Marine Pollution Bulletin 101 (2015) 526–532

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Detection and characterization of integrative and conjugative elements (ICEs)-positive *Vibrio cholerae* isolates from aquacultured shrimp and the environment in Shanghai, China

Yu He, Yuyi Tang [1], Fengjiao Sun, Lanming Chen *

Key Laboratory of Quality and Safety Risk Assessment for Aquatic Products on Storage and Preservation (Shanghai), China Ministry of Agriculture, College of Food Science and Technology, Shanghai Ocean University, 999# Hu Cheng Huan Road, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 20 June 2015
Received in revised form 23 October 2015
Accepted 23 October 2015

Keywords:
Vibrio cholerae
Antimicrobial susceptibility
Heavy metal resistance
ICEs

ABSTRACT

Increasing industrialization and use of antimicrobial agents in aquaculture production, have led to heavy metals and multidrug resistant (MDR) pathogens becoming serious problems. These resistances are conferred in two ways: intrinsic and transfer via conjugation, or transformation by the major transmission mediators. Integrative and conjugative elements (ICEs) are one of the major mediators; however, few studies on ICEs of environmental origin have been reported in Asia. Herein, we determined the prevalence, antimicrobial susceptibility, heavy metal resistance and genotypes of 126 strains of *Vibrio cholerae* isolated from aquatic products and the environment in Shanghai, China. 92.3% of isolates were ICEs-positive from aquaculture water and 89.3% of isolates from shrimp showed MDR. Tracing the *V. cholerae* genotypes, showed no significant relevance of genotype among the antimicrobial resistance strains bearing the ICEs or not. Thus, in aquaculture, ICEs are not the major transmission mediators of resistance to antibiotics or heavy metals.

1. Introduction

Vibrio cholerae is regarded as the etiological agent of the most severe, watery, life-threatening diarrheal disease, causing cholera epidemics, and is particularly prevalent in developing countries with poor sanitary conditions (Rajpara et al., 2015; Zhang and Gou, 2014). An estimated 3–5 million cases and over 100,000 deaths occur each year around the world (http://www.cdc.gov/cholera/general/). Cholera toxin (CT) encoded by *ctxA* and *ctxB*, is the major virulence determinant of *V. cholera*. In addition, the *toxR* gene of *V. cholerae* encodes a transmembrane, DNA-binding protein that positively controls the transcription of the cholera toxin genes, and those encoding the TCP pili and other proteins important in cholera pathogenesis (Boyd et al., 2000; Parsot and Mekalanos, 1990). Unlike clinical strains, most of the environmental strains do not produce CT, but might acquire CT genes under conditions similar to those of the aquatic environment (Faruque and Nair, 2002; Goel et al., 2007); therefore, non-pathogenic *V. cholerae* strains are likely to convert into epidemic strains if they acquire virulence genes, which is a threat to public health. However, multidrug-resistant *V. cholerae* strains that have been reported to cause outbreaks of cholera have become a significant threat to public health and indicate a high incidence and dissemination of antimicrobial resistance (AMR) in bacteria derived from aquatic environments (Chitanand et al., 2010; Teophilo, 2004). Aquaculture ponds with rising pollution of heavy metals and antibiotics have been proved as hotbeds of superbugs. Moreover, a previous study indicated that critical concentrations of heavy metals accumulated can trigger co-selection of antibiotic resistance (Seiler and Berendonk, 2012), which suggests that occurrence of heavy metal and antibiotic resistance has potential relevance. In fact, drug-resistance mechanisms revealed not only the resistance determinants, but also the genetic elements that contribute to the emergence of multidrug resistance (MDR) (Li and Nikaido, 2009; Sjölund-Karlsson et al., 2011). Mutation and acquisition of resistance genes on mobile genetic elements are one way of developing AMR in *Vibrio* spp. (Frost et al., 2005; Kitaoka et al., 2011).

Integrative and conjugative elements (ICEs) are self-transmissible mobile genetic elements (MGEs) that allow bacteria to acquire complex new traits through horizontal gene transfer (HGT). ICEs are found in Gram-positive and Gram-negative bacteria, and are key driving forces in bacterial evolution (Burrus et al., 2002; Wozniak and Waldor, 2010). ICEs are recognized for their role in bacterial genome plasticity, and encode a wide variety of genetic information, including resistance to antibiotics and heavy metals (Davies et al., 2009), and the capacity to degrade aromatic compounds (Ravatn et al., 1998), which may be beneficial under certain environmental conditions. (Bordeleau et al., 2010; Ceccarelli et al., 2013; Wozniak et al., 2009). To date, MGEs with ICE-like properties have been described in several species of *Gammaproteobacteria*, mainly *Vibrios*. On the Indian subcontinent in

* Corresponding author.
E-mail addresses: heyu1120@hotmail.com (Y. He), lmchen@shou.edu.cn (L. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.marpolbul.2015.10.062
0025-326X/

late 1992, a major cholera epidemic was caused by a novel non-O1 serogroup of *V. cholerae* named *V. cholerae* O139, was found to harbor an SXT element, which confers resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin, and has ICE-like properties (Group, Research, & Bangladesh, 8868; Waldor et al., 1996).

To date, few studies on ICEs of environmental origin have been reported in Asia. MGEs play a crucial role in the genomic plasticity and fitness of *V. cholerae*; therefore, in this study, we investigated the prevalence, antimicrobial susceptibility, heavy metal resistance and genotypes of *V. cholerae* isolated from one of the major shrimp production regions in China, to better understand the dynamic of their dissemination and their correlation with resistance to drugs. Our data produced a well-characterized collection of strains that could be used as controls for several molecular-epidemiological approaches to antimicrobial resistance and virulence.

2. Methods and materials

2.1. Bacterial isolation

Shrimp samples were confirmed whether to be free of *V. cholerae* using culture methods by Zhang et al. (Zhang et al., 2015), in accordance with the Bacteriological Analytical Manual standard method (Kaysner and DePaola, 2004) with some modifications. After collection, live shrimps were immediately cooled in iceboxes and transported to our laboratory and homogenized within 2 h. Meanwhile, water samples were collected using 10 L sterile plastic bottles, and immediately transferred on ice to the laboratory as previously described (Tang et al., 2014). Water samples and shrimp samples (*Metapenaeus ensis*, *Macrobrachium rosenbergii* and *Penaeus monodon*; were purchased from Shanghai fish market, China) were collected between March and April 2013 to 2014. Briefly, 25 g of each shrimp samples was rinsed using sterile saline solution (PBS) and then homogenized for 60 s in stomacher bags with 225 mL of sterile alkaline peptone water (APW) containing 3% NaCl. Serial 10-fold dilutions were prepared up to $1{:}10^3$ dilution and 100 μL of each dilution was spread on thiosulfatecitrate-bile salts-sucrose (TCBS; Beijing Land Bridge Technology Company Ltd., Beijing, China) agar plates and incubated at 37 °C, overnight. Bacterial cells of water were separated by standard sequential filtration techniques: each water sample was filtered though 8-μm qualitative filter paper to remove large suspended particles, and 1.0 L filtrate was subsequently filtered through polycarbonate membranes with 0.8- and 0.22-μm pore size (47 mm diameter, Millipore, Corcaigh, Ireland), respectively. Putative *V. cholerae* colonies (yellow on TCBS) were selected from each plate and transferred separately into wells of a sterile 96-well microtiter plate which contained 150 μL alkaline peptone water (APW) of 3% NaCl (pH 8.5 ± 0.2).

2.2. Screening and identification of virulence genes and ICEs genes

Strain taxonomy was determined by conventional biochemical tests and by 16S rRNA gene amplification and sequencing using primers pair 27F and 1492R (Weisburg et al., 1991). Presumptive *V. cholerae* was used for PCR-based screening of the species-specific marker *lolB* gene. Among the *lolB*-positive *V. cholera* strains, virulence-associated genes (*toxR*, *ctxAB*, *rtxA*, *tcpA*, *sto*, *ace* and *zot*) and SXT/R391-like ICE conserved genes (*int*, *attR*, *traC*, *setR*, *traI*) were also detected by PCR. All the primers used in this study are listed in Table 1.

2.3. PCR conditions

As previously described by Song et al. (Song et al., 2013), genomic DNA was prepared using the MiniBest bacterial genomic DNA extraction kit ver. 2.0 (Japan TaKaRa BIO, Dalian Company, Dalian, China). The concentration of DNA in the samples was determined using a multi-mode microplate reader, BioTek Synergy™ 2 (BioTek Instruments, Inc., Winooski, VT, USA). PCR amplification was performed in a 20 μL reaction volume, PCR for the *lolB* gene was carried out with the primers VHMF and VHA-AS5 under conditions as described by Lalitha et al. (Lalitha et al., 2008): initial denaturation of 95 °C for 3 min; followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s; and one cycle at 60 °C for 30 s, followed by final elongation at 72 °C for 5 min. The other PCR reactions were performed with appropriate annealing temperatures and elongation times according to melting temperatures of primer pairs and the predicted lengths of PCR products. All amplifications were performed in a Mastercycler® pro PCR thermal cycler (Eppendorf, Hamburg, Germany). A sample (5 μL) of each amplification reaction was analyzed

Table 1
Oligonucleotide primers used in this study.

Primer	Nucleotide sequence (5′ to 3′)	Target genes	Reference
27F	AGAGTTTGATCCTGGCTCAG	16S rDNA	Weisburg et al. (1991)
1492R	GGTTACCTTTTACGACTTG		
traI-F	GCAAGTCCTGATCCGCTATC	*traI*	Bani et al. (2007)
traI-R	CCAGGGCATCTCATATGCGT		
traC-F	TTGACGCTGTTTTCACCAACG	*traC*	(Bani et al., 2007)
traC-R	GGCACGACCTTTTTTCTCCC		
setR-F	ACGGCGGAGATGTTTTTGT	*setR*	Bani et al. (2007)
setR-R	GTGCGCCAATGCTCAGTT		
attR-F	GGTTTAGCCACAGTTGTTC	*attR*	Song et al. (2013)
attR-R	CGTCAGGGTGCGAGAT		
Int-F	GACGCATTTCATCCAGG	*int*	Song et al. (2013)
Int-R	GCAACAGCGGGTAGACA		
ctxAB-F	GCCGGGTTGTGGGAATGCTCCAAG	*ctxAB*	Goel et al. (2007)
ctxAB-R	CATGCGATTGCCGCAATTAGTATGGC		
toxR-F	CCTTCGATCCCCTAAGCAATAC	*toxR*	Rivera et al. (2001)
toxR-R	AGGGTTAGCAACGATGCGTAAG		
VHMF	TGGGAGCAGCGTCCATTGTG	*lolB*	Lalitha et al. (2008)
VHA-AS5	CAATCACACCAAGTCACTC		
rtxA-F	GGGATACAATGCCCTCTGGCA	*rtxA*	Kumar et al. (2010)
rtxA-R	TGGGTTGGCGGTTGGATTTTAC		
tcpA-F	ATGCAATTATTAAAACAGCTTTTTAAG	*tcpA*	Kumar et al. (2010)
tcpA-R	TTAGCTGTTACCAAATGCAACAG		
zot-F	TCGCTTAACGATGGCGCGTTTT	*zot*	Rivera et al. (2001)
zot-R	AACCCCGTTTCACTTCTACCCA		
ace-F	TAAGGATGTGCTTATGATGGACACCC	*ace*	Shi et al. (1998)
ace-R	CGTGATGAATAAAGATACTCATAGG		