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Isolation and Identification of Pathogenic *Vibrio* Species in Black Rockfish *Sebastes schlegeli*

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Abstract: Four pathogenic *Vibrio* species were isolated from three diseased black rockfish *Sebastes schlegeli* in Yantai, Shandong Province, China. The strains were identified based on physiological and biochemical characteristics and 16S rDNA sequencing and named SF-2, SF-3, SF-5, and SF-6, respectively. SF-2 was *Vibrio scophthalmi*, SF-3 was *V. harveyi*, SF-5 was *V. alginolyticus*, and SF-6 was *V. parahaemolyticus*. This is the first time that *V. scophthalmi* was isolated from black rockfish. The present research shows that *V. scophthalmi* is a potential pathogen. Detection of virulence genes using polymerase chain reaction showed that SF-3, SF-5, and SF-6 carried *FlaB*; SF-5 and SF-6 carried *TcpA*; and SF-2, SF-5, and SF-6 carried ToxS. *Tdh*, *Trh*, *Tlh*, ToxR, and Zot were not detected. SF-3, SF-5, and SF-6 all had protease, gelatinase, lipase, and lecithinase. They were all intermediately sensitive to erythromycin, whereas SF-2, SF-5, and SF-6 were sensitive to spectinomycin, and SF-3 was sensitive to cotrimoxazole and chloramphenicol. They were resistant to most antibiotics and multidrug resistance was obvious.

Keywords: *Sebastes schlegeli*; virulence factors; *Vibrio scophthalmi*; opportunistic pathogen

Key Contribution: This is the first time that *V. scophthalmi* was isolated from *S. schlegeli*. Multiple pathogenic bacteria were found in fish disease.

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1. Introduction

The black rockfish *Sebastes schlegeli* has become an important marine aquaculture species along the northeastern coasts of China, Japan, and South Korea [1]. Daqindao, Changdao City, Shandong Province is the largest sea-cage fish culture area in northern China [2]. In recent years, offshore marine fishery resources have gradually become scarce, and the output of wild black rockfish is unable to meet consumer demand. Now, a large proportion of black rockfish sold in the market is cultured in cages and has become one of the most important types of fish in sea-cage culture in China.

Owing to the high density of cages and high density of cultured fish, caged fish are prone to diseases. Nutritional levels, physiological stages, drugs, and environmental stress can alter their intestinal flora composition to varying degrees [3]. The health status of black rockfish is susceptible to these external factors.

Parasites and bacteria are the main pathogens isolated from black rockfish [4,5]. The parasites in black rockfish include *Microcotyle sebastis*, *Heteraxine* spp., *Opecoelusnipponicus*, and parasites from the Anisakidae family. Common pathogenic bacteria include *Vibrio*, *Lactococcus garvieae*, β-hemolytic *Streptococcus*, and *Aeromonas salmonicida*(*A. salmonicida*). The *Vibrio* core group consists of eight species including *V. harveyi*, *V. alginolyticus*, *V. rotifer*, *V. parahaemolyticus*, *V. campbell*, *V. natrigens*, *V. mytili*, and *V. azureus* [6]. These are recognized aquatic animal pathogens. Intensive farming is associated with problems such as bacterial diseases caused by various *Vibrio* spp. [7]. After being infected by *Vibrio* spp., black rockfish mainly exhibit symptoms such as a black body color, red and swollen epidermis, irregular swimming, poor feeding, and muscle rot in severe cases [8].

In the study, black rockfish were found to have died from a disease at a cage farm in Yantai, Shandong Province, China. Different pathogenic *Vibrio* spp. were isolated. In this study, the determination of bacterial morphology, identification of physiological and biochemical characteristics, 16S rDNA sequence amplification and sequencing analysis, identification of the virulence gene and virulence-related factors, and tests for antibiotic resistance were performed.

2. Materials and Methods

2.1. Fish and Clinical Signs

Diseased black rockfish were obtained from a farm in Yantai City, Shandong Province, China in September 2020. The mortality rate for diseased black rockfish was 30–40%. Three sick fish were randomly selected for the experiment. The diseased fish had skin ulcers and their eyes and mouth were congested. An autopsy revealed fluid accumulation in the abdominal cavity and liver congestion.

2.2. Isolation and Purification of Pathogenic Strains

Under aseptic operation, the liver tissues of three fish were smeared on the corresponding Thiosulfate–Citrate–Bile Salts–Sucrose (TCBS) medium with am inoculation ring and cultured at 28 °C. Colony morphology was observed after incubation for 24 h. Four dominant bacterial colonies were isolated and purified by repeated streaking on their corresponding agar plates and named SF-2, SF-3, SF-5, and SF-6.

2.3. Identification of Strains

2.3.1. Biochemical Characteristics

The purified strains were obtained for biochemical identification. Single colonies of different purified bacteria were inoculated into bacterial trace biochemical reaction tubes and cultured at 37 °C for between 24–48 h. The change in the reaction tube was observed after culturing.

2.3.2. 16S rDNA Amplification of the Isolated Strains

Purified bacteria isolated from each sample were identified using 16S rDNA gene sequencing and alignment. The forward primer (27F) was 5′-AGAGTTTGATCCTGGCTCAG-3′, and the reverse primer (1492R) was 5′-GGCTACCTTGTTACGACTT-3′. The polymerase chain reaction (PCR) mixture consisted of 5 μL of PCR buffer (10×), 3 μL of $MgCl_2$ (25 mmol/L), 1.25 μL of dNTP (10 mmol/L), 1 μL of Taq enzyme (5 U/μL), 1 μL of DNA template, and 5 μL of each primer. Sterile nanopure water was added to bring the final reaction volume to 50 μL. PCR was performed as follows: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1.5 min for a total of 35 cycles, followed by a final extension step at 72 °C for 10 min.

PCR products were electrophoresed in 1.0% agarose gel. The 16S rDNA purification and sequencing of the strain were entrusted to General Biosystems (Anhui). The 16S rDNA was analyzed and compared using the BLAST search program (http://www.ncbi.nlm.nih.gov, accessed on 25 November 2021) of GenBank. The evolutionary tree was constructed using Mega 7. Using the Neighbor Joining method, it is a bootstrap analysis with 1000 repeats.

2.4. Determination of Virulence-Related Factors

2.4.1. Determination of Extracellular Enzyme Activity

Extracellular proteinases play an important role in the virulence of *Vibrio* spp. [9]. The determination of extracellular proteinases detection was according to the method reported by Bunpa et al. [10]. Extracellular enzymes were used in the agar plate assay. Four purified strains were inoculated on the corresponding detection plate and cultured at 28 °C for 24 h, and the changes around the colony were observed.

2.4.2. Detection of Virulence Genes

The virulence genes of four different isolates were amplified. Eight main virulence genes, including thermostable direct hemolysin (*Tdh*), Tdh-related hemolysin (*Trh*), thermolabile hemolysin (*Tlh*), flagella-related genes toxin-coregulated pilus major subunit A (*TcpA*), and flagellin B subunit (*FlaB*) and the virulence regulatory proteins ToxS, ToxR, and zonula occludens toxin (Zot), were targeted. The primers for these genes are listed in Table 1. The PCR mixture consisted of 1 μL of PCR buffer (10×), 0.6 μL of $MgCl_2$ (25 mmol/L), 0.25 μL of dNTP (10 mmol/L), 0.2 μL of Taq enzyme (5 U/μL), 1 μL of DNA template, and 0.5 μL of each primer. Sterile nanopure water was added to make up the final reaction volume to 10 μL. PCR was performed as follows: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing for 30 s, extension at 72 °C for 1 min for a total of 30 cycles, followed by a final extension step at 72 °C for 7 min. PCR products were identified using 1.0% agarose gel electrophoresis. The annealing temperatures are shown in Table 1.

Table 1. Primer sequences for the detection of virulence genes in *Vibrio* spp.

virulence Gene	Primer Sequence	Primer Sequence (bp)	Product Size (°C)	Reference
Tdh	GTAAAGGTCTCTGACTTTTGGAC TGGAATAGAACCTTCATCTTCACC	269	60	[11]
Trh	TTGGCTTCGATATTTTCAGTATCT CATAACAAACATATGCCCATTTCCG	500	60	[11]
Tlh	GCTACTTTCTAGCATTTTCTCTGC AAAGCGGATTATGCAGAAGCACTG	450	60	[11]
Zot	CACTGGGCGAGAAAGGAC CGCCCATAGACCACGATA	737	58	[12]
TcpA	ACCGTGGTCTAGGTAATT CAACGCCGAATGGAGCAG	431	58	[12]
ToxS	CCACTGGCGGACAAAATAACC AACAGTACCGTAGAACCGTGA	640	52	[12]
ToxR	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTTCATG	368	52	[11]
FlaB	AACGTATCAGCGATGACC TTGAAACGGTTCTGGAAT	928	50	[12]

2.5. Antibiotic Resistance Tests

The strains were tested for antibiotic resistance using the disc diffusion method. The antibiotics used in this experiment mainly included gentamicin, cotrimoxazole, ampicillin, penicillin G, erythromycin, norfloxacin, chloramphenicol, amikacin, ciprofloxacin, pioneer V, spectinomycin, enrofloxacin, cefotaxime, levofloxacin, cefoxitin, rifampicin, doxycycline, imipenem, clindamycin, metronidazole, amoxicillin, and bacitracin. The concentration of the bacterial suspension was adjusted to 10^7 colony-forming units/mL and spread on the surface of a 2216 E agar medium. Up to five sheets of antibiotic-containing paper disks were pasted per plate. The diameters of the bacteriostatic circles of each drug were measured after 24 h of culture at 28 °C. The test was performed three times, and the average value was calculated to determine the sensitivity of the strains to antibiotics.

3. Results

3.1. Isolation and Biochemical Identification of Strains

Four different bacteria were isolated, and all four isolates were Gram-negative, short, rod-shaped bacteria. All isolates could grow on TCBS medium, forming yellow, round colonies with neat edges. The growth cycle of SF-2 was longer than that of other bacteria, and the colony morphology gradually emerged after 18 h of culture. SF-6 colonies were cultured on TCBS medium, and the colonies were bluish green after about 14 h of culturing. The physiological and biochemical results are shown in Table 2.

Table 2. Physiological and biochemical findings.

Physiological Identification	SF-2	SF-3	SF-5	SF-6
Gram stain	−	−	−	−
Oxidase enzyme	+	+	+	+
Methyl red	+	+	−	−
VP	+	−	+	−
H_2S	−	+	−	−
Citrate	−	+	−	+
Glucose gas production	−	−	−	−
Salt-free tryptone water	−	−	−	−
6% tryptone water	−	+	+	+
8% tryptone water	−	−	+	+
10% tryptone water	−	−	+	+

"+" positive; "−" negative.

3.2. 16S rDNA Amplification of the Isolated Strains

A 1448-bp fragment from all four isolates was identified using 16S rDNA PCR. Sequences of the isolated strains were compared using the blast search function of NCBI, and the results showed that all four isolates belonged to the *Vibrio* genus. Electrophoresis results of the isolated bacteria and the phylogenetic trees constructed with 16S rDNA sequences are shown in Figures 1 and 2, respectively.

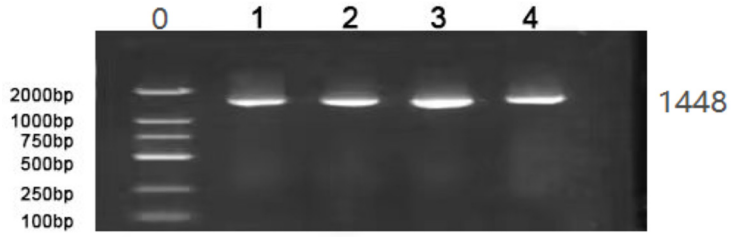

Figure 1. Results from 16S rDNA PCR. Lane 0, DNA bp ladder (100–2000 bp). The 1448-bp fragments of the isolates are represented accordingly: Lane 1 SF-2, Lane 2 SF-3, Lane 3 SF-5, Lane 4 SF-6.

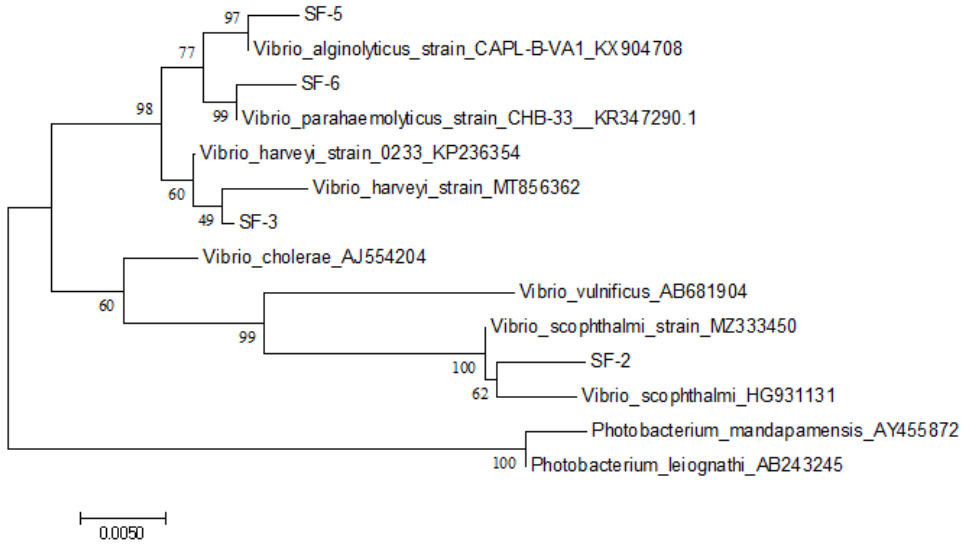

Figure 2. Neighbor-joining phylogenetic trees based on 16S rDNA gene sequences showing the relationship between isolates. The numbers at the nodes indicate the levels of bootstrap based on 1000 replicates. Sequences from related species were obtained from the GenBank database, and their accession numbers are indicated after the species name.

Based on the phenotypic characteristics, biochemical characteristics, and 16S rDNA analysis of the isolated strains, isolate SF-2 was determined to be *V. scopthalmi*; SF-3 was determined to be *V. harveyi*; SF-5 was determined to be *V. alginolyticus*; and SF-6 was determined to be *V. parahaemolyticus*.

3.3. Determination of Extracellular Enzyme Activity

Four extracellular enzymes were detected in this experiment, including caseinase, gelatinase, lipase, and lecithinase. The activity of extracellular enzymes was detected in SF-3, SF-5, and SF-6, whereas no extracellular enzyme activity was detected in SF-2. The results are shown in Table 3.

Table 3. Extracellular enzyme activity.

Enzymes	SF-2	SF-3	SF-5	SF-6
Caseinase	−	+	+	+
Lecithinase	−	+	+	+
Lipase	−	+	+	+
Gelatinase	−	+	+	+

"+" extracellular enzyme activity detected, "−" extracellular enzyme activity not detected.

3.4. Detection of Virulence Genes

The flagella-related gene *FlaB* was found in SF-3, SF-5, and SF-6; toxic force regulatory protein ToxS was found in SF-2, SF-5, and SF-6; and the flagellum-related gene *TcpA* was found in SF-5 and SF-6. Other virulence genes such as *Tdh*, *Trh*, *Tlh*, *ToxR*, and *Zot* were not detected. The results are shown in Figure 3.

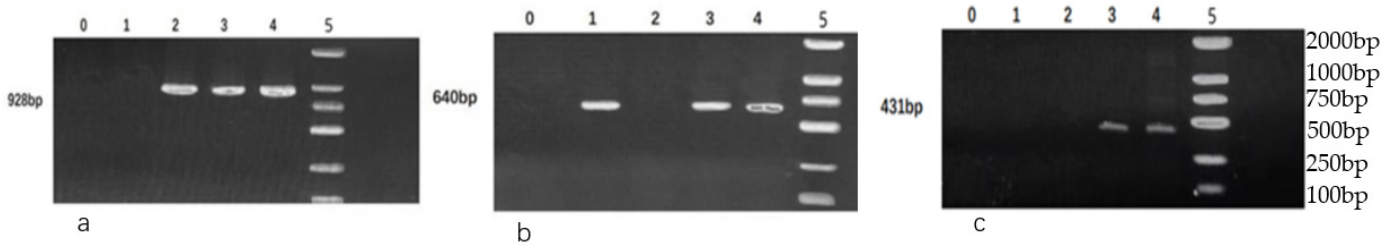

Figure 3. PCR results for virulence genes. (**a**) Virulence gene *Flab*; (**b**) virulence gene *ToxS*; (**c**) virulence gene *TcpA*. Lane 0, negative control; Lane 1: SF-2, Lane 2: SF-3, Lane 3: SF-5, Lane 4: SF-6, and DNA bp ladder (100–2000 bp).

3.5. Antibiotic Resistance Tests

CLSI documents are the documents of the Clinical Microbiology Laboratory of the United States research center and reflect the regulatory requirements of the United States. It is different from the verification method of the domestic drug sensitivity test system. The sensitivity was determined according to the criteria for inhibition zones of the disk-method test provided by the Hangzhou Tianhe microbial reagent Company. All *Vibrio* spp. showed multidrug resistance, with SF-2, SF-5, and SF-6 being sensitive to only one antibiotic. SF-3 was sensitive to only two of the 22 antibiotics tested; SF-5 was intermediately sensitive to two antibiotics; SF-2 and SF-6 were intermediately sensitive to three antibiotics; and SF-3 was intermediately sensitive to five antibiotics. All four *Vibrio* spp. showed intermediate sensitivity to erythromycin. Test results for antibiotic resistance are shown in Table 4.

Table 4. Disk diffusion test results for the four *Vibrio* isolates from black rockfish.

Antibiotic	SF-2	SF-3	SF-5	SF-6
Gentamicin	R	I	I	I
Cotrimoxazole	R	S	R	R
Ampicillin	R	R	R	R
Penicillin G	R	R	R	R
Erythromycin	I	I	I	I
Norfloxacin	R	I	R	R
Chloramphenicol	R	S	R	R
Amikacin	R	R	R	R
Ciprofloxacin	R	R	R	R
Pioneer V	R	R	R	R
Spectinomycin	S	R	S	S
Enrofloxacin	I	I	R	R
Cefotaxime	I	R	R	I
Levofloxacin	R	I	R	R
Cefoxitin	R	R	R	R
Rifampicin	R	R	R	R
Doxycycline	R	R	R	R
Imipenem	R	R	R	R
Clindamycin	R	R	R	R
Metronidazole	R	R	R	R
Amoxicillin	R	R	R	R
Bacitracin	R	R	R	R

"S" sensitive, "I" intermediate sensitivity, "R" resistant.

4. Discussion

In this study, we identified *V. scophthalmi*, *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*. *V. alginolyticus* and *V. parahaemolyticus* were isolated from the same diseased fish. Various bacteria have been isolated during similar disease outbreaks. Such co-infections often cause more serious economic losses than single infections with either pathogen [13]. *V. anguillarum* and *V. scophthalmi* can jointly cause ascites disease in turbot larvae (*Scophthalmus maximus*) [14]. In addition, the presence of two pathogens was also found in striped mullet *Mugil cephalus* that died of septicemia. *A. hydrophila* and *V. parahaemolyticus* were isolated from diseased striped mullet [15]. Co-infection is one of the reasons for the high mortality rate of striped mullet in summer. Erythematous dermatitis, necrotizing dermatitis, and dermal ulcers in fish have also been caused by a combination of pathogens, including *Aeromonas* spp., *Alcaligenes* spp., *Pseudomonas* spp., and *Vibrio* spp. However, no genus was consistently dominant in cultures obtained from any of the lesion types [16]. Co-infections by two or more pathogens often occur in fish culture. Experimentally infected zebrafish showed significantly higher mortality after co-infection with *A. hydrophila* and *A. veronii* [17]. Numerous pathogenic bacteria, including *Enterovibrio nigricans*, *Photobacterium swingsii*, *V. owensii*, *V. harveyi*, and *V. rotiferianus*, have also been isolated from diseased fish with hemorrhaging and festering fins and skin and swollen internal organs [18]. The presence of multiple pathogenic bacteria in a single diseased fish is common and more likely to cause outbreaks.

Both extracellular protease and virulence genes are virulence factors of *Vibrio*. The pathogenicity of *Vibrio* depends on the presence of virulence factors. The potential pathogenicity of *Vibrio* was determined by detecting its virulence factor [19]. Various extracellular enzymes are important factors contributing to the virulence of *Vibrio*. Extracellular proteases including caseinase, gelatinase, lipase, and lecithinase are common in bacteria and play an important role in bacterial virulence [10]. Caseinase and gelatinase can decompose the collagen protein component to facilitate the dissemination of the hemolytic toxin throughout the body. The hemolysis factor causes blood cells to rupture, disabling oxygen transport in the body and leading to tissue necrosis; death can occur in severe cases. Lecithinase and lipase decompose host tissue elements, such as cell membranes, causing

widespread tissue damage [20]. The presence of these extracellular enzymes can cause host tissue damage, leading to disease. *V. fluvialis* secretes extracellular enzymes such as protease and lipase. Extracellular toxins, including protease, are the main factors of *V. fluvialis* responsible for pustule disease and death in abalone [21]. *Vibrio* spp. that are pathogenic to black rockfish can produce extracellular enzymes and cause hemorrhage and ascites [11]. Similar results were observed in our study; four extracellular enzymes were detected in *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*, but not in *V. scopthalmi.* Thus, *Vibrio* spp. secreting these extracellular enzymes are pathogenic to black rockfish.

The pathogenicity of *Vibrio* depends on several virulence factors encoded by their virulence genes [22]. The presence of virulence genes also helps judge the pathogenicity of *Vibrio*. Tdh is thermostable direct hemolysin, Trh is Tdh-related hemolysin, and Tlh is thermolabile hemolysin [11]. *V. parahaemolyticus* has also been found to be pathogenic in the absence of the *Tdh* and *Tlh* genes [23]. Thus, these two genes are not the only criteria responsible for the pathogenicity of *V. parahaemolyticus*. Zot can affect the tight junction structure or bond bridge between cells, thereby enhancing the permeability of the small intestinal mucosa [12]. *FlaB*-encoding flagellin regulates the ability of bacteria to attach to the surface and enables strong adhesion of *Vibrio* spp. Flagella are responsible for conferring virulence in some *Vibrio* spp [24]. In *V. cholerae*, motile isolates have been reported to be more virulent than the nonmotile mutants [25]. TcpA toxin co-regulates the pilus-encoded protein to regulate the main subunits of toxin-coregulated pilus (TCP). TCP helps in microcolony formation on the epithelial surface [26]. ToxR is a transmembrane regulatory protein that plays a key role in the virulence expression system of *V. cholerae*. *ToxR* activity is dependent on the inner membrane protein ToxS [27,28], and ToxS has been detected in pathogenic *V. alginolyticus* and *V. cholera* [26]. The existence and expression of virulence genes can affect the pathogenicity of bacteria to the host. *Vibrio* spp. with virulence genes are more likely to cause disease in black rockfish. While identifying virulence genes, *FlaB* and *TcpA* were detected in *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*, whereas *ToxS* was detected in *V. alginolyticus* and *V. parahaemolyticus*. These three *Vibrio* spp. have been found as pathogens in black rockfish and reported to contain several extracellular virulence factors and virulence genes. *Vibrio* spp. that carry *FlaB* genes are more likely to attach to their hosts. The excretion of extracellular products contributes to successful bacterial colonization and host invasion. Therefore, it is concluded that they may have certain toxicity.

The current study is the first report of *V. scophthalmi* in black rockfish. *V. scophthalmi* has earlier been reported to be one of the main bacterial species in the diseased olive flounder *Paralichthys olivaceus* [29]. *V. scophthalmi* isolated in our study did not secrete extracellular enzymes. There was evidence of the pathogenicity of *V. scophthalmi* without the secretion of extracellular enzymes. High-virulence *V. scophthalmi* strains were found to produce lipase and gelatinase but not caseinase and lecithinase. Some strains were found to produce extracellular enzymes, similar to those observed in the case of high-virulence strains, but caused fewer incidences of olive flounder death. Other moderately virulent strains did not produce these enzymes but were still pathogenic to the olive flounder. Thus, they suggest that some toxins in *V. scophthalmi* were not produced during experimental conditions, or that these extracellular enzymes were not the only virulence factors [29,30]. *V. scophthalmi* may also be considered an opportunistic disease-causing pathogen in aquatic animals. Early studies have reported that *V. scophthalmi* was a common microorganism present in the intestine of turbot larvae. However, it has recently been found that *V. scophthalmi* can cause diseases in marine animals and is an opportunistic pathogen in olive flounder and turbot larvae. Hosts that are immune-suppressed or stressed because of physiological or environmental factors are more likely to be infected and suffer from severe disease. The stress factors here include water temperature and invasion by pathogenic bacteria [30–33]. Studies suggest that the physiological mechanisms of *V. scophthalmi* may be complex. Changes in the culture environment or invasion by other bacteria can affect the virulence expression of *V. scophthalmi*. The main symptom of the Japanese eel *Anguilla japonica* infected with *V. scophthalmi* was skin and peritoneal hemorrhage [34]. This is similar to the

symptoms exhibited by black rockfish infected with *V. scophthalmi* and is likely because the medium used for laboratory culture is rich in nutrients and quite different from the natural growth environment of bacteria. The laboratory environment weakens bacterial competitiveness and reduces their ability to secrete extracellular proteins. In our study, although no extracellular enzymes were detected, the complex aquaculture environment might be stressful to fish, allowing even a low pathogenic strain of *V. scophthalmi* to infect and cause disease. At the same time, virulence regulatory proteins of *ToxS* were also detected. *V. alginolyticus* and *V. parahaemolyticus*, which produce extracellular enzymes, were also found to have *ToxS* genes. Thus, the *V. scophthalmi* isolated in our study may have had potential toxicity. Experiments showed that all four *Vibrio* spp. carried certain extracellular enzymes or virulence genes, which are the virulence factors of *Vibrio*. Virulence factor is closely related to the incidence of *vibrio* [35]. *Vibrio* carrying flagella genes are more likely to attach to the host tissue and break host cells and tissues by secreting extracellular enzymes, followed by proliferation and invasion into the internal organ systems via blood circulation. *Vibrio* spp. are a group of common bacteria that are natural constituents of freshwater, estuarine, and marine environments [36]. The presence of a variety of *Vibrio* spp. in the bodies of fish indicates the presence of numerous *Vibrio* spp. in the aquaculture environment. The presence of multiple *Vibrio* strains is more likely to cause disease than the presence of a single pathogenic strain [12,37].

The antibiotic resistance test revealed all four *Vibrio* strains to be multidrug resistant. SF-2, SF-5, and SF-6 were sensitive to spectinomycin, whereas SF-3, SF-5, and SF-6 were intermediately sensitive to gentamicin. All four species of *Vibrio* were intermediately sensitive to erythromycin. The unreasonable use of antibiotics in aquaculture has led to the emergence of antibiotic resistance in *Vibrio* spp. [38,39]. The multidrug-resistant phenotype has been found in *V. scophthalmi* [40]. *V. alginolyticus* strains were found to have multiple antimicrobial-resistance genes [41]. Additionally, bacteria were isolated from aurus and Penaeus indicus collected from local markets in Hail region, Saudi Arabia. Drug sensitivity tests showed that there was multidrug resistance [42]. The use and misuse of antimicrobials can lead to a rapid increase in the frequency of bacterial resistance; antibiotic resistance has become a major problem in veterinary and human medicine [43–45]. The emergence of bacterial resistance in aquaculture has led to the reduced ability of drugs to control diseases in aquatic animals [46]. Drug susceptibility is related to the region, temperature, salinity, and culture conditions, as well as to the use of drugs in the farm. The extensive use of antibiotics leads to residues in the environment. This, in turn, will lead to the selection of resistant bacteria and promote the evolution of antibiotic resistance mechanisms, such as reduced intracellular drug concentrations, alternative or protective targets, and direct inactivation of antibiotics, eventually leading to multidrug resistance [47]. This poses a huge challenge in disease prevention and control in the aquaculture industry.

In our study, none of the antibiotics were active against all four *Vibrio* spp. New methods to treat diseases in fish need to be designed. The immune system of fish depends on their nutrition and diet [48], and dietary changes can affect their functional responses. For example, adding methylmercury to the black rockfish diet promotes an active diet, whereas uptake of methylmercury chloride in black rockfish led to higher oxidative stress, which was reflected by significantly increased plasma SOD activity at all tested levels [49]. Some experiments have found that dietary *Chlorella peruviana* could improve the growth and innate immune response of the juvenile rainbow trout *Oncorhynchus mykiss*, which is beneficial in improving production efficiency and resisting bacterial infections [50]. Vaccination is also a useful approach to preventing diseases and is considered one of the most effective tools to combat bacterial and viral diseases, thereby enhancing the survival of fish. Vaccines protect fish from certain diseases by producing antibodies that stimulate their immune systems. Several vaccines including inactivated vaccines, live attenuated vaccines, subunit vaccines, DNA vaccines, and live vector vaccines against *Vibrio* are available [51–53]. Vaccination of fish against vibriosis is one of the most comprehensive and successful developments in disease prevention in modern aquaculture [54]. The

injection or immersion of olive flounder with inactivated *V. anguillarum* can stimulate the response and production of the polymeric immunoglobulin receptor [55]. A *A. salmonicida*-emulsified vaccine can protect turbot larvae without severe side effects on fish growth under field conditions and can also protect fish from chronic infections, which, in turn, reduces infection probability by other pathogens or parasites [56]. In addition to antibiotics, better feed or vaccination are effective approaches to prevent and treat aquatic diseases. These techniques not only improve the survival rate of fish but also protect the environment. Therefore, healthy breeding methods should be implemented, and appropriate farming methods should be sought during the breeding process to reduce disease occurrence.

5. Conclusions

By isolation on agar plates, several types of pathogenic *Vibrio* spp. were found in black rockfish with skin ulcer disease. This is the first study that reports *V. scophthalmi* in black rockfish. After investigating the presence of virulence genes and enzymes, a pathogenic potential was determined in all four isolates. The existence of a variety of *Vibrio* spp. will lead to cross-infection and increase the incidence of disease outbreaks. In the drug sensitivity test, four *Vibrio* spp. exhibited multidrug resistance. This finding makes the prevention and control of fish diseases face greater challenges, thereby urging us to find novel methods to solve the problem.

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