Marine Pollution Bulletin 136 (2018) 164–170

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Antimicrobial resistance and pulsed-field gel electrophoresis typing of *Vibrio parahaemolyticus* isolated from shrimp mariculture environment along the east coast of China

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ARTICLE INFO

Keywords:
Vibrio parahaemolyticus
Antimicrobial resistance
PFGE
Food safety
Mariculture farms

ABSTRACT

Environmental antimicrobial resistance (AMR) has drawn increasing attention due to its great risk to human health. The aim of this study was to investigate AMR and genotyping of *Vibrio parahaemolyticus* isolates (n = 114) recovered from shrimp mariculture environment in China. The isolates exhibited a high rate of resistance to streptomycin (78.9%), ampicillin (64.9%) and gentamicin (53.5%). Furthermore, multi-drug resistance was highly prevalent (61.4%), in which 95.9% of these ampicillin-resistant isolates were primarily mediated by $bla_{\text{CARB-17}}$. Surprisingly, doxycylcine, florfenicol, and trimethoprim/sulfamethoxazole (TMP/SMZ) resistance genes occurred in susceptible isolates. Moreover, 114 isolates were grouped into unique pulsed field gel electrophoresis patterns. These findings suggest the need for the prudent use of antimicrobial agents on mariculture farms, in order to control the dissemination of antimicrobial resistant *V. parahaemolyticus*.

1. Introduction

The prevalence and spread of antimicrobial resistance genes (ARGs) in environmental bacteria has drawn increasing attention in recent decades due to serious threat to human health (Finley et al., 2013). ARGs in the environment could be acquired by human pathogens via horizontal gene transfer, leading to the difficult therapy of infectious diseases and a potential worldwide human health risk (Allen et al., 2010). The prevalence and spread of ARGs in aquaculture environments are closely correlated with the improper or increased application of antibiotics, and is promoted by anthropogenic activities, such as intensive cultivation (Shah et al., 2014) and manure application (Yang et al., 2014). Recent studies have shown that aquaculture environment bacteria are capable of serving as reservoirs of resistance genes, and might facilitate the dissemination of ARGs (Aminov, 2009; Ng et al., 2018). Therefore, monitoring antimicrobial resistance and ARGs in bacteria isolated from aquaculture environments is very important to improve seafood quality and protect human health.

Vibrio parahaemolyticus is well known as the most prevalent food-poisoning bacterium associated with seafood consumption and typically causes acute gastroenteritis (Letchumanan et al., 2015; Su and Liu, 2007). It is naturally distributed in estuarine and marine environments worldwide, which allows the bacterium to be transmitted via seafood (Austin, 2010; Pal and Das, 2010). Naturally, *V. parahaemolyticus* is considered highly susceptible to most clinically used antibiotics (Han et al., 2007; Shaw et al., 2014). However, due to excessive use of antimicrobials in clinical treatment, agriculture, and aquaculture systems, increasingly antimicrobial resistance in *V. parahaemolyticus* has been reported in some countries, such as the USA (Han et al., 2007), Chile (Dauros et al., 2011), and China (Jiang et al., 2014). *V. parahaemolyticus* has been reported to exhibit resistance to penicillin, ampicillin, cefuroxime, and amikacin (de Melo et al., 2011; Sahilah et al., 2014), which compromises clinical therapy and presents a significant threat to human health worldwide. Notably, some strains even obtained resistance to chloramphenicol which has been banned for many years (Letchumanan et al., 2015; Shaw et al., 2014). Furthermore, the extensive use of antimicrobials has also caused the development of ARGs in *V. parahaemolyticus*, such as *catA2*, *aphA-3* (Letchumanan et al., 2015), bla_{TEM},

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https://doi.org/10.1016/j.marpolbul.2018.09.017
Received 7 August 2018; Received in revised form 10 September 2018; Accepted 10 September 2018

sul2, *strA*, *strB* (Jiang et al., 2014), bla_{CMY-2} (Li et al., 2015), and bla_{PER-1} (Wong et al., 2012). Nevertheless, since antimicrobials used in aquaculture are introduced mainly as immersion baths or medicated feed, aquaculture environment including water, sediments, and surrounding biological systems, are often directly and indirectly exposed to the antimicrobials used in aquaculture (Cabello, 2006). To our knowledge, the distribution and dissemination of ARGs on *V. parahaemolyticus* isolated from the aquaculture environment are still poorly investigated.

The eastern coasts of China are important mariculture areas filled with quantities of aquaculture systems providing the vast majority of marine products (Xue et al., 2017). Due to the intensive cultivation of large-scale aquaculture industry, the maricultural animals are especially vulnerable to infectious diseases (Biao and Kaijin, 2007; Diana et al., 2013). Antimicrobials are largely used for disease treatment and prevention on aquaculture, which could result in the development of the antibiotic resistance among the aquaculture bacteria, including potentially pathogenic *V. parahaemolyticus* (Jiang et al., 2014). It is worth mentioning that *V. parahaemolyticus* in maricultural environment is widely considered as the reservoir for antibiotic resistance genes (Chen et al., 2012; Xiong et al., 2015), which can enhance the dissemination of antibiotic resistance. As far as we know, there is little research focusing on the comprehensive reports of the antimicrobial resistance status and the molecular resistance mechanisms of *V. parahaemolyticus* in maricultural environment during the rearing. Additionally, no similar studies have been investigated in Zhejiang and Fujian, which have enormous seafood production and consumption annually.

In the present study, it was crucial to achieve a better understanding about the correlation of the phenotype with the genotype of *V. parahaemolyticus* isolated from maricultural environments in Zhejiang and Fujian Provinces, China during the rearing period. Therefore, the antimicrobial susceptibility and antimicrobial resistance mechanism of *V. parahaemolyticus* collected from shrimp maricultural environments in eastern coastal farms of China were investigated. In addition, pulsed-field gel electrophoresis (PFGE) was employed to explore the diversity and linkage of these isolates from different sources.

2. Materials and methods

2.1. Vibrio parahaemolyticus strains

A total of 260 rearing water samples were collected from 26 conventional shrimp farms in Zhejiang (12 shrimp farms) and Fujian (14 shrimp farms) province in China from September to October 2014 (abbreviated as ZJ and FJ, respectively) (Table 1, Fig. 1).

Table 1
Isolation of *V. parahaemolyticus* from water samples.

Location	Farm of sampling[a]	No. of samples obtained	No. (%)of samples positive to *V. parahaemolyticus*
Zhejiang	T1, T2, T3, T4, T5	5, 6, 6, 7, 11	3(60.0), 4(66.7), 3(50.0), 4(57.1), 4(36.4)
	N1, N2, N3, N4	9, 7, 12, 7	3(33.3), 3(42.9), 4(33.3), 3(42.9)
	W1	27	14(51.9)
	S1, S2, S3	7, 6, 9	3(42.9), 3(50.0), 3(33.3)
	H1	5	2(40.0)
Fujian	Z1, Z2	26, 9	14(53.8), 2(22.2)
	F1, F2, F3, F4	5, 12, 7, 4	1(20.0), 3(25.0), 2(28.6), 1(25.0)
	Q1, Q2	33, 4	20(60.6), 2(50.0)
	A1	10	4(40.0)
	D1	13	5(38.5)
	P1	6	2(33.3)
	J1	7	2(28.6)
Total		260	114

[a] T1-5, N1-4, W, S1-3, H, Z1-2, F1-4, Q1-2, A, D, P, J, represent different shrimp farms in Taizhou, Ningbo, Wenling, Shaoxing, Hangzhou, Zhangzhou, Fuzhou, Fuqing, Fuding, Ningde, Putian, Jinjiang.

These water samples were collected from 15 to 20 cm below the water surface between 10:30 a.m. and 11:30 a.m. in sterile bottles (250 mL) from four corners of each shrimp farm. All samples were cultured onto a thiosulfate-citrate-bile-salts-sucrose (TCBS) agar plate, and incubated at 28 °C for 16–24 h. On the TCBS plate, typical *V. parahaemolyticus* appeared as 2–3 mm in diameter green or blue-green colonies. For each positive plate, three presumptive colonies were collected for further *V. parahaemolyticus* identification, including *16S rRNA* gene sequence analysis (Kim et al., 2010), Gram-staining and API 20E system (Biomerieux Company, France) testing. *V. parahaemolyticus* CICC 21617, which was obtained from the China Center of Industrial Culture Collection (CICC), was used as the positive control strain. For each sample, merely one *V. parahaemolyticus* isolate was selected for subsequent analysis. All confirmed isolates were stored at −80 °C in brain-heart infusion broth (Land-bridge, Beijing, China) containing 20% (*v*/v) glycerol.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the standard agar dilution method, as described by the Clinical and Laboratory Standards Institute (CLSI, 2010). A total of 13 antimicrobials were tested, including ampicillin (AMP), ceftriaxone (CRO), cefepime (FEP), meropenem (MEM), ciprofloxacin (CIP), erythromycin (E), gentamicin (Iwanaga et al.), streptomycin (S), chloramphenicol (C), florfenicol (FFC), doxycylcine (DOX), trimethoprim/sulfamethoxazole (SXT: TMP/SMZ) and rifampicin (RA). Reference strains *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as the quality control organisms. Breakpoints for each antimicrobial agent and the minimum inhibitory concentration (Caburlotto et al.) ranges for quality control (QC) strains are presented in Table S1. A *V. parahaemolyticus* isolate simultaneously resistant to three or more classes of antimicrobials was defined as multi-drug resistant (MDR). Antibiotic sensitivity tests were performed in three repetitions for each isolate.

2.3. Detection of the virulence gene and resistance determinants

The genomic DNA of *V. parahaemolyticus* isolates was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to manufacturer's instructions, and were used as templates for subsequent polymerase chain reaction (PCR). PCR amplification for the detection of *V. parahaemolyticus* virulence genes, thermostable direct haemolysin (*tdh*) and thermostable-related direct haemolysin (*trh*) were performed in a duplex PCR, as previously described (Bej et al., 1999). The presence of genes involved in antimicrobial resistance to β-lactams (bla_{SHV}, bla_{TEM}, $bla_{CARB-17}$, $bla_{CTX-M-2}$), quinolones (*qnrA*, *qnrB*, *qnrS*, *qnrVC*), aminoglycosides (*strA, strB, aac(3)-IV, aadA, aadB*), phenicols (*floR*, *cat1*, *cat2*, *optrA*, *cfr*), macrolides (*ermA*, *ermB*, *mphC*), tetracyclines [*tet*(A), *tet*(B), *tet*(M)], rifampicin (*arr*) and sulfonamides (*sul1*, *sul2*, *sul3*) were detected by PCR. A total of 27 pairs of ologonulecotide primers designed to the resistant genes are listed in Table S2. Then, the PCR products were purified using the Wizard® DNA Clean-Up System (Promega, USA), and subsequently sequenced. The sequence data were compared to the NCBI nucleotide sequence database by means of BLAST.

2.4. Pulsed-field gel electrophoresis

PFGE analysis was performed, as previously described (Li et al., 2015), using *Not*I as the restriction endonuclease and *Salmonella* H9812 as the reference marker (digested with *Xba*I). Then, the PFGE results were analyzed using InfoQuest FP software version 4.5 (Bio-Rad Laboratories), and the banding patterns were clustered using Dice coefficients with a 2% band position tolerance and 1.5% optimization. A PFGE pattern was defined as a group of strains with a Dice coefficient similarity of 85% or greater, and the PFGE pattern represented by