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Identification of pathogenicity, investigation of virulent gene distribution and development of a virulent strain-specific detection PCR method for *Vibrio harveyi* isolated from Hainan Province and Guangdong Province, China

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ABSTRACT

A collection of 46 *Vibrio harveyi* strains were isolated from *Epinephelus* spp., *Lutjanus erythopterus*, and other maricultured fish in coastal areas of Hainan Province and Guangdong Province, China, between 2011 and 2013. Eighteen strains were determined to be pathogenic via artificial infection of healthy *Epinephelus coioides* at 10^7 colony-forming units (CFU) mL^{-1}. Mortality occurred within 2 to 6 h after injection. Genotypic assays of the 46 strains by enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) revealed a similar genotype profile, referred to as the ERIC-1 profile, for all 18 pathogenic strains. This finding indicates that pathogenic *V. harveyi* strains in south China have similar genetic backgrounds and might be representative pathogenic strains of this region. All 46 strains were screened for the presence of virulence genes typical of *V. harveyi*, of zoonotic *Vibrio* species such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* and of the aquatic pathogen *V. anguillarum*. Virulence genes were amplified by PCR using specific primers, and five typical virulence genes of the Harveyi clade, *luxR*, *toxRvh*, *chiA*, *serine protease* and *vhh*, were detected in all pathogenic isolates. Non-pathogenic strains carried only 1 to 4 of these genes, indicating that these five genes might be the main virulence genes of ERIC-1 strains. Strain-specific PCR primers were designed based on the sequences of distinct ERIC-PCR bands for the 18 pathogenic strains. Species-specific primers exhibited high specificity and sensitivity. This study demonstrates that bacteria that are highly important to mariculture could be specifically detected using ERIC-PCR fingerprint-based amplification.

1. Introduction

The southern coastal regions (108°–117°E, 18°–23°N), including Hainan Province, Guangdong Province and the Guangxi Zhuang Autonomous Region, are the major maricultural areas in China. The annual average temperature in these regions ranges from 22 °C to 26 °C, which is suitable for aquaculture of grouper and other tropical marine animals. However, with the development of aquaculture, vibriosis, which is caused by *Vibrio* spp., has become a serious problem, and more than 10 species of *Vibrio*, including *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, and *V. ponticus*, contribute to vibriosis in this region (Xie et al., 2005, 2007). Among them, *V. harveyi* was verified to be the dominant pathogen in recent years (Austin and Zhang, 2006; Haldar et al., 2010; Mei et al., 2010; Rattanama et al., 2012; Xu et al., 2012; Zhou et al., 2012; Cui et al., 2014).

V. harveyi strains are known to differ in their ability to cause aquatic animal diseases (Austin and Zhang, 2006). Several virulence genes are thought to be associated with pathogenicity of vibrios (Ruwandeepika et al., 2010). Previous studies have identified major virulence genes contributing to *V. pathogenicity* and *V. cholera* (Waldor and Mekalanos, 1996; Iida et al., 1998; McCarthy et al., 1999; Zhang et al., 2003). In addition, horizontal gene transfer, a driving force in *Vibrio* evolution and speciation, also appears to be an efficient mechanism for introducing new phenotypes into the bacterial genome (Ochman et al., 2000; Gogarten et al., 2002; Hacker et al., 2003). Although *V. harveyi* has caused severe losses in the aquaculture industry, little is known about its key virulent genes and its pathogenic mechanisms.

Because the pathogenic mechanism of *V. harveyi* is still unknown and no effective method is currently available to control its spread, developing a rapid diagnostic to support a prophylactic approach is particularly important. Although several rapid detection methods have recently been developed for *V. harveyi* (Conejero and Hedreyda, 2003, 2004; Oakey et al., 2003; Pang et al., 2006; Sun et al., 2009), methods

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that detect a specific pathogenic genotype have not been reported. Thus, the design of molecular methods for rapid detection of pathogenic *V. harveyi* is essential for improving industrial culture production. The key step in developing such a method is identification of a target gene or molecular biomarker. Due to the tremendous diversity of bacterial genomic DNA, the sequences of ERIC-PCR bands are often unique to the genome of the strain used for amplification. Therefore, these sequences have been used as biomarkers for designing primers used to discriminate closely related bacterial strains or to detect specific bacteria in various samples, especially with regard to understanding the epidemiology of pathogens and food-contaminating bacteria (Ventura et al., 2003; Alippi et al., 2004; Yan et al., 2007; Ye et al., 2008). To our knowledge, however, this method has not been used in maricultural studies. In this paper, strain-specific PCR primers were designed based on the sequences of specific bands amplified from pathogenic strains of *V. harveyi* by ERIC-PCR fingerprinting. We demonstrate that the pathogenic strains of *V. harveyi* could be specifically detected using ERIC-PCR fingerprint-based amplification. Our technique will help in developing measures to control *V. harveyi* infection.

To explore the pathogenic characteristics of *V. harveyi* isolated from maricultural areas in southern China, the pathogenicity, genotyping and virulent gene distribution of *V. harveyi* strains obtained from these areas were investigated, and a rapid detection method for such pathogenic *V. harveyi* strains was developed in this paper.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 46 *V. harveyi* strains originally isolated from diseased or moribund fish between 2011 and 2013 from mariculture systems of Hainan Province and Guangdong Province, southern China were used in this study. All strains were identified as *V. harveyi* using standard biochemical testing described in Bergey's Manual of Systematic Bacteriology and multilocus sequence analysis (Cano-Gomez et al., 2009). The strains were stored in broth medium containing 25% glycerol (v/v) at −80 °C. The sampling sites are illustrated in Fig. 1, and the sources and origins of these *V. harveyi* isolates are listed in Table 1.

2.2. Artificial infection

To confirm the pathogenicity of *V. harveyi*, experimental challenges were conducted using healthy *Epinephelus coioides* (mean weight 15.0 ± 0.5 g) obtained from culture ponds in Wenchang City, Hainan Province, China. Artificial infection of 46 *V. harveyi* strains was performed by intraperitoneal injection. The artificial infection method and the criterion for classifying a strain as pathogenic was referred to Xie et al. (2005) and Bai et al. (2008), with slight modifications. Six fish were maintained in a 50 L tank containing 40 L filtrated seawater (30 g L^{-1} salinity). The fish were maintained at 30 ± 1 °C and were fed commercial pellets twice a day. Compressed air was pumped continuously into water, and 50% of the water was exchanged daily. Fish were kept for 7 days before artificial infection. All bacterial strains were cultured in Marine Broth 2216E for 24 h with constant shaking at 30 °C. The cultures were diluted with sterile phosphate-buffered saline (PBS, pH 7) to 1×10^7 CFU mL^{-1}, as determined by plate counting. Fish was infected intraperitoneally with 100 μL of the diluted bacterial suspension, and the same volume of PBS was injected as the control. Two parallel experiments were performed per treatment. After artificial injection, the symptoms caused by virulent bacteria and the mortality of the fish were recorded for 7 days. The injected isolate was considered the cause of death only if it could be re-isolated as single colonies in pure culture from the liver, spleen and brain of a moribund or dead fish. Histopathological examination of liver, spleen and brain was also performed to diagnose the results of infection. The strains which could cause all the infected fish to death in 7 days were defined as pathogenic strains and the other strains were non-pathogenic.

To determine the toxicity of the *V. harveyi* culture supernatant, experimental challenges with the supernatant of strain GDH11385 were conducted in *E. coioides*. Briefly, strain GDH11385 cells were grown in Zobell 2216E broth to the logarithmic phase and then serially diluted 10-fold to obtain 1×10^7 CFU mL^{-1}. The cultures were centrifuged to collect the supernatants, which were then filtered through a 0.2-μm syringe filter to remove all remaining cells. Experimental challenge of six fish with 100 μL of the supernatant was performed by intraperitoneal injection. As a positive control, the same volume of an untreated 10^7 CFU mL^{-1} GDH11385 culture was injected; as a negative control, the same volume of the 2216E broth filtered through a 0.2-μm syringe filter was injected. Two parallel experiments were performed per treatment.

2.3. LD_{50} assays

To determine the 50% lethal dose (LD_{50}) of the pathogenic *V. harveyi* strain GDH11385, bacterial doses ranging from 10^3 to 10^8 CFU mL^{-1} were used for intraperitoneal inoculation. Three tanks (6 fish per tank) were used for each treatment. The LD_{50} value was calculated by the classical Bliss method (Bliss, 1935).

2.4. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

All isolated strains of *V. harveyi* (n = 46) were grown overnight at 28 °C on Zobell 2216E agar plates (Sigma). One colony of each strain was suspended in 5 mL Zobell 2216E broth (Sigma) and grown for 24 h at 28 °C. Chromosomal DNA was extracted using a TaKaRa

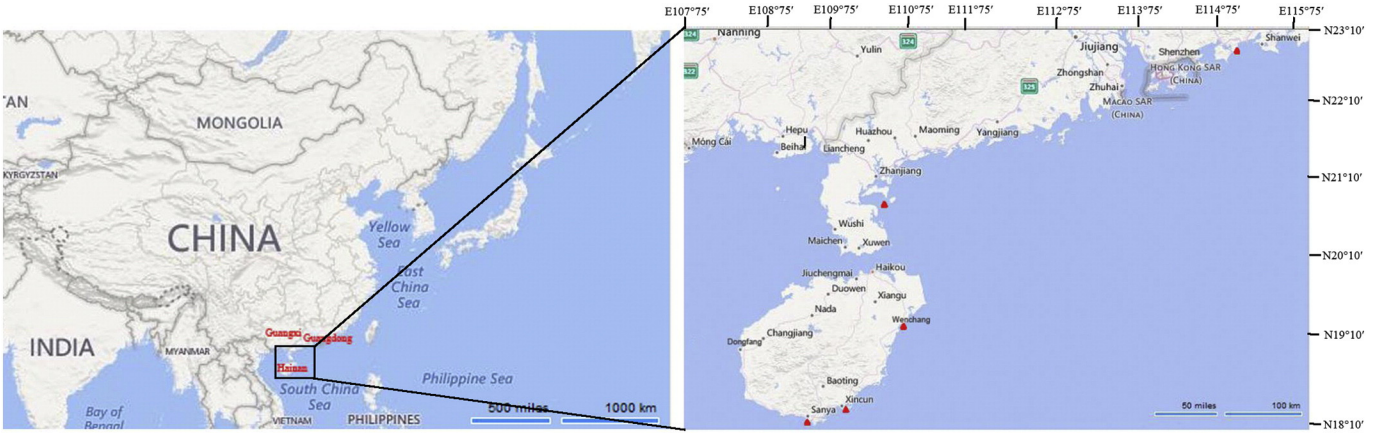

Fig. 1. Sampling area and the locations of sampling sites (red triangles). (http://www.worldmapfinder.com/BingMaps/Cn.html). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)