Received: 22 February 2018 | Revised: 27 June 2018 | Accepted: 27 June 2018

DOI: 10.1111/tbed.12967

ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

Vibrio vulnificus in aquariums is a novel threat to marine mammals and public health

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Funding information
State Forestry Administration of China; Chinese Academy of Sciences, Grant/Award Number: CZBZX-1

Abstract

Vibrio vulnificus is a Gram-negative, curved, obligate halophilic marine bacterium that exclusively exists in coastal seawaters. Previous studies revealed that *V. vulnificus* is one of the most dangerous foodborne zoonotic pathogens for human beings. However, it remains unknown whether marine mammals can be infected by *V. vulnificus*. In May 2016, a captive spotted seal (*Phoca largha*) died due to septicemia induced by *V. vulnificus*. Upon post-mortem examination, *V. vulnificus* was isolated, identified, and named as BJ-PH01. Further analysis showed that BJ-PH01 belongs to biotype 1 and the Clinical genotype. Furthermore, we performed an epidemiological investigation of *V. vulnificus* in six aquariums in northern China. As a result, *V. vulnificus* was successfully isolated from all investigated aquariums. The positive rates ranged from 20% to 100% in each investigated aquarium. During the investigation, 12 strains of *V. vulnificus* were isolated, and all 12 isolates were classified into biotype 1. Eleven of the 12 isolates belonged to the Clinical genotype, and one isolate belonged to the Environmental genotype. All 12 isolated *V. vulnificus* strains showed limited antibiotic resistance. Overall, our work demonstrated that *V. vulnificus* is frequently distributed in aquariums, thus constituting a threat to captive marine mammals and to public health.

KEYWORDS
epidemiology, *Phoca largha*, Septicemia, *Vibrio vulnificus*

1 | INTRODUCTION

Vibrio vulnificus (*V. vulnificus*) is a Gram-negative bacterium naturally existing in estuarial and coastal environments throughout the world. *V. vulnificus* infection is a highly lethal disease, which is responsible for 95% of all seafood-related deaths in the United States, with a fatality rate of approximately 50%. Due to its capacity to cause rapid death among high-risk populations, *V. vulnificus* is considered one of the most dangerous foodborne pathogens for humans (Baker-Austin & Oliver, 2016; Oliver, 2005).

Vibrio vulnificus causes human infection through oral ingestion and wound infection (Kashimoto et al., 2015; Madiyal et al., 2016). Oral ingestion mainly leads to primary septicemia when people consume contaminated raw or uncooked seafood (Kim, Bae, Ma, & Kim, 2015). Symptoms of primary septicemia include fever, chills, nausea, and hypotension. Wound infection is generally acquired through the exposure of a preexisting wound to contaminated seawater or shellfish, resulting in fulminant necrotizing skin and soft tissue infections (Huang et al., 2016; Kotton, Soboh, & Bisharat, 2015). Previous reports indicated that *V. vulnificus* can only affect humans and other primates. Although *V. vulnificus* accumulates in oysters due to their filter feeding of particles from seawater, *V. vulnificus* infection causes no clinical signs in shellfish. Therefore, it is difficult to identify *V. vulnificus*-containing oysters without laboratory detection.

The incidence of *V. vulnificus* infection in humans is associated with multiple risk factors, including (a) seawater temperature and

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salinity, (b) season, (c) gender and age, (d) preexisting chronic diseases, and (e) bacterium virulence. As reported, the majority of human infections occur in the subtropical regions from April to November. People over the age of 40 are predominantly infected. Moreover, males are more susceptible than females (Ito et al., 2012), perhaps due to the role of estrogen in protecting against the bacterium's endotoxins (Miyamoto et al., 1999; Soucy, Boivin, Labrie, & Rivest, 2005). As mentioned previously, patients with underlying chronic diseases, including alcoholism, diabetes, cancer, or renal diseases, are more susceptible to *V. vulnificus* (Bross, Soch, Morales, & Mitchell, 2007a). Several other risk factors contribute to the high pathogenicity of *V. vulnificus* in humans, such as capsule, iron, and the *vcg* gene (Jones & Oliver, 2009).

Marine mammals are important inhabitants of tropical and temperate regions, especially offshore areas of the sea (Schipper et al., 2008). There is an overlap in the distributions of *V. vulnificus* and marine mammals. No evidence has shown that marine mammals can be infected by *V. vulnificus*; however, the concerns cannot be excluded. Here, we provide evidence that marine mammals can also be infected by *V. vulnificus*. Further investigation showed that *V. vulnificus* is ubiquitous in aquariums, thus revealing a novel threat to captive marine animals and human beings in aquariums.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Animal studies were performed in strict accordance with the Guidelines for the Care and Use of Animals in Research, which is issued by the Institute of Zoology, Chinese Academy of Sciences. This study was evaluated and approved by the Animal Ethics Committee of Institute of Zoology, Chinese Academy of Sciences. All experiments were conducted in a Biosafety Level 2 (BSL-2) facility.

2.2 | Tissue sample collection and handling

2.2.1 | Sample collection

An autopsy was performed after the spotted seal died. Pathological lesions were observed and recorded. Tissue samples (i.e., lung, liver, stomach, spleen, intestine, kidney, and heart) were sterilely collected. A festered sample was collected from the trauma injury. All samples were transported on ice and analyzed immediately.

2.2.2 | Sample handling

Tissue samples were cultured on blood agar (Oxoid, UK), MacConkey agar (Oxoid, UK), chocolate agar (Oxoid, UK), and thiosulphate-citrate-bile-salt-sucrose (TCBS) agar (Oxoid, UK). All plates were placed in aerobic or anaerobic conditions at 37°C for 18–24 hr. From each plate, at least 24 single colonies were selected for identification, and all the selected colonies were preliminarily identified by 16S rDNA sequencing. The results were further confirmed by the BD Phoenix automated Microbiology System (BD Diagnostic Systems, Sparks, MD.) (Stefaniuk, Baraniak, Gniadkowski, & Hryniewicz, 2003) and species-specific PCR amplification. Several known viral pathogens that have been reported to infect marine mammals were also detected by virus-specific PCR, including type A influenza virus (IAV), phocine distemper virus (PDV), coronavirus, and rotavirus. The primers used in this study are listed in Table 1 (Chatzidaki-Livanis, Jones, & Wright, 2006; Gómara, Wong, Blome, Desselberger, & Gray, 2002; Hill et al., 1991; Lau et al., 2005; Miller et al., 2011; Reynaud, Pitchford, De Decker, Wikfors, & Brown, 2013; Sea, 2015; Warner & Oliver, 2008).

2.3 | Epidemiological investigation of *V. vulnificus*

2.3.1 | Sample collection

Samples (including water samples and animal body surface swabs) were collected from six aquariums in northern China. In detail, 100-ml water samples were collected from the pools in which the marine

TABLE 1 Primers used in this study

Gene	Sequences	Target (bp)
vvhA	CGCCACCCACTTTCGGGCC	519
	CCGCGGTACAGGTTGGCGC	
NanA	GCGGTGATCGATCAAATTGCTG	618
	CCCTTGGTTGAACGCCTCAAT	
mtlABC	GCCCAACATCGGGGCATTTA	569
	GGCCAGCTTCTGAAGCCTG	
Ary	CCAGACCCGAGCGGATATGC	638
	GCGTGTGCGGGCCCCAGA	
SerE	TGTTGTTCTTGCCCACTCTC	665
	CGCGCTTAGATTTGTCTCACC	
Bt2	AGAGATGGAAGAAACAGGCG	344
	GGACAGATATAAGGGCAAATGG	
vcgC	AGCTGCCGATAGCGATCT	277
	R:CGCTTAGGATGATCGGTG	
vcgE	CTCAATTGACAATGATCT	277
	CGCTTAGGATGATCGGTG	
CPS1	TCGCGTTATCTGATCAACCA	294
	CGATGGAATCGTGTGATCAGT	
CPS2	GAACCTTCTGCGATGTTTGATGG	381
	CGATGGAATCGTGTGATCAGT	
IAV	GACCAATCCTGTCACCTCTGA	251
	GTATATGAGGCCCATRCAACT	
CDV	GTGACTGCTCCTGATACTGC	477
	ACCAACTCCCATAGCATAAC	
Rotavirus	GACGGVGCRACTACATGGT	382
	GTCCAATTCATNCCTGGTG	
Coronavirus	GGTTGGGACTATCCTAAGTGTGA	440
	CCATCATCAGATAGAATCATCATA	

animals (i.e., spotted seal, turtle, dolphin, shark, whale, and saltwater fish) were kept (Table 2). Animal (i.e., turtle, spotted seal) body surface swabs were collected using medical degreasing cotton. Freshwater samples and freshwater fish body surface swabs were also collected as negative controls. In total, 54 samples were collected. All the samples were stored on ice, transported to the Institute of Zoology, Chinese Academy of Sciences, and analyzed immediately.

2.3.2 | Sample handling

All samples were examined using procedures in the Bacteriological Analytical Manual of The Food and Drug Administration (FDA) (Kaysner & DePaola, 2004). In detail, water samples were serially diluted and cultured by 1% NaCl alkaline peptone water (APW) at 37°C for 18–24 hr. Body surface swabs were diluted in a 5-ml volume of sterile phosphate-buffered saline (PBS) followed by vortexing for 45 s. The supernatant was cultured in 1% NaCl alkaline peptone water (APW) at 37 °C for 18–24 hr. The resulting products were diluted and cultured on blood agar and then detected by *V. vulnificus vvhA* gene-specific PCR (Warner & Oliver, 2008), 16S rDNA sequencing, and the BD Phoenix Automated Microbiology System (BD).

2.4 | Biotyping of *V. vulnificus*

Biotyping of isolated *V. vulnificus* was performed as previously reported (Bisharat, Agmon, Finkelstein, Raz, Ben-Dror, Lerner, Soboh, Colodner, Cameron, & Wykstra, 1999). Briefly, ONPG testing, indole production, 1% NaCl ornithine decarboxylase testing, D-sorbitol fermentation, D-mannitol fermentation, and lactose fermentation analysis were performed to identify the biotypes of the isolated *V. vulnificus*.

2.5 | Genotyping of *V. vulnificus*

Genotyping was conducted to identify the virulence genes of the isolated *V. vulnificus*. In our study, *vcg* (virulence-correlated gene), *serE* (serovar E) gene, *cps* (capsular polysaccharide) gene, *bt2* (biotype 2) gene, *ary* (arylsulfatase) gene, *mtlABC* (mannitol/fructose-specific phosphotransferase system IIA protein) gene, and *nanA* (N-acetylneuraminate lyase) gene were detected using virulence gene-specific PCR as listed in Table 1.

TABLE 2 *Vibrio Vulnificus* can be detected from all the six investigated aquarium

Aquariums	No. of collected samples[a]	No. of positive samples	Positive rate (%)
BJ-BZ	12	4	33.30
BJ-PH	10	10	100
BJ-SAR	4	4	100
BJ-FG	2	1	50
SJZ	10	2	20
XA	16	4	25

Note. [a]Samples collected from fresh water were not included in this table.

2.6 | Animal experiment

The *V. vulnificus* isolated from the seal (BJ-PH01) was cultured in 1% APW and quantified by a colony forming unit (CFU) assay on blood agar. The inoculum was serially diluted to 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/ml 6-week-old female BALB/c mice were intraperitoneally (for each group, $n = 4$) (Strom & Paranjpye, 2000) or intramuscularly (for each group, $n = 4$) (Hor, Chang, Chang, Lei, & Ou, 2000; Lee & Chuang, 2010) injected with 0.1 ml of inoculum. The mice were observed each day after inoculation, and dead mice were examined. Pathological information was observed and recorded. Tissue samples of the dead mice were also collected.

Susceptibility tests were conducted by the Kirby-Bauer method according to the recommendations of the National Committee for Clinical Laboratory Standards (M45) (Clinical & institute, 2015). In detail, *V. vulnificus* was cultured in Mueller-Hinton broth for 14–16 hr. The direct colony suspension method was adopted to produce the inoculum suspension. The inocula were cultured on Mueller-Hinton Agar for disk diffusion. The plates were placed at 35°C for 16–20 hr. The selected antibiotics included amikacin, levofloxacin, chloramphenicol, gentamicin, piperacillin/tazobactam, trimethoprim-sulfamethoxazole, cefepime, ceftazidime, ciprofloxacin, piperacillin, cefotaxime, and tetracycline.

3 | RESULTS

3.1 | A spotted seal died due to *V. vulnificus* infection

Since March 2016, a captive spotted seal (*Phoca largha*) had suffered from a traumatic injury on the tail, which had not healed over a 2-month treatment. The mental condition and health status were not clearly affected during the course of treatment. In May 2016, the seal began vomiting, became depressed, and died within 24 hr of the onset of clinical manifestations. Upon necropsy, severe pneumonia (Figure 1a), extensive intestinal hemorrhage (Figure 1b), and liver necrosis (Figure 1c) were observed. No lesions were noted on the heart, kidney, stomach, or spleen. We preliminarily speculated that the seal had died due to sepsis induced by trauma fester or other lethal infections.

To explore the cause of death, we conducted bacteriological analysis (Figure 2a) and virological testing. Consequently, we failed to detect any known viral pathogens from the dead seal. In addition, *V. vulnificus* was successfully isolated from the fester, blood, and several tissue samples (i.e., lung, liver, intestine, and spleen) (Figure 2b). The result was confirmed by 16S rDNA sequencing, *V. vulnificus vvhA* (cytolysin/hemolysin) gene-specific PCR amplification (Figure 2c), the BD Phoenix Automated Microbiology System, and electron scanning microscope (*SEM*) analysis (Figure 2d). The isolated *V. vulnificus* was named BJ-PH01. These results suggested that the seal died due to septicemia induced by *V. vulnificus*.

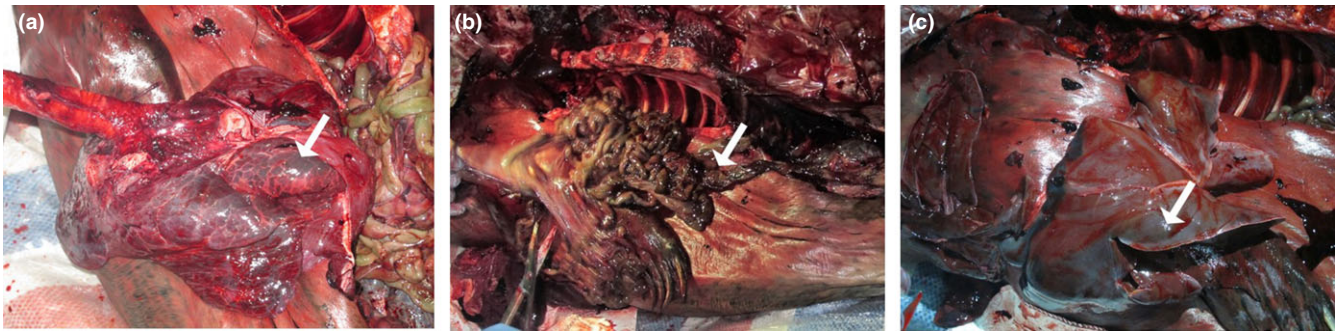

FIGURE 1 Autopsy of dead spotted seals. (a) Swelling and hemorrhage of lung tissues; (b) extensive hemorrhage was observed in the intestine; (c) severe necrosis was observed in the liver [Colour figure can be viewed at wileyonlinelibrary.com]

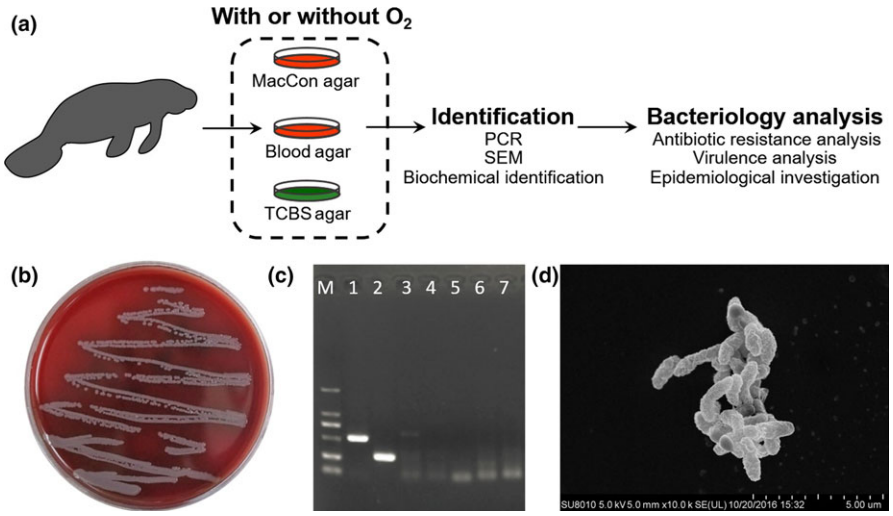

FIGURE 2 Isolation and identification of *Vibrio vulnificus*. (a) Scheme of bacteria isolation and identification from the dead seal; (b) *V. vulnificus* forms opaque colonies on blood agar; (c) *V. vulnificus*-specific gene detection by PCR. The result showed that BJ-PH01 was positive for the *vvhA* gene. Further analysis showed that BJ-PH01 belongs to the *vcgE* genotype. Lane M: DL2000 marker; lanes 1-7: *vvhA*, *vcgC*, *vcgE*, *serE*, *cps0*, *cps1*, and *bt2*, respectively. (d) Observation of BJ-PH01 using electron scanning microscopy [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Virulence of the isolated *V. vulnificus*

Several virulence factors of *V. vulnificus* have been reported previously, such as toxin, LPS, and capsule (Jones & Oliver, 2009). Possession of an antiphagocytic capsule is one of the absolute requirements for virulence. Encapsulated cells produce opaque colonies, and only opaque cells are able to utilize transferrin-bound iron. As shown in Figure 2b, the *V. vulnificus* isolate BJ-PH01 grew opaque colonies on blood agar. This result prompted us to consider that BJ-PH01 is virulent in animal models (Simpson, White, Zane, & Oliver, 1987). Several animal models have been constructed to explore the increased susceptibility to *V. vulnificus* infections after the injection of iron-containing compounds. Here, we conducted an animal experiment using intraperitoneal (i.p.) injections or intramuscular (i.m.) injections and concurrent i.p. injections with PBS. Six- to eight-week-old female BALB/c mice were included in the experiment. The results showed that, when infected by 1.4×10^6 PFU *V. vulnificus*, the mice died within 12 hr post-infection. The median lethal dose of the isolated *V. vulnificus* BJ-PH01 was 3.16×10^5 CFU. Severe hemorrhage was observed at the inoculation site and tails (Figure 3a). Furthermore, extensive edema and hemorrhage were noted in the lungs (Figure 3b) and intestines of the dead mice (Figure 3c), and *V. vulnificus* could be re-isolated from the dead mice.

3.3 | Epidemiological investigation of *V. vulnificus* in aquariums in northern China

Having demonstrated that *V. vulnificus* can be detected in aquariums, we next asked whether this lethal bacterium is widespread in aquariums. To explore the prevalence of *V. vulnificus* in aquariums, we performed an epidemiological investigation of *V. vulnificus*. In this analysis, we successfully collected 54 samples from six aquariums in northern China (Table 2). The detection of *V. vulnificus* was performed as described above. *V. vulnificus* was isolated from all six of the investigated aquariums. The average positive rate was 44.4%, ranging from 20% to 100% (Table 2). Finally, 12 strains of *V. vulnificus* were successfully isolated and identified. In addition to *V. vulnificus*, other bacteria of the genus *Vibro* were also frequently isolated, such as *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio fluvialis*.

The result indicated that *V. vulnificus* is widely distributed in coastal environments as well as aquariums, which are artificial saltwater environments. Together with other species of *Vibrio*,

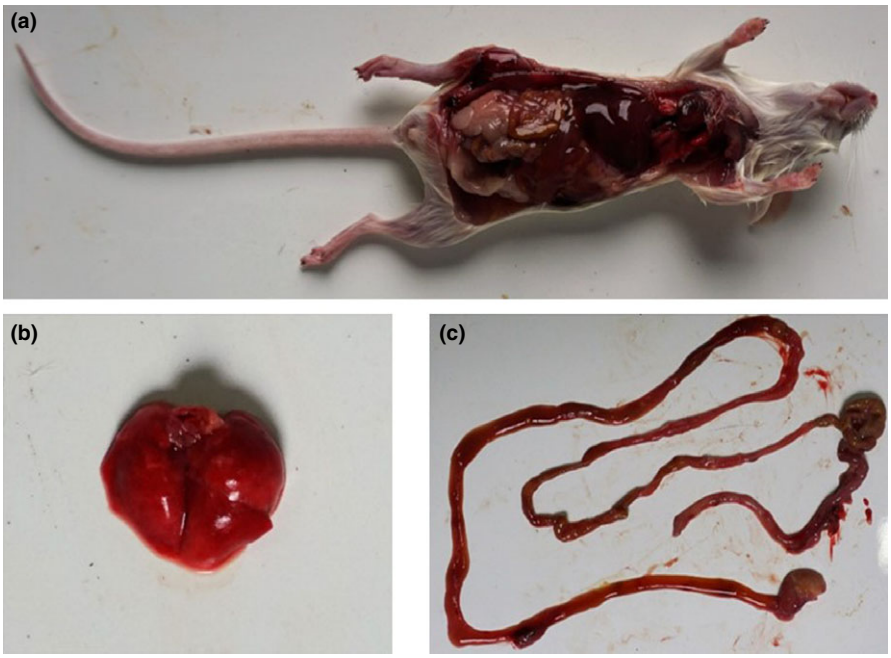

FIGURE 3 Experimental infection of mice using the isolated *Vibrio vulnificus* BJ-PH01. (a) The mouse died within 12 hr post-injection; upon necropsy, swelling and hemorrhage of the lung (b) and intestine (c) were observed [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Biotype of isolated *Vibrio Vulnificus*

	Results											
Test	BJ-PH01	BJ-PH02	BJ-PH03	BJ-PH04	BJ-PH05	BJ-PH06	BJ-SAR01	BJ-BZ01	SJZ01	XA01	XA02	XA03
ONPG test	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	–	–	–	–	–	–	–	–	–	–	–	–
Indole production	+	+	+	+	+	+	+	+	+	+	+	+
D-sorbitol fermentation	–	–	–	–	–	–	–	–	–	+	–	–
D-mannitol fermentation	–	–	–	–	–	–	–	–	–	–	–	–
Lactose fermentation	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	–	–	–	–	–	–	–	–	–	–	–	–
Biotype	1	1	1	1	1	1	1	1	1	1	1	1

V. vulnificus in aquariums poses a novel threat to captive marine animals and humans.

3.4 | Biotyping and genotyping of isolated *V. vulnificus*

Vibrio vulnificus can be classified into three biotypes based on biochemical characteristics. To explore the biotype of the isolated *V. vulnificus*, we performed an assay of biochemical identification as previously reported (Bisharat, Agmon, Finkelstein, Raz, Ben-Dror, Lerner, Soboh, Colodner, Cameron, Wykstra, et al., 1999). All the tested *V. vulnificus* samples were positive for the indole test, ONPG test, and ornithine decarboxylase test (Table 3). The results indicated that all 12 isolated strains belonged to biotype 1. In addition, biotype 1 *V. vulnificus* was classified into Clinical (C) or Environmental (E) genotypes based on its virulence-correlated gene (*vcg*) (Warner & Oliver, 2008); our analysis showed that 11 of the 12 isolated *V. vulnificus* strains were classified into the C genotype, and only one of the 12 belonged to the E genotype (Table 4).

A previous study demonstrated that genotypic markers cannot unequivocally predict virulence, although several genes were putatively reported to be linked to virulence. Here, we investigated the genes *ary* (arylsulfatase), *mtlABC* (mannitol/fructose-specific phosphotransferase system IIA protein), and *nanA* (N-acetylneuraminate lyase) by PCR in our study (Table 4). The *ary* gene has been demonstrated to be associated with virulence of clinical strains by providing a pathogen with sulfur within the host, thus providing an immune evasion approach (Morrison et al., 2012). Positive *Ary* PCRs were obtained in all of the isolated strains. Although *mtlABC* appears to be linked to pathogen virulence, the precise role of *mtlABC* is not

TABLE 4 Genotype of isolated *Vibrio vulnificus*

Strain	Source	Virulence genes							
		vvhA	*vcg*	*serE*	CPS type	*bt2*	*ary*	*mtlABC*	*nanA*
BJ-PH01	Spotted seal	+	C	–	0	–	+	+	+
BJ-PH02	Turtle limbs	+	C	–	0	–	+	+	+
BJ-PH03	Turtle limbs	+	C	–	0	–	+	+	+
BJ-PH04	Turtle limbs	+	C	–	0	–	+	+	+
BJ-PH05	Turtle limbs	+	C	–	0	–	+	+	+
BJ-PH06	Turtle shell	+	C	–	0	–	+	+	+
BJ-SAR01	Turtle shell	+	C	–	0	–	+	+	+
BJ-BZ01	Water	+	C	–	1	–	+	+	+
SJZ01	Water	+	E	–	0	–	+	+	+
XA01	Water	+	C	–	1	–	+	+	+
XA02	Water	+	C	–	1	–	+	+	+
XA03	Water	+	C	–	1	–	+	+	+

yet understood (Reynaud et al., 2013). In our experiments, we obtained positive *mtlABC* results for all of the isolated strains. The *nanA* gene has been demonstrated to be involved in sialic acid metabolism and is essential for *V. vulnificus* virulence (Kim, Hwang, Kim, & Choi, 2011). Positive *nanA* gene PCR results were obtained for all of the isolated strains. These results indicate that the 12 isolated *V. vulnificus* strains display a potential threat to mammals, including marine animals and humans.

3.5 | Antibiotic analysis of *V. vulnificus*

The disease progression of *V. vulnificus* infection is often acute, and it is therefore important to provide timely treatment with proper antibiotics. We conducted a further analysis to understand its sensitivity to major antibiotics. The result showed that all isolated *V. vulnificus* strains were sensitive to antibiotics. In detail, *V. vulnificus* was sensitive to levofloxacin, tetracycline, piperacillin, and gentamicin (Table 5).

TABLE 5 Antibiotic susceptibility tests of the BJ-PH01 using the agar disk disffusion method

Test	Zone diameter (mm)	Result[a]
Amikacin	≥16	I
Levofloxacin	≥20	S
Chloramphenicol	≥17	I
Gentamycin	≥21	S
Piperacillin/Tazobactam	≥21	S
Sulfamethoxazole trimethoprim	≥21	S
Cefepime	≥21	S
Ceftazidime	≥22	S
Ciprofloxacin	≥23	S
Piperacillin	≥21	S
Cefotaxime	≥20	I
Tetracycline	≥26	S

Note. [a]S: susceptible; I: intermediate; R: resistant.

4 | DISCUSSION

Vibrio vulnificus is a foodborne pathogen of humans. Here, we provide evidence that marine mammals can also be infected by *V. vulnificus*. Epidemiological investigations of *V. vulnificus* showed that this lethal bacterium can frequently be detected in aquariums, thus constituting a novel threat to marine animals, workers, and tourists in relevant aquariums. Antibiotic drug resistance is an increasing concern due to the overuse of antibiotics. Fortunately, all of the isolated *V. vulnificus* isolates in our study were sensitive to levofloxacin, tetracycline, piperacillin, and gentamicin. No drug-resistant *V. vulnificus* isolates were isolated.

The minimum dose capable of causing human infection is currently unknown (Strom & Paranjpye, 2000). Previous studies have suggested that the dose may be fewer than 1,000 organisms. Animal experiments have been helpful in researching disease syndromes produced by *V. vulnificus*. However, they have no instructive value for determining the infectious dose 50 (ID_{50}) for human infections (Strom & Paranjpye, 2000). In our research, we found that the median lethal dose of isolated *V. vulnificus* BJ-PH01 was 3.16×10^5 CFU. These data have limited value in determining virulence for humans at present, but they may contribute to future research in studying the correlation between infectious dose for animal models and humans.

Marine mammals are a diverse group of species that includes cetaceans, pinnipeds, sirenians, sea otters, and polar bears. Protection for marine mammals from multiple threats has increased since the enactment of the *Marine Mammal Protection Act* in 1972. However, the lack of scientific data, confusion about permitting requirements, failure to adopt appropriate management, and inappropriate human activities have impaired our protection efforts (Schipper et al.,

2008). Infectious disease is one of the most important risks to wildlife animals. Scientific research on marine mammal infectious diseases has been relatively insufficient. Previously demonstrated pathogens of marine mammals include viruses (e.g., adenovirus, calicivirus, herpesvirus, morbillivirus, poxvirus, influenza virus, retrovirus) (Goldstein et al., 2009; Maness et al., 2011; Ohishi et al., 2010; Ramis, van Riel, van de Bildt, Osterhaus, & Kuiken, 2012; Rivera, Nollens, Venn-Watson, Gulland, & Wellehan, 2010), bacteria (e.g., *Actinomycetes*, *Brucella*, *Clostridia*, *Erysipelothrix rhusiopathiae*, *Pasteurella multocida*, and *Mycoplasma*) (Waltzek, Cortés-Hinojosa, Wellehan, & Gray, 2012), and parasites (e.g., Acanthocephalans, Toxoplasma gondii, Sarcocystis, Coccidia, and *Parafilaroides decorus*) (Hernandez-Orts et al., 2015; Jensen, Aars, Lydersen, Kovacs, & Åsbakk, 2010). Here, we demonstrated that spotted seals can be infected by *V. vulnificus*. As no host tropism of *V. vulnificus* has been reported, one can speculate that other marine mammals might also be infected with *V. vulnificus*. Therefore, we should pay more attention to abnormal deaths of wild marine mammals to accumulate scientific data.

In addition to *V. vulnificus*, other opportunistic pathogens of the *Vibrio* genus were also detected in this investigation, such as *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio fluvialis*. *Vibrio parahaemolyticus* is a marine organism native to estuarine waters around the world. In Asia, *Vibrio parahaemolyticus* is a common cause of foodborne disease (Sakata, Yonekita, & Kawatsu, 2018), while *Vibrio alginolyticus* is implicated in wound infections and otitis. *Vibrio alginolyticus* was recently recognized as a human pathogen after excessive exposure to seawater (Gao et al., 2017). *Vibrio fluvialis* has been considered an emerging pathogen that induces foodborne diarrhea (Ramamurthy, Chowdhury, Pazhani, & Shinoda, 2014). These results suggest that *V. vulnificus* is not the only threat to marine animals and public health in aquariums.

The major limitation of our research is that we failed to perform Koch's postulate test because it was impossible for us to confirm the infection using a healthy seal. Due to limited background information, the typical clinical manifestations of *V. vulnificus* infected seals are poorly understood. We provided a diagnosis mainly based on the detection and isolation of *V. vulnificus* from the visceral tissues and blood, which indicated that *V. vulnificus* induced septicemia (Bross, Soch, Morales, & Mitchell, 2007b).

Previous studies have demonstrated that *V. vulnificus* can survive under nutrient starvation in seawater for years without losing its pathogenic potential for animals and humans (Marco-Noales, Biosca, & Amaro, 1999). Thus, it is difficult to eliminate *V. vulnificus* without scientific measures, and it is necessary to take measures to control the water quality in aquariums.

We demonstrated that *V. vulnificus* can infect and kill marine mammals. However, it was difficult to evaluate the risk of infection of marine mammals by *V. vulnificus*: (a) *V. vulnificus* is an opportunistic pathogen, and one or more predisposing factors are required to initiate disease; (b) the minimum dose for *V. vulnificus* to cause an infection is poorly understood, even in humans; (c) the interface between *V. vulnificus* and marine mammals in the wild has never been studied. Nevertheless, the prevalence of *V. vulnificus* is a persistent threat to marine mammals.

ACKNOWLEDGEMENTS

This work was funded by grants from the State Forestry Administration of China, Chinese Academy of Sciences (CZBZX-1).

CONFLICT OF INTEREST

No conflict of interest exists in the submission, and all authors approved the publication.

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How to cite this article: Li M, Zhao L, Ma J, et al. *Vibrio vulnificus* in aquariums is a novel threat to marine mammals and public health. *Transbound Emerg Dis*. 2018;65:1863–1871. https://doi.org/10.1111/tbed.12967