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Isolation and characterisation of *Vibrio harveyi* as etiological agent of foot pustule disease in the abalone *Haliotis discus hannai* Ino 1953

R. X. WANG, T. YAO, X. J. LIU AND J. Y. WANG*

South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou - 510 300, China
**Key Laboratory of South China Sea Fishery Resources Exploitation and Utilisation, Ministry of Agriculture, P. R. China*
e-mail: wrxlxwlld@163.com

ABSTRACT

Foot pustule disease is an important disease affecting abalones. In the present study, microbiological and histopathological investigations were carried out in the Japanese abalone *Haliotis discus hannai*, affected by foot pustule disease. Diseased abalones became lethargic, weak and eventually died. The remarkable symptoms were apparent foot pustules and atrophy of the foot muscle. A predominant bacterium designated as BV2 was isolated from the pustules of diseased abalones. Experimental infection by immersion challenge showed that BV2 was virulent to abalones and caused symptoms of foot pustule disease with median lethal dose value of $\approx 7.76\times10^5$ cfu ml^{-1}. BV2 was infectious to all tested abalones with high mortality. Histopathological investigations showed degeneration and collapse of foot muscles as well as connective tissues. Tissues in round pustules were necrotic and disorganised. The BV2 bacterium was identified as *Vibrio harveyi* based on the results of phenotypic and biochemical tests as well as 16S rRNA gene sequence analysis.. The bacterium was found resistant to kanamycin and clindamycin, and sensitive to other 11 antibiotics tested.

Keywords: Abalone, Antibiotics, Foot pustule, *Haliotis discus hannai* Pathogen, *Vibrio harveyi*

Introduction

Abalones are marine gastropods that can be found worldwide in temperate and tropical waters from intertidal zones to a depth of 40 m. Abalone aquaculture began in the 1960s in the United States and Japan (McBride, 1998) and has since become a well-established industry in China, Korea, Australia, Chile, USA, Mexico and New Zealand (Flores-Aguilar *et al.*, 2007; Allsopp *et al.,* 2012). More than 90% of the world abalone production is based on farming (Jo *et al.,* 2014). China is one of the world's primary abalone-producing countries, with production of 110380 t during 2013 (Chinese fishery statistical yearbook, 2014). *Haliotis discus hannai* Ino 1953 and their hybrids are the predominant abalone species cultured onshore in coastal areas of China. Abalone aquaculture is an intensive industry, which is often land-based, with tanks stocked at high density. Given the crowding and suboptimal environmental conditions compared with conditions in the wild (Shepherd, 1973; Goodsell *et al.,* 2006; Gorski and Nugegoda, 2006), abalones in aquaculture systems are vulnerable to infection by microorganisms that are often ubiquitous (Jo *et al.,* 2014).

Chinese abalone farmers face significant challenges as the financial commitments are high to bring temperate abalones to market size which can take up to 3 years. Deteriorating environmental conditions and infectious diseases have also become major challenges that have long threatened the abalone aquaculture industry, especially during summer (Wang *et al.,* 2008). Outbreaks of infectious diseases leading to mass mortality events of cultured abalones, result in catastrophic losses to aquaculturists. *Vibrio* has been described as a predominant pathogenic bacterium of cultured abalones (Gauger and Gomez-Chiarri, 2002; Wang *et al.,* 2006; Travers *et al.,* 2010; Wang *et al.,* 2010; Schikorski *et al.,* 2013). Vibriosis is the most common disease caused by pathogenic bacteria in halophilic environments and this disease affects several groups of organisms (Cardinaud *et al.,* 2015; Ananda Raja *et al.,* 2017a, b). Mortality in physiologically depressed abalone, *H. tuberculata*, in seawater at temperatures higher than 16°C has been attributed to the bacterial pathogen *Vibrio harveyi* (Travers *et al.,* 2009; Cardinaud *et al.,* 2014). Abalone foot pustule disease is a serious and chronic disease caused by *Vibrio* spp. infection in both natural and farmed populations; which was recorded for the first time in 1993 (Li *et al.,* 1997; Huang, 2005). Almost all infected abalones display obvious white pustules and present signs of atrophy in the feet as well as a malformed appearance. The epidemic nature of this disease leads to high rate of mortality. Furthermore, this disease has been associated with serious economic losses (Chen *et al.,* 2011).

The current study reports the results of investigations on an epizootic foot pustule disease resulting in mass mortality of cultured abalone *H. discus hannai* hybrids (*H. discus hannai* from Shandong province x *H. discus hannai* from Fujian province), from Guangdong Province, China.

Materials and methods

Clinical signs of diseased abalones

Diseased abalones *H. discus hannai* (n = 40; 50 ± 3 mm, 2.5 years old) were collected from an abalone farm (seawater temperature 28.5°C) in Guangdong Province, China in June 2014. The abalones were transported to the laboratory in about 3 h. To keep the abalones alive during transport, they were placed in polystyrene foam boxes containing ice packs. Clinical signs of diseased abalones were recorded and lesions measured and sampled for analyses.

Isolation and identification of bacteria

Samples collected aseptically from the foot muscle (focusing on the pustules), visceral mass and haemolymph were respectively streaked on brain-heart infusion (BHI) agar and thiosulfate citrate bile salts sucrose (TCBS) agar plates. The plates were incubated at 28°C for 96 h. Dominant colonies were purified using gradient dilution and streak plate technique. Pure isolates were then stored at -80°C in normal sterile saline (NSS, 0.85%) with 20% glycerol (Wang *et al*., 2013b).

The dominant bacterial isolate designated as BV2 was identified by phenotypic traits using API 32E system (BioMerieux, France) as per Wang *et al*. (2013b) as well as by electron microscopy. The 16S rDNA gene of the isolate was amplified by PCR using the bacterial universal primers, 27f 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492r 5′-GGTTACCTTGTTACGACTT- 3′ (Polz and Cavanaugh 1998). The *tox*R gene was also amplified using the primers *tox*R-f 5'-GAAGCAGCACTCACCGAT-3' and *tox*R-r 5'-G GTGAAGACTCATCAGCA-3'(Conejero *et al*., 2003). DNA extraction, PCR amplification and sequencing were done as per Kumar *et al*. (2004) and Wang *et al*. (2013b). Similar sequences from the GenBank database were selected for alignment, using CLUSTALX software (Thompson *et al*., 1997). The resulting alignment was checked and corrected manually using BioEdit (Hall, 1999). The neighbour-joining method and the maximum parsimony (MP) method were used to create phylogenetic tree, in MEGA3.1 (Kumar *et al*., 2004), using Kimura two-parameter model and pairwise deletion for gaps. MP analyses were conducted with PAUP*4.0b10. Clade support for both analyses was assessed by bootstrapping with 1000 replicates.

Sensitivity to antibacterial agents

The strain BV2 was tested for susceptibility against a total of 13 different antibiotics by the Kirby-Bauer (K-B) diffusion method (Wang *et al*., 2013b).

Pathogenicity of dominant bacterial isolate

The bacteria was cultured on BHI agar for 18-24 h (28°C) and suspended and diluted in NSS. The bacterial density in each suspension was estimated by serial dilution and plating on BHI agar plates in triplicates. After 24 h incubation, colonies on the plates were counted. Abalones *H. discus hannai* from quarantined stocks certified as disease-free were used to assess pathogenicity of the isolate. The abalones were acclimated for 3-4 days in 50 l tanks filled with oxygenated and filtered seawater at 28°C. During this period, the abalones were not fed. Following acclimation, 12 groups (five experimental groups and one control, each group had 2 replicates) were formed, each containing 30 abalones (average weight = 3.5±0.5 g, average shell length = 3.5±0.5 cm, in 12 tanks each filled with 50 l seawater). The abalones in the experimental groups were exposed to seawater containing the bacterial suspensions at concentrations of 8.7×10^3, 8.7×10^4, 8.7×10^5, 8.7×10^6, and 8.7×10^7 CFU ml^{-1} for 16 days, with sterile seawater used for preparation of bacterial suspension. The control group animals were maintained in normal seawater. The abalones were maintained for 16 days (the conditions identical to the acclimation period), during which disease symptoms and mortality were monitored daily. Samples collected aseptically from the feet and viscera of infected abalones were used to inoculate TCBS plates and BHI agar plates, which were then incubated at 28°C for 96 h.

Histopathology

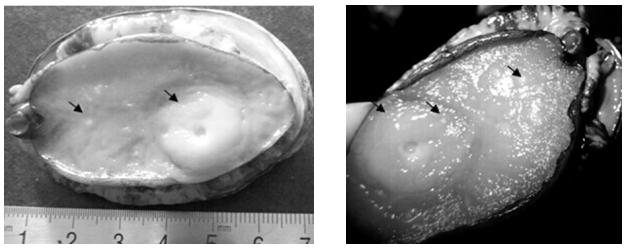
Infected tissues from the diseased abalones and healthy ones were fixed in 10% neutral buffered formalin, dehydrated in ascending series of alcohol, embedded in paraffin, sectioned at 4-5 µm and stained using hematoxylin and eosin (H&E).

Results and discussion

Characteristics of diseased abalones and pathogenicity of the dominant bacteria

Naturally infected abalones (shell length = 50 mm) exhibited foot pustules and died in large numbers, with a mortality rate of approximately 60% in the farm in Shenzhen. During the early stages of infection (after 5-7 days), the foot contracted and presented small white spots. As the disease progressed, the white spots enlarged and developed into pustules with maximum diameter of 2 cm each in 12 or 15 days. At this stage, the pustules tended

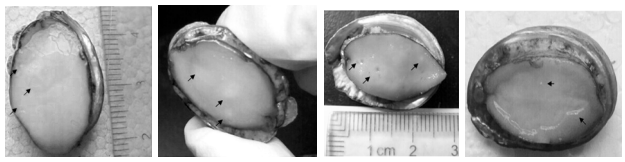
to rupture, releasing white pus and leaving holes 2~5 mm deep (Fig. 1). The dominant bacterium isolated from the nidus (pustules) of diseased abalones on BHI agar plates was designated as strain BV2. Pathogenicity assays by immersion challenge in healthy abalones revealed that strain BV2 was highly virulent (Table 1, Fig. 2).



(a) (b)

Fig. 1. Initial symptoms of pustules (arrows) scattered in the foot of naturally infected abalones

infected abalones showed pustules in the foot (Fig. 3), which were similar to those in the naturally infected abalones. Similar bacterial strain as BV2 was re-isolated from the foot and entrails of moribund abalones. Histopathological examination showed that foot muscles and connective tissues degenerated and collapsed. Tissues in the round pustule were necrotic and disorganised (Fig. 4).



(a) (b) (c) (d)

Fig. 3. Pustules (arrows) scattered in the foot of diseased abalone challenged by strain BV2

Table 1. Pathogenicity of strain BV2 to abalone *H. discus hannai*

Group	Final concentration of BV2 in seawater (CFU ml^{-1})	No. of abalones in each group	Average mortality rate (Immersion challenge for 16 days)
1	8.7×10^3	30	60%
2	8.7×10^4	30	75%
3	8.7×10^5	30	85%
4	8.7×10^6	30	90%
5	8.7×10^7	30	100%
Control	Normal seawater	30	0

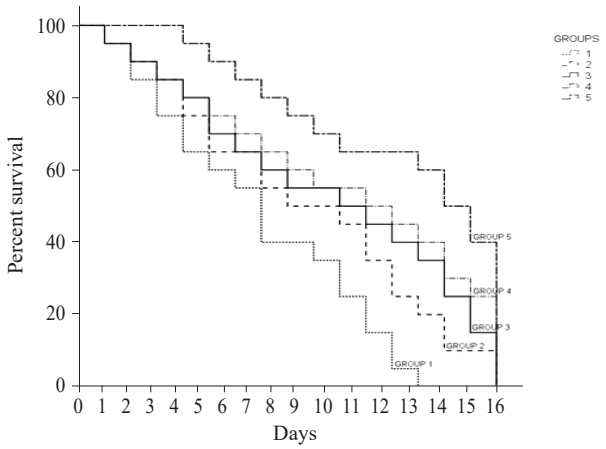
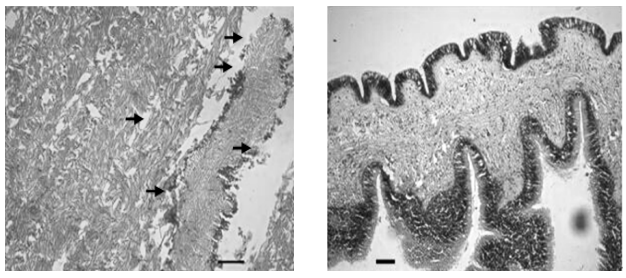


Fig. 2. Kaplan-Meier survival analysis of diseased abalone, immersion challenged with strain BV2: GROUP 1, 8.7×10^7 CFU ml^{-1}; GROUP 2, 8.7×10^6 CFU ml^{-1}; GROUP 3, 8.7×10^5 CFU ml^{-1}; GROUP 4, 8.7×10^4 CFU ml^{-1}; GROUP 5, 8.7×10^3 CFU ml^{-1}. (n = 30 per group)

According to the improved Karber's method, the LD_{50} value of BV2 for immersion was 7.76×10^5 CFU ml^{-1} of seawater (temperature = 29.5°C, pH 8.15, 16 days after immersion exposure). Experimentally

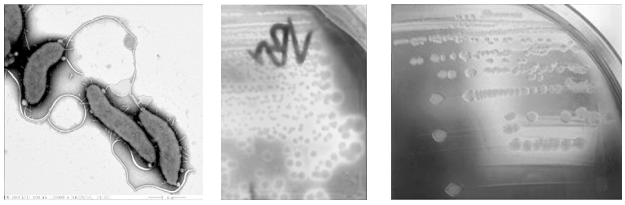


(a) (b)

Fig. 4. Histological section of foot tissue from (a): Diseased abalone (scale bar = 50 µm); (b): Healthy abalone (scale bar = 90 µm) (H&E). Arrows indicate necrotic and disorganised foot pustules

Characterisation and identification of bacterial strain BV2

The isolate BV2 was found to be a Gram-negative, rod-shaped, and curved organism with one long flagellum and many pili (Fig. 5a). This strain was sensitive to the *Vibrio* inhibitor O/129 (10 and 150 µg ml^{-1}). The BV2 isolate also demonstrated obvious ß-hemolysin activity (Fig. 5b). Colonies of BV2 on TCBS agar were golden yellow, orbicular and smooth. The average diameter of the colonies was 2-3 mm after being cultured for 24 h (Fig. 5c).



(a) (b) (c)

Fig. 5. Morphology of strain BV2. a: Electron micrograph of BV2 after negative staining; b: strong ß-hemolysis by BV2 on blood agar; c: BV2 cultured on TCBS plate

Strain BV2 presented a relatively narrow salinity tolerance range and did not grow in 0 or 10% NaCl but grew in 1, 2, 6 and 8% (WPV) NaCl. Strain BV2 was identified as *V. harveyi* using the API 32E kit (%id: 99.9, T: 0.51) which presented similar phenotypic traits as that of type strain *V. harveyi* ATCC14126 (Table 2). The strong ß-hemolysin (an important virulence factor) activity of BV2, indicates its virulence and all the infected abalones were kraurotic compared with the controls.

The edited and assembled 16S rDNA nucleotide sequences of the isolate BV2 resulted in a full-length sequence of 1420 bp. BLAST search of the GenBank database revealed that the gene sequence showed high similarity to members of *V. harveyi* (99% similarity, Fig. 6). Based on the similarity in more than 28 biochemical indices (employing API 32E system), with the type strain *V. harveyi* ATCC14126; and based on 16S rDNA sequence analysis, the strain BV2 was identified as *V. harveyi*.

Various diseases commonly caused by bacterial pathogens belonging to the genus *Vibrio* (Liu *et al*., 2003; Cai *et al*., 2007; Travers *et al*., 2008; Haldara *et al*., 2010; Wang *et al*., 2013a) have threatened the sustainable development of the abalone industry. Vibrios

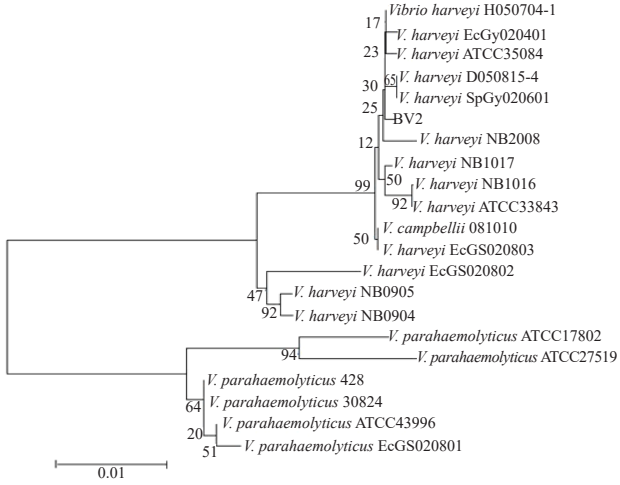


Fig. 6. Phylogenetic tree based on 16S rDNA gene sequence of BV2

belonging to the *harveyi* clade comprises important pathogens of numerous marine animals. *V. harveyi* has been widely recognised as a common pathogen of many commercially cultured fish and shellfish species worldwide, including the Asian seabass *Lates calcarifer* (Tendencia, 2002; Ransangan *et al*., 2012), sea horse *Hippocampus kuda* (Tendencia, 2004), sea bream *Sparus aurata*

Table 2. Important phenotypic traits of BV2 in comparison with reference strain, *V. harveyi* ATCC14126

Biochemical tests	BV2	*V. harveyi* ATCC14126	Biochemical tests	BV2	*V. harveyi* ATCC14126
D-glucose	+	+	TDA	-	+
Saccharose	+	+	Catalase	-	-
Xylose	-	-	Urease	+	+
Lactose	-	-	Gelatinase	+	+
Raffinose	-	-	Arginine dihydrolase	+	+
L-arabinose	-	-	Ornithine decarboxylase	-	-
D-arabite	-	-	Lysine decarboxylase	-	-
L-rhamnose	-	-	L-asparagic acid arylamine	-	-
α-maltobiose	-	-	α- galactosidase	-	-
D-maltobiose	+	+	β-galactosidase	-	-
D-cellobiose	+	+	β- glucosidase	+	+
D-mycose	+	+	β-glucuronidase	+	+
Antiquity-sugar	-	-	V-P	-	-
D-mannite	-	-	Indole	-	-
Inose	+	+	H_2S	+	+
Adonitol	-	-	Nitrate	-	-
Sorbierite	+	+	Citrate	-	-
Salicin	-	-	Malonate	-	-
Oxidase	+	+	D-Galactose acid	-	-
ONPG	+	+	β-acet-glucosamine	-	-

(Haldar *et al*., 2010), red drum *Sciaenops ocellatus,* cobia *Rachycentron canadum* (Liu *et al*., 2001; 2004), shrimp *Penaeus chinensis* (Vandenberghe *et al*., 1998), Arabian surgeon fish *Acanthurus sohal* (Mahmoud and Manal, 2013) and abalones *H. tuberculata* and *H. diversicolor* (Nicolas *et al*., 2002; Huang *et al*., 2005; Sawabe *et al*., 2007; Jiang *et al*., 2010; Schikorski *et al*., 2013). However, only less than 5% of *V. harveyi* strains are believed to be capable of initiating disease in humans (Kanga *et al*., 2014).

Foot pustule disease is a serious concern for abalone aquaculture, which results in mass mortalities of abalones in China, especially during summer. The present study demonstrated that *V. harveyi* was a single pathogen that caused foot pustule disease in *H. discus hannai* with LD_{50} value of 7.76×10^5 CFU ml^{-1} in immersion challenge. In recent years, though *V. harveyi* has frequently been reported as a causative bacterium for disease in mollusks, including abalones and oysters, affirming its pathogenicity is difficult because the symptoms are not visible (Nicolas *et al*., 2002; Wang *et al*., 2010; Wang *et al*., 2012; Wang *et al*., 2013a; Cardinaud *et al*., 2015; Sweet and Bateman, 2015). The LD50 values of many *V. harveyi* strains were reported to range from 10^4 to 10^6 CFU ml^{-1} (Sawabe *et al*., 2007). In this study, symptoms caused by BV2 was different from that caused by other pathogenic *V. harveyi* strains (Sawabe *et al*., 2007; Travers *et al*., 2009; Qiang, 2013).

Sensitivity to antibiotics

The pathogenic bacterium BV2 was found resistant to kanamycin and clindamycin while it was sensitive to all other antibiotics tested (Table 3), suggesting the possibility of employing chemotherapeutic agents to control this pathogen. However, selective pressure when using antibiotics may increase or even induce antibiotic resistance among bacteria (Alderman *et al*., 1998; Smith, 2008; Heuer *et al*., 2009; Wang *et al*., 2014; Wang *et al*., 2015) and hence use of antibiotics in aquaculture facilities should be controlled strictly. Other control methods such as the use of probiotics and prebiotics (Ravi *et al*., 2007), UV treatment of seawater, disinfection and frequent cleaning of facilities and also lowering stocking density to avoid stress to abalones can be tried preemptively before diseases occur.

Table 3. Antibiotic susceptibility of BV2 to 13 different antibiotics

Antibiotic	Antibiotic concentration per disc	Susceptibility
Penicillin	10 u	I
Sulfamethoxazole/ Trimethoprium	23.75/1.25 µg	S
Erythromycin	15 µg	I
Kanamycin	30 µg	R
Gentamicin	10 µg	I
Florfenicol	30 µg	S
Tetracycline	30 µg	I
Neomycin	30 µg	I
Streptomycin	10 µg	S
Clindamycin	20 µg	R
Doxycycline	30 µg	S
Azithromycin	15 µg	S
Cefixime	30 µg	S

R: Resistant, I: Intermediate, S: Sensitive

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