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Antimicrobial resistance, virulence and genetic relationship of *Vibrio parahaemolyticus* in seafood from coasts of Bohai Sea and Yellow Sea, China

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ABSTRACT

Vibrio parahaemolyticus is an important foodborne pathogen which commonly inhabits estuarine and marine environments and seafood. In the present study, 90 *V. parahaemolyticus* isolates from the main seafoods from three coastal provinces surrounding Bohai Sea and Yellow Sea, China were analyzed to elucidate their antimicrobial resistance, virulence and genetic relationship by multilocus sequence typing (MLST). The results showed that the virulence genes *tdh* and *trh* were detected in one isolate and five isolates respectively. Most of isolates showed resistance to ampicillin (86/90) and cephazolin (75/90). Some isolates were resistant to amikacin (27/90), cefuroxime sodium (18/90), tetracycline (16/90), sulphamethoxazole/trimethoprim (16/90) and streptomycin (13/90). Forty isolates (44.4%) possessed multiple antimicrobial resistance to at least three antimicrobials. The *V. parahaemolyticus* population was composed of 68 sequence types, of which 41 were novel to the pubMLST database, displaying a high level of genetic diversity. The phylogenetic relatedness of *V. parahaemolyticus* isolates was irrelevant to the collection sources. Moreover, there were no associations of antimicrobial resistance and *trh* positive virulence with genetic population of *V. parahaemolyticus* isolates. These results indicated that the diversity of antimicrobial-resistant or pathogenic *V. parahaemolyticus* isolates from coasts of Bohai Sea and Yellow Sea, China could pose a potential risk to human health.

1. Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium which commonly inhabits estuarine and marine environments and seafood. Since it was discovered in the 1950s in Japan, this organism has been isolated from all over the world and considered as an important foodborne pathogen which can cause gastroenteritis, wound infections and septicemia (Deepanjali et al., 2005; Fujino et al., 1953; Nair et al., 2007; Okuda et al., 1997; Wagley et al., 2008; Yang et al., 2017; Zhao et al., 2011). The consumption of raw or undercooked seafood is the main reason of *V. parahaemolyticus* infection. In addition, the contact with the contaminated seafood, mariculture sources or processing places can also result in the dissemination of this pathogen. In China, the public health and commercial burdens associated with *V. parahaemolyticus* contamination are very high especially in the coastal regions. According to the official surveillance statistics from national foodborne disease surveillance system of China, *V. parahaemolyticus* is the leading cause of foodborne bacterial poisoning in China (Liu et al., 2008; Mao et al., 2013).

At present, more and more antimicrobial resistance problems have occurred due to the abuse of antimicrobials in the treatment and control of pathogens infection in clinic or in aquaculture. Since antimicrobial resistance could render many known antimicrobials ineffective, if the antimicrobial resistant pathogens enter the human body or antimicrobial resistant genes are transferred to the intestinal bacteria, there will be a threat to human health (York, 2017). It has been reported that many *V. parahaemolyticus* isolates from seafood have developed the antimicrobial resistance to ampicillin, streptomycin, kanamycin, tetracycline, ciprofloxacin, etc., and even to chloramphenicol which has been banned for many years (Devi et al., 2009; Elmahdi et al., 2016; Jiang et al., 2014; Kitiyodom et al., 2010; Kang et al., 2016; Raissy et al., 2012; Wong et al., 2012; Xie et al., 2017). Therefore, the

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surveillance of antimicrobial resistance for *V. parahaemolyticus* isolates in seafood is necessary.

Multilocus sequence typing (MLST) is a tool for molecular epidemiology and population genetic studies of pathogens (Maiden, 2006; Urwin and Maiden, 2003). Although there are other molecular typing methods, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE), MLST can provide the consistent typing results of bacterial isolates in different laboratories and facilitate determination of the global distribution and genetic population structure of pathogens. González-Escalona et al. (2008) had developed a successful MLST scheme for *V. parahaemolyticus* using sequences of internal fragments of seven housekeeping genes in a study of 100 isolates of global origin. Many studies subsequently utilized this method to successfully determine the genetic diversity of *V. parahaemolyticus* globally or geographically restricted isolates and demonstrated the epidemicity and genetic population structure of *V. parahaemolyticus* (Han et al., 2014, 2015; Li et al., 2015; Li et al., 2016; Theethakaew et al., 2013; Turner et al., 2013; Urmersbach et al., 2014).

Bohai Sea and Yellow Sea are the important mariculture areas in the north of China, where there are variety of seafoods. Although there were some reports on surveillance or antimicrobial resistance analysis of *V. parahaemolyticus* isolates from seafoods in these areas, however, the previous studies just focused on the specific area or sample type (Jiang et al., 2013, 2014; Li et al., 2017; Yuan et al., 2017; Zheng et al., 2015). Furthermore, no studies on genetic relationship on *V. parahaemolyticus* isolates from coasts of Bohai Sea and Yellow Sea by MLST were reported as far as we know. In the present study, *V. parahaemolyticus* isolates from the main seafoods in Shandong Province, Hebei Province and Liaoning Province surrounding Bohai Sea and Yellow Sea were analyzed to elucidate their antimicrobial resistance and virulence. Moreover, the genetic relationship of these isolates were investigated by MLST analysis to reveal the sequence polymorphisms and evolutionary relationships among *V. parahaemolyticus* isolates from different sources and the probable association of antimicrobial resistance and virulence with genetic population of these isolates was analyzed.

2. Materials and methods

2.1. Bacterial strains

A total of 90 *V. parahaemolyticus* isolates recovered from different seafoods, including shrimp, shellfish, sea cucumber and half-smooth tongue sole were analyzed in this study. The seafoods were sampled from some major mariculture farms in Shandong Province, Hebei Province and Liaoning Province surrounding Bohai Sea and Yellow Sea, China from 2009 to 2016. The samples were enriched in alkaline peptone water (Land Bridge Technology, Beijing, China), and then the resultant culture was streaked onto CHROMagar™ Vibrio chromogenic agar (CHROMagar Microbiology, Paris, France) and thiosulphate-citrate-bile salts-sucrose agar (Land Bridge Technology) plates. The typical suspected colonies were selected and confirmed by VITEK automatic microbial identification system (BioMérieux, France). All the isolates were preserved in LBS medium (10 g/L peptone (Oxoid, Hampshire, England), 5 g/L yeast extract (Oxoid), and 30 g/L sodium chloride) containing 20% (v/v) glycerol at −80 °C until use. Additionally, *V. parahaemolyticus* ATCC 17802 was served as reference strain for process control.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed by Kirby-Bauer disc agar diffusion method according to the instruction of Clinical and Laboratory Standards Institute (CLSI, 2006, 2011). Nineteen antimicrobial discs (Oxoid) were used including ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid 2:1 (AMC, 30 μg), cephazolin (CFZ, 30 μg), cefuroxime sodium (CXM, 30 μg), ceftriaxone (CRO, 30 μg), cefepime (FEP, 30 μg), amikacin (AMK, 30 μg), streptomycin (STR, 10 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), ofloxacin (OFX, 5 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NAL, 30 μg), tetracycline (TET, 30 μg), sulphamethoxazole/trimethoprim 19:1 (SXT, 25 μg), chloramphenicol (CHL, 30 μg) and nitrofurantoin (NIT, 300 μg). *V. parahaemolyticus* isolates were cultured in LBS to 0.5 McFarland turbidity, and 0.3 mL of the culture was spread on Mueller-Hinton Agar (Land Bridge Technology) supplemented with 8.5 g/L NaCl plates. The antimicrobial discs were attached to the plates after the culture was absorbed by the agar. The zones of inhibition were measured using the MF2 multi-functional machine (Shineso, Hangzhou, China) after incubation at 36 °C for 16–20 h. *Escherichia coli* ATCC 25922 was used as a quality control strain.

2.3. Genomic DNA extraction

The isolates were inoculated in 5 mL LBS and incubated at 36 °C overnight. The bacterial pellets were collected after centrifugation at 12,000*g* for 5 min and the genomic DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). The concentration and purity of genomic DNA were evaluated by Nanophotometer spectrophotometer (Implen, German).

2.4. Detection of virulence genes

The virulence genes including *tdh* and *trh* were tested by polymerase chain reaction (PCR) amplification. The sequences of primers for *tdh* (251 bp) and *trh* (250 bp) were as follows: tdh-F: 5′-CCACTACCACTC TCATATGC-3′, tdh-R: 5′-GGTACTAAATGGCTGACATC-3′, trh-F: 5′-GGCTCAAAATGGTTAAGCG-3′, trh-R: 5′-CATTTCCGCTCTCATA TGC-3′ (Tada et al., 1992). The PCR was carried out in a final volume of 20 μL containing 1 × PCR buffer (containing $MgCl_2$) (Takara, Dalian, China), 0.5 mM dNTPs (Takara), 0.25 μM forward primer and reverse primer (Songon, Shanghai, China), 1.0 U Taq polymerase (Takara), and 50 ng genomic DNA. The PCR amplification were conducted under the following condition: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s, and an additional 7 min extension at 72 °C. The amplicons were analyzed by agarose gel electrophoresis and photographed in the gel imaging system (Vilber Lourmat, France). The amplicons were gel purified by TIANgel Midi Purification Kit (Tiangen) and submitted to Sangon Biotech Ltd. for sequencing to confirm their identities. The sequence data were compared to the NCBI nucleotide sequence database by means of Basic Local Alignment Search Tool (BLAST). *V. parahaemolyticus* ATCC 33846 (tdh^+trh^-) and ATCC17802 (tdh^-trh^+) were used as positive control.

2.5. MLST analysis

Seven housekeeping genes, *dnaE*, *gyrB*, *recA*, *dtdS*, *pntA*, *pyrC* and *tnaA* were chosen as target genes for MLST analysis. The primers and PCR amplification protocol were described on the *V. parahaemolyticus* pubMLST website (http://www.pubmlst.org/vparahaemolyticus). The amplicons were analyzed by agarose gel electrophoresis and photographed in the gel imaging system. The amplicons were cleaned by TIANgel Midi Purification Kit (Tiangen) and submitted to Sangon Biotech Ltd. for sequencing in both directions with primers M13F and M13R. The Numbers for alleles and sequence types (STs) were queried by submitting sequences of seven housekeeping genes to the *V. parahaemolyticus* pubMLST website. If novel alleles or STs were identified to mismatch any preexisting alleles in the database, the forward and reverse sequences or the new alleles profile were submitted to the database curator to obtain a new serial number for the alleles or STs.