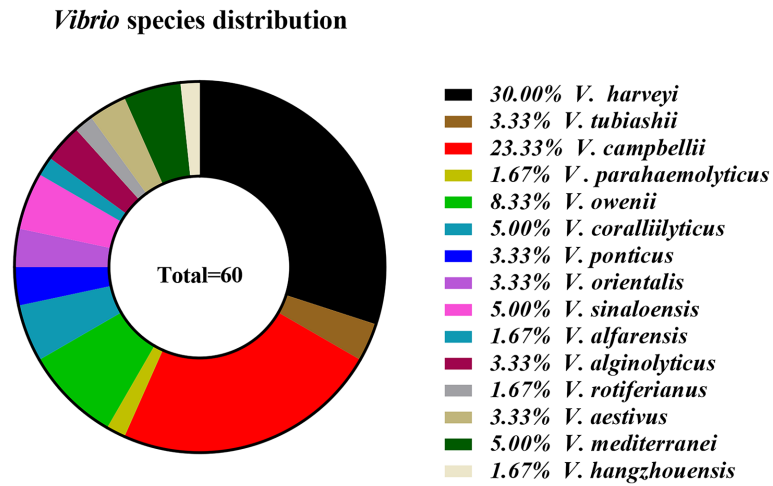


FIGURE 2
The *Vibrio* species composition (%).

prevalent strain found was *V. harveyi*, which accounted for 30% of the total. *V. harveyi*, *V. alginolyticus*, *V. rotiferianus*, *V. campbellii*, and *V. parahaemolyticus* were among the isolates, with 36 isolates belonging to the Harveyi clade, which is the most important pathogen clade in aquatic organisms (60%) (Darshanee Ruwandepika et al., 2012). The Harveyi clade, particularly *V. harveyi*, prefers warm temperatures (Bossart, 2007; Igbinsosa & Okoh, 2008), and is prevalent in marine fish farming in China, resulting in substantial losses (Deng et al.,

2020). Warming temperatures can also directly induce the expression of virulence genes (Bouza & Cercenado, 2002). Guijarro et al. reported that when the temperature rose from 24 to 27°C, virulence factors involved in motility, host degradation, secretion, antimicrobial resistance, and transcriptional regulation were increased 16 times in *V. coralliilyticus* (Guijarro et al., 2015). Yuan et al. mentioned that the risk of *Vibrio* infection in Shenzhen was higher (Yuan & Yuan, 2014), posing a danger to the people and fishery industry

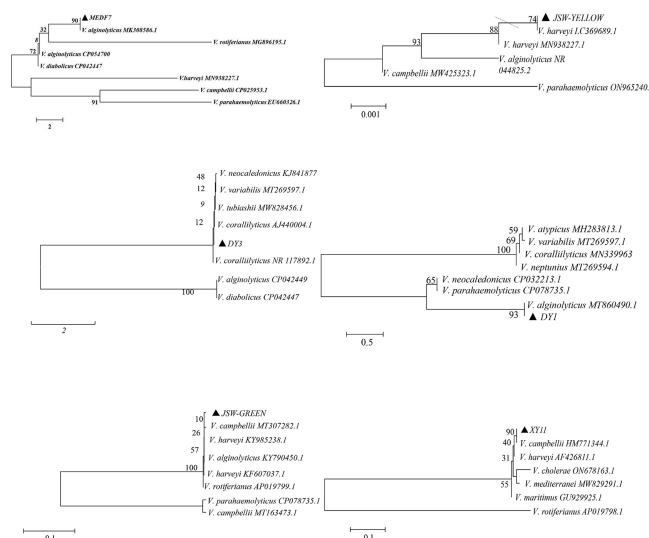


FIGURE 3
The 16S rRNA phylogenetic tree. Phylogenetic tree construction using MEGA6.0 and neighbor-Joining method (1000-fold bootstrap).

TABLE 3 Physiochemical water parameters of the 8 studied beach.

| SitesIndicator | TotalAmmonia | Salinity (%) | Pondus Hydrogenii | Dissolved O ₂ (mg L ⁻¹) | Temp. (°C) | Concentration of Bacteria (cfu/ml) | Concentration of <i>Vibrio</i> (cfu/ml) |
|--------------------|--------------|--------------|-------------------|------------------------------------------------|------------|------------------------------------|-----------------------------------------|
| Dameisha beach | 0.321 | 25 | 8.16 | 5.92 | 30.9 | 2.430×10 ⁴ | 5.50×10 ² |
| Xiyong beach | 0.289 | 23 | 8.25 | 5.34 | 31.5 | 1.075×10 ⁴ | 2.15×10 ³ |
| Guan lake beach | 0.288 | 22 | 7.98 | 5.53 | 31.2 | 1.340×10 ⁴ | 3.00×10 ² |
| Jinshawan beach | 0.311 | 26 | 8.19 | 5.26 | 30.5 | 5.450×10 ³ | 2.35×10 ³ |
| Jiaochangwei beach | 0.289 | 25 | 7.98 | 5.72 | 32.2 | 6.050×10 ³ | 1.50×10 ³ |
| Nan'ao beach | 0.299 | 23 | 8.31 | 5.37 | 31.6 | 1.075×10 ⁴ | 5.75×10 ³ |
| Westyong beach | 0.278 | 22 | 7.95 | 5.23 | 31.3 | 2.050×10 ³ | 2.00×10 ² |
| Jidiaosha beach | 0.285 | 23 | 8.29 | 5.66 | 30.8 | 3.750×10 ³ | 9.50×10 ² |
| Average value | 0.295 | 24 | 8.13 | 5.50 | 31.3 | 1.209×10 ⁴ | 2.407×10 ³ |

in the city. In order to assess bacterial pathogenicity, virulence factors must be identified. These factors allow bacteria to infect and damage hosts (Md Yasin et al., 2019). Include potential toxins, accessory colonization factors, outer membrane proteins, proteases, hemolysins, hemagglutinins, and a capsular polysaccharide, all of which could help *Vibrio* species survive and reproduce in the host (Shinoda, 2005; Gutierrez et al., 2013; Yokochi et al., 2013). Serine protease had the maximum one-time detection rate (71.67%), followed by *chiA* (56.67%), and *luxR* (53.33%). Extracellular protease: Serine protease is a virulence factor found in many pathogenic organisms. It is an essential hydrolytic enzyme. *Vibrio* species produce a variety of extracellular hydrolytic enzymes that are important in the

invasiveness and establishment of infections (Yuan & Yuan, 2014). Lee and Cheng found a serine protease that was toxic to CHO, Hela, and Vero cells and could degrade various host proteins, resulting in tissue bleeding and death (Lee et al., 2002). Furthermore, the quorum-sensing regulator *luxR*, and the chitinase *chiA* were all discovered in *V. harveyi*, which was in line with previous findings (Xiandong et al., 2017; Zhu et al., 2017). In the high-risk strain MEDF7, six atypical virulence factors, *luxR*, *toxR*, *vhhA*, *flaA*, *chiA*, and *trh*, were found, accounting for 54.54%. These findings suggest that virulence genes are transferred among *Vibrio* species and that acquiring atypical virulence genes would increase *Vibrio*'s virulence to hosts (Deng et al., 2020). In aquatic environments, *Vibrio* species

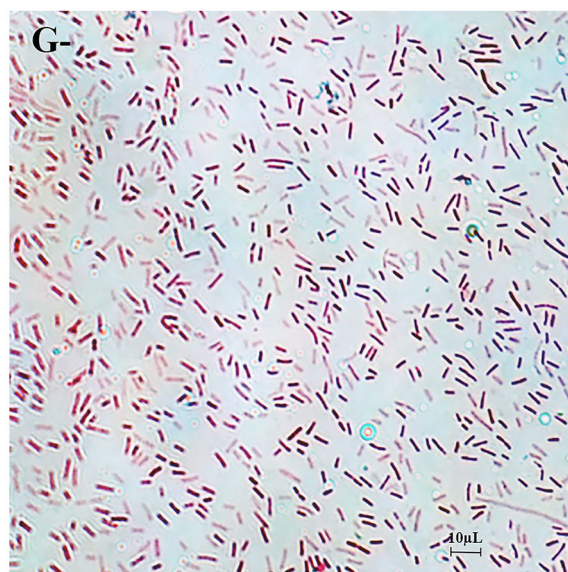


FIGURE 4
The gram stain. Gram stain shows negative, short rod-shaped bacteria. The scale used in the Figure is 10µm.

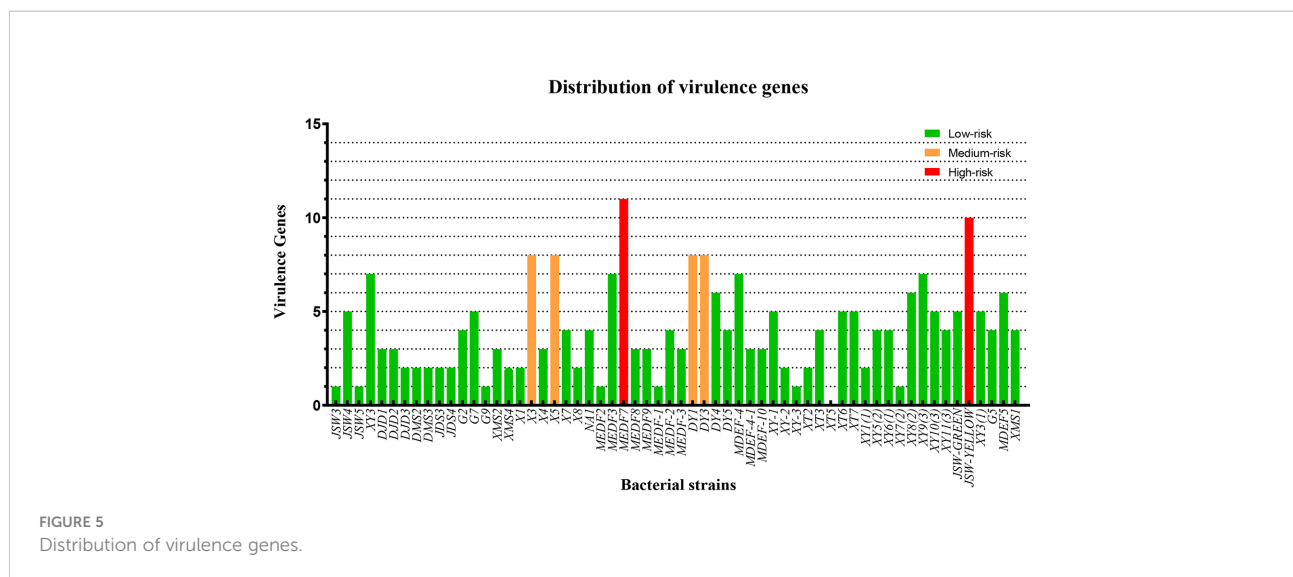


FIGURE 5
Distribution of virulence genes.

are a significant reservoir of potential virulence and bacterial DNA material exchange (Igbinsosa, 2015). The emergence of new pathogenic *Vibrio* from non-harmful species may be possible (Balakrishnan et al., 2019). Moreover, all six experimental strains caused gill filament necrosis and increased the number of inflammatory cells in zebrafish. Especially the MEDF7 strain, which has a high mortality rate and can cause death in a short time. MEDF7 has the lowest LD₅₀ (5.67 × 10⁴ CFU/fish). It is shown that, to some extent, the superposition of virulence genes, especially the increase in the number of atypical virulence genes,

has an important effect on the pathogenicity of *Vibrio*. This is consistent with the conclusion of related studies (Deng et al., 2020).

Antibiotics are widely used to prevent or treat bacterial diseases in aquaculture. Their indiscriminate use has led to an increase in antibiotic resistance in bacteria. The yearly use of raw antibiotic ingredients in China is estimated at about 180,000 t (R. Zhang et al., 2012). Therefore, monitoring the spread of antibiotic resistance is particularly important for assessments involving *Vibrio* species (Letchumanan et al., 2015). According to Wenguang and Xiong,

TABLE 4 The LD₅₀ of 3 strains of *Vibrio*.

| Bacterial Isolate | | MEDF7 | | DY1 | | JCW-GREEN | |
|-------------------|------------------------------|---------------------------------|---------------|---------------------------------|---------------|---------------------------------|---------------|
| Group | Inoculum | Dead fish | Mortality (%) | Dead fish | Mortality (%) | Dead fish | Mortality (%) |
| 1 | Phosphate buffer saline | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 2 × 10 ⁷ CFU/fish | 6 | 85.71 | 5 | 71.42 | 4 | 57.14 |
| 3 | 2 × 10 ⁶ CFU/fish | 5 | 71.42 | 5 | 71.42 | 3 | 42.85 |
| 4 | 2 × 10 ⁵ CFU/fish | 4 | 57.14 | 3 | 42.85 | 3 | 42.85 |
| 5 | 2 × 10 ⁴ CFU/fish | 3 | 42.85 | 2 | 28.57 | 1 | 14.28 |
| LD ₅₀ | | 5.67 × 10 ⁴ CFU/fish | | 3.72 × 10 ⁵ CFU/fish | | 4.31 × 10 ⁶ CFU/fish | |

TABLE 5 Experiment of artificial infection of 6 *Vibrio* strains.

| Group | Bacterial Isolate | Number of experimental fish | Inoculum | Water °C | Dead Fish no. | Mortality % |
|-------|-------------------|-----------------------------|------------------------------|----------|---------------|-------------|
| 6 | MEDF7 | 10 | 2 × 10 ⁸ CFU/fish | 27 | 10 | 100 |
| 7 | JCW-YELLOW | 10 | 2 × 10 ⁸ CFU/fish | 27 | 9 | 90 |
| 8 | DY1 | 10 | 2 × 10 ⁸ CFU/fish | 27 | 8 | 70 |
| 9 | DY3 | 10 | 2 × 10 ⁸ CFU/fish | 27 | 6 | 60 |
| 10 | JCW-GREEN | 10 | 2 × 10 ⁸ CFU/fish | 27 | 6 | 50 |
| 11 | XY11 | 10 | 2 × 10 ⁸ CFU/fish | 27 | 5 | 40 |

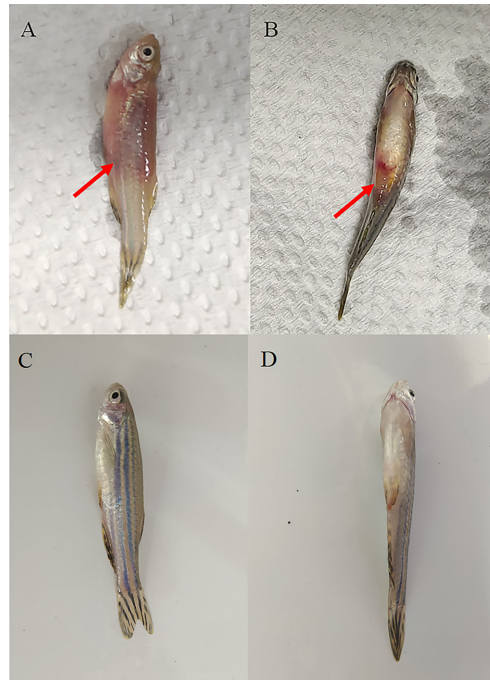


FIGURE 6
Clinical signs of *Vibrio* infection. Symptoms of *Danio rerio* fingerlings experimentally infected with MEDF7 (red arrow). (A, B): Diseased fish group; (C, D): Healthy fish group.

the presence of potentially resistant and pathogen-associated taxonomic groups (*Acinetobacter*, *Arcobacter*, and *Clostridium*) may indicate a risk to human health (Xiong et al., 2015). In our study, the six strains of *Vibrio* were almost resistant to neopencillin

(β -lactams) and doxycycline (tetracycline). β -lactams and tetracycline are commonly used in animals as feed additives or veterinary drugs to promote growth and prevent disease (Zhao et al., 2009). For example, extensive intrinsic resistance to

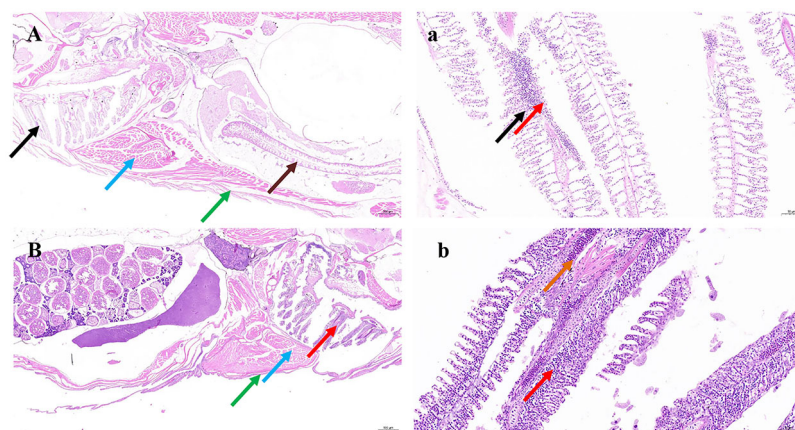


FIGURE 7
Pathological tissue section results. Tissue sections of *Danio rerio* fingerlings experimentally infected with MEDF7. (A-a): Necrosis foci are occasionally seen in the gill, with some gill filament necrosis, cell nuclei fragmentation or dissolution (black arrow), accompanied by inflammatory cell dot infiltration (red arrow); Intestinal epithelial exfoliation and lamina propria exposure (brown arrow); Note the regular arrangement of muscle fibers (blue arrow); There are more mucus cells in the skin (green arrow), H&E, X =2.0–20.0. (B-b): The gill filaments are short and thick with extravasation (orange arrows) and more inflammatory cells (red arrows). Loose .

TABLE 6 Results of antimicrobial susceptibility test of 6 *Vibrio* strains.

| AntibioticPathogen | MEDF7 | | JCW-YELLOW | | DY1 | | DY3 | | JCW-GREEN | | XY11 | | Breakpoints (mm) | | |
|-------------------------------|------------|------|------------|------|------------|------|------------|------|------------|------|------------|------|------------------|-------|-----|
| | Z. D. (mm) | Int. | Z. D. (mm) | Int. | Z. D. (mm) | Int. | Z. D. (mm) | Int. | Z. D. (mm) | Int. | Z. D. (mm) | Int. | R | I | S |
| Ampicillin | 0 | R | 0 | R | 20 | S | 0 | R | 16 | I | 18 | S | ≤14 | 15–17 | ≥18 |
| Streptomycin | 20 | S | 18 | S | 0 | R | 18 | S | 20 | S | 12 | I | ≤10 | 11–14 | ≥15 |
| Erythromycin | 15 | I | 18 | S | 0 | R | 11 | R | 14 | I | 12 | R | ≤13 | 14–17 | ≥18 |
| Chloramphenicol | 24 | S | 31 | S | 31 | S | 20 | S | 21 | S | 28 | S | ≤12 | 13–17 | ≥18 |
| Ciprofloxacin | 15 | S | 21 | S | 20 | S | 15 | S | 15 | S | 17 | S | ≤10 | 11–14 | ≥15 |
| Trimethoprim-Sulfamethoxazole | 25 | S | 0 | R | 24 | S | 0 | R | 22 | S | 20 | S | ≤10 | 11–14 | ≥15 |
| Neopenicillin | 0 | R | 23 | S | 14 | R | 0 | R | 15 | R | 0 | R | ≤12 | 13–16 | ≥17 |
| Macrodantin | 16 | S | 15 | S | 22 | S | 17 | S | 17 | S | 19 | S | ≤10 | 11–14 | ≥15 |
| Gentamicin | 15 | S | 13 | I | 12 | R | 15 | S | 0 | R | 7 | R | ≤12 | 13–14 | ≥15 |
| Florfenicol | 17 | I | 20 | S | 25 | S | 22 | S | 18 | S | 20 | S | ≤14 | 15–18 | ≥19 |
| Doxycycline | 0 | R | 0 | R | 0 | R | 0 | R | 0 | R | 0 | R | ≤10 | 11–13 | ≥14 |
| Rifampicin | 10 | R | 12 | I | 15 | S | 15 | S | 15 | S | 15 | S | ≤10 | 11–14 | ≥15 |
| Meropenem | 0 | R | 21 | S | 25 | S | 0 | R | 20 | S | 20 | S | ≤10 | 11–14 | ≥15 |

ZD, Zone Diameter in millimeter; Int, interpretation; R, resistant; I, intermediate; S, susceptible.

doxycycline and neopenicillin has been reported (Saha et al., 2020). This may be related to the use of biofertilizers, which contain high levels of residual antibiotic compounds, resistance factors, and drug-resistant bacteria (Aedo et al., 2014). Even the Chinese Ministry of Agriculture and Rural Affairs has banned the use of antibiotics in the aquaculture industry. Most antibiotics, including these banned antibiotics are commonly accessible and inexpensive (Wang et al., 2014), and their discharge from aquaculture activities is not currently regulated. This study focused on and investigated the drug resistance of pathogenic *Vibrio* in Shenzhen bathing beaches.

Conclusions

The warm climate conditions in Shenzhen provide favorable conditions for the growth and reproduction of *Vibrio*, and the high abundance of *Vibrio* in the coastal water leads to a high potential incidence of vibriosis. In addition, the transfer of atypical virulence factors and antibiotic resistance has led to the increasing pathogenic capacity of *Vibrio*, which poses a great threat to aquaculture and human health. The results of our study will provide theoretical support for the prevention and control of

Vibrio coastal water in Shenzhen, and the virulence gene detection classification will offer indications for the risk prevention of *Vibrio* coastal water in Shenzhen. It will provide scientific guidance on the management of coastal ecology and sustainable development of the Shenzhen region.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

ZH: bacterial isolation and identification, pathogenicity and challenge tests, and antimicrobial susceptibility tests, wrote the manuscript, and analyzed and processed data. ZH, JL, and MA: review and editing. ZH and YL designed the study. YL and JC: funds and experimental reagents. All authors read and agreed to the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.980593/full#supplementary-material>

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