Isolation and determination of *Vibrio* spp. pathogen from hemorrhagic disease in *Sciaenops ocellatus* in cage culture

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Research Article

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Abstract

In this study, 18 strains of *Vibrio* bacteria were identified from 27 samples of Red drum fish (*Sciaenops ocellatus*) suffering from the haemorrhagic disease from cage culture in Vietnam. The bacterial strains were identified with the 16S *rRNA* sequencing method and checked for morphological, physiological, and biochemical characteristics by using the API 20E KIT. Twelve strains of *V. alginolyticus*, three strains of *V. fluvialis*, and three strains of *V. orientalis* were recorded. All *Vibrio* strains have gene similarities with those on the gene bank ranging from 98 to 100%. The biochemical characteristics of these 18 isolates were similar. These bacteria are susceptible to tetracycline and doxycycline and entirely resistant to ampicillin, amoxicillin, and erythromycin.

1. Introduction

Red drum (*Sciaenops ocellatus*) is a marine fish species with high economic value, commonly farmed in Texas and Florida, USA, to provide alternatives to wild-caught fish and stock enhancement efforts (FAO, 2009). Red drums have also become the primary fish breed in China since 1991 (Hong & Zhang, 2003; Shen, Qian, Xu, Gu, & Shao, 2005) and that was imported in Vietnam in 1999, this fish is cultured mainly in the central coastal provinces from Ba Ria Vung Tau to Quang Ninh (Ministry of Fisheries, 2005). Vibriosis is an intestinal disease. *Vibrio* is considered one of the most common pathogens in farmed marine fish and water environments (Novriadi, 2016). *Vibrio* bacterium is also identified as a pathogen in marine fish cultures, such as *Lates calcarifer* (Gibson Kueh, Terence, Chew, Uichanco, & Shen, 2021), (Hoa, Oanh, & Phuoc, 2018) and *Mugil cephalus* (Raju & Sreeramulu, 2017). Some studies have shown that the causative agent of disease on Red drum fish is *Streptococcus iniae* infection in cage-cultured Red drums in Eastern China (Zhou et al., 2008), (Mmanda et al., 2014). *Vibrio harveyi* (*V. carchariae*) causes infectious gastroenteritis in cultured Red drums in Taiwan (P. C. Liu, Chuang, & Lee, 2003). *V. vulnificus*, *V. brasiliensis*, *V. cholera*, and *V. parahaemolyticus* cause Red drums' haemorrhagic disease (Hoang Tan Quang et al., 2020). According to our research in 2020, the causative agent of haemorrhagic disease on *S. ocellatus* in Thuan An, Thua Thien Hue, Vietnam is mainly *V. alginolyticus* (50%), *V. azureus* (27.67%), *V. fluvialis* (16.67%), and *V. orientalis* (6.67%) (Yen, Linh, & Tram, 2021). The first evidence of Red drums infected by *Nocardia seriolae* is from a farm in Mexico (Rio-Rodriguez et al., 2021). In addition, *V. alginolyticus* bacterium is an opportunistic pathogen related to infections in humans and marine animals. *V. alginolyticus* and *V. parahaemolyticus* strains are also pathogens in coastal aquaculture systems in Guangdong, China (Xie Z.Y., Hu C.Q., Chen C., Zhang L.P., & C.H, 2005). The first study to develop vaccine VaBGs can cause a stronger humoral and cellular immune response to protect mice and fish from *V. alginolyticus* compared with the conventional FKC vaccine (Cao et al., 2018). *V. alginolyticus* and *S. iniae* have also been identified as the causative agent of *Cobia* with the haemorrhagic disease, ulcers, fin erosion, and blindness (Nguyen Bao Trung & Dung, 2018).

2. Materials And Methods

2.1 | Sampling and preparation

Thirty-five specimens of Red drums with signs of haemorrhage were collected from four cage culture sites in Hai Duong commune, Huong Tra, Thua Thien Hue, Vietnam. The sample were collected from January to March, 2020. The geographic coordinates of those four locations are as follows: (1) 107°36'39.3984" East and 16°34'0.558012" North; (2) 107°36'51.89544" East and 16°33'57.93696" North; (3) 107°37'3.20988" East and 16°33'58.190796" North; and (4) 107°37'15.12192" East and 16°33'59.01696" North (Fig. 1). After collection, the live fish were transported by styrofoam boxes (with manual aerator, temperature from 15 to 18 °C) to the laboratory for bacterial isolation.

2.2 | Physical and chemical water quality

The pH (HANNA HI98107 pH handheld pH meter, Romania), temperature (mercury thermometer), salinity (refractometer), and dissolved oxygen (sera test KIT, Virtue) of the sites were also recorded.

2.3 | Bacteria isolation and identification

The fish were washed thoroughly under tap water and dried. The outer surface of the fish was disinfected with 70% ethanol, and the fish was opened with a sterilized scalpel. The internal pathological signs were recorded. The damaged tissues from the brain, liver, kidney, and spleen were separated with sterilized culture rods. They were then inoculated in the thiosulfate citrate bile salts sucrose medium (TCBS, Himedia, India) and cultured at 28 °C for 24 hours. The prevalent, loose colonies were further cultured in the TCBS medium under the same conditions for total DNA extraction. The presence of toxin genes was identified, and their morphological, physiological, and biochemical characteristics and antibiotic susceptibility were determined.

2.4 | *Vibrio* molecular identification

Total DNA extraction

The *Vibrio* bacterial cell lines isolated from Red drum fish with haemorrhagic signs were grown proliferatively in the trypto-casein soy broth (TSB) supplemented with 2% NaCl and shaken at 180 rpm at 30 °C for 24 hours. Cell biomass was obtained by centrifugation at 8,000 rpm/min for 2 min at 4 °C. The total DNA of *Vibrio* cell lines was extracted with the modified phenol/chloroform method according to Neumann et al. (1992). The step using SDS/lysozyme or proteinase K was eliminated, and the bacterial cells were directly extracted with phenol (Neumann, Pospiech, & Schairer, 1992) (Table 1).

16S *rRNA* gene amplification with PCR and nucleotide sequence analysis

The 16S *rRNA* gene regions of bacterial strains isolated by using PCR with specific primer pairs according to Frank et al. (2008) are 27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT (Jeremy A Frank et al., 2008). The PCR was performed on an Applied Biosystems–Life Technologies–Thermo Fisher Scientific–USA system with the reaction components presented in Table 1 and the thermal cycle shown in Table 2.

The PCR product was subjected to electrophoresis on 1% agarose gels stained with ethidium bromide, and the electrophoretic images were analyzed with a DyNA Light, Dual Intensity UV Transilluminator system. Then, the PCR product of the 16S *rRNA* gene region was purified and directly sequenced with the Sanger method on an ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems) system at Maccrogen Company, Korea (dna.macrogen.com). The nucleotide sequence of the genomic region was determined and aligned by using the program Clustal-X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and edited by using the BioEdit 7.0.5 software (Hall, 1999). The nucleotide sequences of the genomic region were compared with the 16S *rRNA* sequences of the microorganisms published on the World GenBank (GenBank) with the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>). The phylogenetic tree was generated according to the maximum likelihood algorithm of the MEGA X software.

2.5 | Physiological and biochemical characterization

The physiological and biochemical characteristics include Gram staining, oxidase, catalase, oxidase fermentation, bacterial motility, and hemolysis. The bacterial motility was determined with the hanging drop method and hemolysis by culturing on agar supplemented with 5% horse blood, with the Buller's method (2014) (Buller, 2004). The bacterial strains were tested by using the API 20E KIT (Bio-Mérieux, France) following the manufacturer's instructions. The reactions were carried out at 28 °C, and the results were read after 24 hours.

In addition, several biochemical reactions were also carried out to fully understand the characteristics of the isolated strains of *Vibrio*, namely, the Voges-Proskauer test and the fermentation of carbohydrates with the purple Broth Base (Difco, UK) supplemented with 5% glucose, fructose, galactose, glucose, glycerol, maltose, mannose, or ribose. The viability of *Vibrio* strains at different salt concentrations was conducted on the TSB medium supplemented with 0, 1, 6, 8, and 10% NaCl. The *Vibrio* strains isolated in this study were compared with those isolated by Buller (Buller, 2004).

2.6 | Antibiotic susceptibility test

The antibiotic susceptibility of *Vibrio* spp. was tested with the agar plate diffusion method following Bauer et al. (1966) (Bauer, 1966). The bacterial strains *Vibrio* spp. were grown proliferatively in the TSB medium supplemented with 2% NaCl and inoculated at 28 °C for 24 h in a culture cabinet with a shaking speed of 150 rpm. Then, the bacterial cell density was determined by determining the optical density (OD) at 600 nm on a UV-VIS spectrophotometer (U2900, Hitachi, Japan). The preparation was diluted to a concentration corresponding to OD 1 and further to 10⁶ CFU/mL for the following experiments.

One hundred micro-litre of the 10⁶ CFU/mL bacterial solution was evenly spread on the Muhler Hilton agar-agar medium (MHA, Himedia, India), supplemented with 1.5% NaCl and dried at ambient temperature for 30 minutes. Next, the paper plates impregnated with antibiotics were transferred to the media with a pair of sterilized forceps. The antibiotics in the study are tetracycline 30 ug (Te), amoxicillin 10 ug (Ax), neomycin 30 ug (Ne), ampicillin 10 ug (Am), kanamycin 30 ug (Kn), doxycycline 30 ug (Dx), enrofloxacin 5 ug (Ef), erythromycin 15 ug (Er), and cefotaxime 30 ug (Ct). The plates were placed in an incubator at 28°C. The diameter of the sterile ring was measured after 24 hours.

The bacteria's antibiotic susceptibility or resistance to antibiotics was evaluated from the diameter of the sterile ring following the standards of the Clinical and Laboratory Standards Institute (2018) (Sahu, Jain, Mishra, & Prasad, 2018).

3. Results And Discussion

3.1 | Physical, chemical water parameters and sample characteristics

Table 3 displays water pH, temperature, salinity, and dissolved oxygen. The values are within the acceptable range for nurturing Red drums. Out of 35 samples collected from the sites, 27 are infected, accounting for 77.14%. The fish show the following symptoms: tail amputation, haemorrhaging in gills, body, and skin and fluid accumulation in the abdominal cavity. The fish weight ranges from 23 to 25 g with a length between 11 and 16 cm (Table 4).

3.2 | Isolation and identification

In haemorrhaged Red drums (Fig. 2), blue and yellow colonies of *Vibrio* spp. appear on the TCBS medium (Fig. 3) after 24 h of culture. Different parts of the fish body exhibit different degrees of infection, from 11.11% in the brain to 38.89% in the liver (Table 5).

3.3 | Molecular identification and phylogenetic tree generation

PCR product electrophoresis

The 16S *rRNA* region's PCR amplification of all 18 bacterial strains isolated from haemorrhaged Red drums collected at the study sites gives a single band with an amplification rate of 100%. All PCR products of bacterial strains are highly concentrated and sharp. The size of the PCR product is approximately 1,500 bp, consistent with the original expected size (Fig. 4).

Nucleotide sequence of the 16S rRNA gene region

The nucleotide sequence analysis in the *16S rRNA* gene region of the bacterial strains shows that the PCR amplification rate and the successful nucleotide sequence analysis rate equal 100%. The sequence edited with the Bioedit software results in the gene regions sized from 1381 to 1448 bp with a mean at 1441 bp. The occurrence rate of each type of nucleotide in the region indicates that guanidin (G) occupies the first place (31.43–32.04%, mean 31.86%), followed by adenine (A) (24.93–25.35%, mean 25.19%) and timin (T) (uracin, (U)) being the last place (20.51–20.86%, mean 20.64%). The prevalence (G + C) accounts for 53.89–54.37%, mean 54.17% (Table 6).

The *16S rRNA* gene region analysis with the MEGA X software shows that the conserved area for bacterial populations isolated from Red drums has 83/1448 modified nucleotide positions, accounting for 5,732% of the total length of the gene (Table 7).

Molecular identification

The BLAST results from the world gene bank show that the 18 bacterial strains belong to the genus *Vibrio*, with *V. alginolyticus* being the most significant occurrence (67%), followed by *V. fluvialis* and *V. orientalis* with the same rate (17%). Their similarities range from 98.05 to 100%. All nucleotide sequences of the bacterial strains are registered on the world gene bank (Genbank Database) with corresponding reference codes (Table 8).

Phylogenetic tree

The phylogenetic tree generated with the UPGMA method shows the genetic relationships of the 18 strains of *Vibrio* bacteria isolated from Red drums (Fig 5).

Phylogenetic tree shown genetic relationships of 18 strains of *Vibrio* bacteria isolated from hemorrhagic red drum divided into five groups, in which group I includes 3 strains of *Vibrio* bacteria (YHD4, YHD6 and YHD9) these bacterial strains are in the same branch as *V. fluvialis* (CP046756.1 and CP051126.1), group II includes 5 strains (YHD3, YHD8, YHD17, YHD1 and YHD12), these bacteria strains are in the same branch with species of bacteria *V. alginolyticus* (CP046814.1 and CP051109.1), group III includes 3 strains (YHD11, YHD15 and YHD2). These strains belong to the same branch as *V. orientalis* (MN945276.1), group IV consists of 2 strains (YHD13 and YHD14), these strains belong to the same branch as *V. alginolyticus* (KT986139.1 and MN874162.1) and 1 strain belongs to *V. orientalis* (MN86083.1), Group V includes 5 strains (YHD10, YHD18, YHD7, YHD5, and YHD16) all of these strains belong to the same branch and are closely related to *V. alginolyticus* (MN938185.1, MK102585.1, MN843961.1, and KT986138.1) and *V. azureus* species (KT986135.1) (Fig 5).

3.4 | Biochemical characteristics of isolated bacterial strains

The 18 bacterial strains identified in Table 10 were subjected to their morphological, physiological, and biochemical characterization (Fig 6, Table 9).

The physiological and biochemical test with the API 20E KIT (Bio Mérieux, France) indicates that among the 18 strains causing haemorrhage in Red drums, 12 strains belong to *V. alginolyticus*, 3 to *V. fluvialis*, and 3 to *V. orientalis*. According to the Buller's taxonomy key, these strains correspond to codes 414725, 3246126, and 4066106 (Table 8). Specifically, 67.67% of the strains have yellow colonies on the TCBS medium, belonging to the group of Gram-negative bacteria. They are comma-shaped (Fig. 6. A, D), indole unproductive, capable of degrading nitrate, producing catalase, oxidase, and positive Voges–Proskauer reaction. They can also ferment glucose, sucrose, mannitol, sorbitol, and arabinose. One of these strains is capable of producing H₂S (8.33%).

Three over eighteen (16.67%) bacterial strains have yellow colonies on the TCBS medium, belonging to the group of Gram-negative. They are short rod-shaped (Fig. 6. B, E), oxidase-positive, capable of producing catalase and indole, fermenting glucose, mannitol, sucrose, and arabinose but not sorbitol. The rest 3/18 (16.67%) bacterial strains have blue colonies. They appear as short rods and belong to the group of Gram-negative bacteria (Fig. 6. C, F), and they can grow at the NaCl concentration of 1, 6, and 8% but fail in 0 and 10% saline. The reaction is oxidase, catalase, mannitol, and arabinose positive but glucose, sorbitol, sucrose, and H₂S-negative.

3.5 | Antibiotic susceptibility of *Vibrio* strains

All 18 strains of *Vibrio* are entirely resistant to ampicillin, amoxicillin, and erythromycin (Table 10). Generally, the Am resistance of *Vibrio* strains isolated from the marine environment is high (Stabili, Gravili, Boero, Tredici, & Alifano, 2010) Nguyen and Tu (2018) also reported that *V. alginolyticus* causing disease in the caged Cobia cultured in Kien Giang, Vietnam, is resistant to ampicillin, streptomycin, and erythromycin (Nguyen Bao Trung & Dung, 2018).

The isolates are also sensitive to tetracycline and doxycycline. Tetracycline is an antibiotic allowed in aquaculture, so farmers often use it to prevent and treat the diseases caused by bacteria in fish and shrimp farms. Our findings agree well with those of Nguyen and Tu (Nguyen Bao Trung & Dung, 2018). Tetracycline has long been the most commonly used antibiotic in aquaculture in Korea, especially for the species severely infected by *Vibrio* (J.-W. Liu et al., 2006; Morris & Tenney, 1985). The less tetracycline-resistant *Vibrio* strains have also been reported in other studies. According to Liu et al. (2004), the *V. alginolyticus* strains causing disease in Cobia in Taiwan are sensitive to doxycycline, erythromycin, nalidixic acid, oxolinic acid, oxytetracycline, streptomycin, sulphonamide, and tetracycline but resistant to ampicillin and neomycin (P.-C. Liu, Lin, Hsiao, & Lee, 2004).

These 12 strains of *V. alginolyticus* are sensitive to cefotaxime (75%), enrofloxacin (66.7%), kanamycin (50%), and neomycin (41.7%); 3 strains of *V. fluvialis* and 3 strains of *V. orientalis* are sensitive to cefotaxime (66.7%), enrofloxacin (66.7%), kanamycin (66.7%), and neomycin (33.3%). Kang et al. (2016) (Kang, Shin, Jang, Jung, & So, 2016) reported that 15 *V. alginolyticus* strains isolated from oysters in Korea are completely resistant to ampicillin, highly sensitive to tetracycline (100%), cefotaxime (86.7%), and kanamycin (73.3%), while moderately resistant to erythromycin (100%).

4. Conclusions

Our findings indicate twelve *V. alginolyticus* strains, three *V. fluvialis* strains, and three *V. orientalis* strains isolated from the haemorrhaged Red drums cultured in cages in Thua Thien Hue, Vietnam. These *Vibrio* strains have 16S rRNA gene homology on the GenBank, ranging from 98.05 to 100% and correspond to codes 4147125, 3246126, and 4066106. They are all susceptible to tetracycline and doxycycline. The *V. fluvialis* and *V. orientalis* strains are entirely resistant to ampicillin, amoxicillin, and erythromycin.

Declarations

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CONFLICT OF INTEREST

There are no any conflicts of interest regarding to this article and all of authors agreed to submit this manuscript for publishing.

DATA AVAILABILITY STATEMENT

The authors declare that we do not have any shared data available

ANIMAL ETHICS STATEMENT

The use of experimental animals follows the regulation of ethics for animal use in scientific work and was approved by international, and rules in Vietnam. The Hue University's scientific Committee agreed for setting up the experiments and monitoring the outcomes by No. DHH2021-02-153.

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Tables

TABLE 1 PCR components

Element	Volume (µL)
2 × PCR master mix (2.4 mM dNTP each, 0.3 units Taq DNA polymerase)	25
10 pmol of 27F primer	1
10 pmol/µL of 1492 primer	1
DNA (50 ng/µL)	1
Sterile distilled water	22
Total	50

TABLE 2 Thermal cycle of PCR amplified *16S rRNA* gene region

Temperature, °C	Time	Cycle
95	5 min	1
95	1 min	30
57	50 s	
72	1 min	
72	10 min	Lengthen
4	Storage temperatures	

Table 3. Physical and chemical water parameters

Parameter	1	2		3		4		
	Mean $\pm$ SD	Min-Max	Mean $\pm$ SD	Min-Max	Mean $\pm$ SD	Min-Max	Mean $\pm$ SD	Min-Max
pH	7.95 $\pm$ 0.19	7.8–8.2	7.75 $\pm$ 0.31	7.5–8.2	7.83 $\pm$ 0.21	7.6–8.1	7.75 $\pm$ 0.24	7.5–8.0
T (° C)	26.40 $\pm$ 0.64	25.5–27.0	26.85 $\pm$ 1.01	26.0–28.0	26.23 $\pm$ 1.32	25.0–27.0	26.40 $\pm$ 1.14	25–27.6
Salinity (‰)	26.75 $\pm$ 4.27	21.0–30.0	25.00 $\pm$ 2.16	23.0–28.0	28.00 $\pm$ 1.41	27.0–30.0	28.75 $\pm$ 0.96	28–30
DO (mg/L)	6.00 $\pm$ 0.41	5.5–6.5	6.13 $\pm$ 0.25	6.0–6.5	6.38 $\pm$ 0.48	6.0–7.0	6.63 $\pm$ 0.85	5.5–7.5

TABLE 4 Fish infection rate and physical characteristics

Point	Samples	Infection rate (%)	Length (cm)	Weigth (g)
1	10	80.00	11.65 $\pm$ 0.84	24.77 $\pm$ 1.13
2	7	71.00	15.21 $\pm$ 1.65	26.14 $\pm$ 1.57
3	6	66.67	12.13 $\pm$ 0.55	24.31 $\pm$ 1.16
4	12	83.33	13.89 $\pm$ 1.64	24.75 $\pm$ 1.43
Total	35	77.14	13.22 $\pm$ 1.32	24.99 $\pm$ 0.56

TABLE 5 Ratio of bacterial strains isolated from the organs of haemorrhaged Red drums fish cultured in Thua Thien Hue province

Organ of isolation	Number of bacteria	Ratio (%)
Brain	2	11.11
Liver	7	38.89
Kidney	5	27.78
Spleen	4	22.22
Total	18	100

TABLE 6 Nucleotide sequence analysis of each 16S rRNA gene region

No.	Sample code	T (U) (%)	C (%)	A (%)	G (%)	G + C (%)	Gen length (bp)
1	YHD1	20.64	22.59	25.34	31.43	54.02	1381
2	YHD2	20.57	22.29	25.28	31.86	54.16	1444
3	YHD3	20.51	22.31	25.35	31.84	54.14	1448
4	YHD4	20.86	22.24	24.93	31.98	54.21	1448
5	YHD5	20.53	22.33	25.10	32.04	54.37	1442
6	YHD6	20.86	22.24	24.93	31.98	54.21	1448
7	YHD7	20.72	22.25	25.16	31.88	54.12	1443
8	YHD8	20.51	22.31	25.35	31.84	54.14	1448
9	YHD9	20.86	22.24	24.93	31.98	54.21	1448
10	YHD10	20.72	22.25	25.16	31.88	54.12	1443
11	YHD11	20.57	22.29	25.28	31.86	54.16	1444
12	YHD12	20.76	22.43	25.35	31.46	53.89	1440
13	YHD13	20.55	22.35	25.19	31.90	54.26	1445
14	YHD14	20.55	22.35	25.19	31.90	54.26	1445
15	YHD15	20.57	22.29	25.28	31.86	54.16	1444
16	YHD16	20.53	22.33	25.10	32.04	54.37	1442
17	YHD17	20.51	22.31	25.35	31.84	54.14	1448
18	YHD18	20.72	22.25	25.16	31.88	54.12	1443
	Mean	20.64	22.31	25.19	31.86	54.17	1441

TABLE 7 Some characteristics based on *16S rRNA* gene regions of bacterial strains in PCR populations

PCR success rate (%)	Sequencing success rate (%)	Total genomic length (bp)	Percentage of polymorphic nucleotide sites (%)
100	100	1381–1448	5.732

TABLE 8 Phylogenetic affiliation of isolates based on *16S rRNA* gene sequencing by using BLAST program in GenBank database based on sequence similarity

No	Isolated	Genbank code	GenBank reference	Similarity (%)
1	<i>V. alginolyticus</i> strain, YHD1	MZ753696	MH298564.1	98.05
2	<i>V. alginolyticus</i> strain, YHD3	MZ753698	CP051109.1	99.59
3	<i>V. alginolyticus</i> strain, YHD5	MZ753700	MN843961.1	99.72
4	<i>V. alginolyticus</i> strain, YHD7	MZ753702	MN938185.1	99.86
5	<i>V. alginolyticus</i> strain, YHD8	MZ753703	CP051109.1	99.59
6	<i>V. alginolyticus</i> strain, YHD10	MZ753705	MN938185.1	99.86
7	<i>V. alginolyticus</i> strain, YHD12	MZ753707	MH298564.1	98.05
8	<i>V. alginolyticus</i> strain, YHD13	MZ753708	MN938360.1	99.65
9	<i>V. alginolyticus</i> strain, YHD14	MZ753709	MN938360.1	99.65
10	<i>V. alginolyticus</i> strain, YHD16	MZ753711	MN843961.1	99.72
11	<i>V. alginolyticus</i> strain, YHD17	MZ753712	CP051109.1	99.59
12	<i>V. alginolyticus</i> strain, YHD18	MZ753713	MN938185.1	99.86
13	<i>V. fluvialis</i> strain, YHD4	MZ753699	CP051126.1	100
14	<i>V. fluvialis</i> strain, YHD6	MZ753701	CP051126.1	100
15	<i>V. fluvialis</i> strain, YHD9	MZ753704	CP051126.1	100
16	<i>V. orientalis</i> strain, YHD11	MZ753706	MN945276.1	100
17	<i>V. orientalis</i> strain, YHD2	MZ753697	MN945276.1	100
18	<i>V. orientalis</i> strain, YHD15	MZ753710	MN945276.1	100

Genbank registration Code No: <https://submit.ncbi.nlm.nih.gov/subs/?search=SUB10184933>

TABLE 9 Physiological and biochemical parameters of *Vibrio* spp. isolated from diseased Red drums

No.	Indicator	<i>V. alginolyticus</i> (Buller, 2014)	<i>V. alginolyticus</i> n = 12	<i>V. fluvialis</i> (Buller, 2014)	<i>V. fluvialis</i> n = 3	<i>V. orientalis</i> n = 3 (Buller,2014)	<i>V. orientalis</i> n = 3
1	Gram staining	–	–	–	–	–	–
2	Form	Comma	Comma	Rod	rod	rod	rod
3	Colonies on TCBS	Yellow	Yellow	Yellow	Yellow	Green	Green
4	Colonies on TSA	ND	ND	Milky white	Milky white	ND	Milky white
5	Growth in NaCl solution (%)						
	0	–	–	–	–	–	–
	1	ND	+	+	+	+	+
	6	+	+	+	+	+	+
	8	+	+(10/12)	–	–	+	+
	10	–/+	–/+	–	–	–	–
6	API 20E	414725	414725	3246126	3246126	4066106	4066106
7	Oxidase	+	+	+	+	+	+
8	Catalase	+	+	+	+	+	+
9	H ₂ S synthesis	–	– (11/12)	–	–	–	–
10	NO ₃ decomposition	+	+	+	+	+	+
11	Indol	–	–	+	+	+	+
12	Voges–Proskauer	+	+	–	–	–	–
13	Citrate use	+	+	+	+	–	–
14	Glucose	+	+	+	+	–	–
15	Mannitol	+	+	+	+	+	+
16	Sorbitol	+	+	–	–	–	–
17	Sucrose	+	+	+	+	–	–
18	Arabinose	–	–	+	+	+	+

TABLE 10. Antibiotic susceptibility rate of *Vibrio* strains isolated from diseased Red drums

Antibiotics	Rate (%) <i>V. alginolyticus</i> , n = 12			Rate (%) <i>V. fluvialis</i> , n = 3			Rate (%) <i>V. orientalis</i> , n = 3		
	S	I	R	S	I	R	S	I	R
β-lactams									
Cefotaxime (Ct, 30 ug)	75	25	0	66.7	33.3	0	66.7	33.3	0
Ampicillin (Am, 10 ug)	0	0	100	0	0	100	0	0	100
Amoxicillin (Ax, 10 ug)	0	0	100	0	0	100	0	0	100
Quinolones									
Enrofloxacin (Ef, 5 ug)	66.7	33.3	0	66.7	33.3	0	66.7	33.3	0
Aminoglycosides									
Kanamycin (Kn, 30 ug)	50	33.3	16.7	66.7	33.3	0	66.7	33.3	0
Neomycin (Ne, 30 ug)	41.7	33.3	25	33.3	33.3	33.3	33.3	33.3	33.3
Macroid									
Erythromycin (Er, 15 ug)	0	0	100	0	0	100	0	0	100
Tetracyclines									
Tetracycline (Te, 30 ug)	100	0	0	100	0	0	100	0	0
Doxycycline (Dx, 30 ug)	100	0	0	100	0	0	100	0	0

S: Sensitive; I: Intermediate; R: Resistant

Figures

Figure 1

Sampling sites

Figure 2

Characteristic signs of haemorrhagic disease: red spots on fins, torn tail, and haemorrhage (arrows, Figures A, B); mucus accumulation in abdominal cavity (arrow, Fig. B)

Figure 3

Bacterial colonies isolated on TCBS medium

Figure 4

Electrophoresis of PCR product 16S rRNA
M: DNA mass scale (HyperLadder™ 1 kb (200 bp to 10,037 bp), Bioline, Meridian Bioscience

Figure 5

Phylogenetic tree of *Vibrio* spp.

Figure 6

Colony appearance on TCBS: A: *V. alginolyticus*; B: *V. fluvialis*; C: *V. orientalis*; Gram staining: D: *V. alginolyticus*; E: *V. fluvialis*; F: *V. orientalis*; identification of bacteria isolated from Red drums with API 20E KIT: G: *V. alginolyticus*; H: *V. fluvialis*; I: *V. orientalis*.