International Journal of Food Microbiology 290 (2019) 116–124

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

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

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ARTICLE INFO

Keywords:
Vibrio parahaemolyticus
Seafood
Antimicrobial resistance
Virulence
Genetic relationship
Multilocus sequence typing

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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https://doi.org/10.1016/j.ijfoodmicro.2018.10.005
Received 9 May 2018; Received in revised form 11 September 2018; Accepted 5 October 2018
Available online 06 October 2018

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.

2.6. Clonal complexes and phylogenetic analysis

The clonal complexes of the *V. parahaemolyticus* isolates were identified using the program eBURST v 3.0 (http://eburst.mlst.net). The clonal complex was restrictively defined as that isolates had to share 100% identity at least in six of the seven alleles in the same group (Feil et al., 2004). The statistical confidences for the ancestral types were assessed using 1000 bootstrap resamplings. Two different STs are considered single-locus variant (SLV) when they differ from each other at a single locus. Double-locus variants (DLVs) refer to two STs differing in only two of the seven loci. The "population snapshot" with the group definition 0 of 7 loci shared to all STs was constructed by eBURST v 3.0 (Francisco et al., 2009). The phylogenetic relationship based on UPGMA generated from concatenated sequences of seven housekeeping genes was constructed by START v 2.0 (Jolley et al., 2001).

2.7. Population genetic analysis

DnaSP v 5.0 was used to calculate the nucleotide diversity (Librado and Rozas, 2009). The START v 2.0 was used to calculate the number of alleles, the number of polymorphic sites, GC content, the ratio of nonsynonymous to synonymous substitutions (d_N/d_S), and the standardized index of association (I_A^S) (Jolley et al., 2001). For the d_N/d_S, the value < 1 means that the relative gene was mainly affected by purifying selection during the population evolution, the value $= 1$ means neutral selection and the value > 1 means positive selection. The extent of recombination within populations of bacteria can be reflected by standardized index of association (I_A^S). $I_A^S = 0$ indicates alleles are in linkage equilibrium (alleles are independently distributed at all loci) and recombination occurred frequently.

3. Results

3.1. Bacterial isolates

A total of 90 *V. parahaemolyticus* strains were isolated from seafoods, of which, 26 from sea cucumber, 24 from shrimp, 37 from shellfish and 3 from half-smooth tongue sole, 32 from Liaoning province, 54 from Shandong province and 4 from Hebei province (Table 1). Each strain was originally from different samples and was named from Vp01 to Vp90. The virulence gene *tdh* was detected in one isolate (1.1%) and *trh* was found in five isolates (5.5%).

3.2. Antimicrobial resistance of V. parahaemolyticus strains

In the present study, 19 antimicrobials belonging to β-lactams, aminoglycosides, carbapenems, quinolones, tetracyclines, sulphonamides, chloramphenicols and nitrofurans were used for testing the antimicrobial susceptibility of *V. parahaemolyticus* isolates. As results shown in Table 1, a large number of isolates showed resistance to AMP (86/90) and CFZ (75/90). Some isolates were resistant to AMK (27/90), CXM (18/90), TET (16/90), SXT (16/90) and STR (13/90). Very few of isolates exhibited resistance to NIT (3/90), OFX (2/90), CIP (2/90), NAL (2/90), CHL (2/90) and AMC (1/90). All isolates were sensitive to CRO, FEP, IPM and MEM. Forty isolates (44.4%) showed multiple antimicrobial resistance (MAR) to three or more than three antimicrobials. Among these MAR isolates, ten were resistant to four antimicrobials, five were resistant to five antimicrobials, four were resistant to six antimicrobials, one was resistant to seven antimicrobials, one was resistant to eight antimicrobials, and two were resistant to nine antimicrobials. The tdh^+ isolate was resistant to AMP, CFZ and AMK. Of the trh^+ isolates, two showed resistance to AMP and CFZ, two were resistant to AMP, CFZ and AMK, and one was resistant to AMP, CFZ, AMK and STR.

3.3. Nucleotide diversity of each MLST locus

The nucleotide sequence data for the seven housekeeping gene fragments for the 90 *V. parahaemolyticus* isolates were summarized in Table 2. The numbers of alleles observed for each MLST locus were distributed as follows: 44 (*dnaE*), 49 (*gyrB*), 47 (*recA*), 52 (*dtds*), 32 (*pntA*), 44 (*pyrC*) and 34 (*tnaA*). There were 19 new alleles found in six loci including 2 *dnaE*, 4 *gyrB*, 3 *recA*, 3 *dtds*, 2 *pntA* and 5 *pyrC* (Table 1). The most frequency found alleles per locus were *dnaE98* (11), *gyrB4* and *gyrB106* (12), *recA31* (9), *dtdS74* (8), *pntA*26 (15), *pyrC11* (13), *tnaA23* and *tnaA26* (14). The number of polymorphic sites varied per locus from 25 (*tnaA*) to 183 (*recA*). The nucleotide diversity ranged from 0.0113 to 0.0358, with the highest degrees of diversity observed for *recA* (0.0358) and *dtdS* (0.0224). The GC contents varied per locus from 43.81% (*pntA*) to 50.29% (*dtds*). The value of d_N/d_S was lower than 1 for each locus analyzed using a selection test for neutrality, among which, no nonsynonymous change was detected at *gyrB*.

3.4. ST diversity

A total of 68 STs were identified in the 90 *V. parahaemolyticus* isolates, and 41 (60.3%) of them were novel, since they had not been recorded in the pubMLST database previously (Table 1). Twenty-one STs were present in 26 isolates from sea cucumber, 13 (61.9%) of which were novel. Nineteen STs were present in 24 isolates from shrimp, 6 (31.6%) of which were novel. Thirty-two STs were present in 37 isolates from shrimp, 21 (65.6%) of which were novel. Three isolates from half-smooth tongue sole represented 3 novel STs. Fifty-six of the STs contained single isolates, while 12 STs included between 2 and 6 isolates. ST-564 was most frequent (6/90) and was composed of isolates originated from three kinds of aquatic products and two provinces. Other STs were also composed of more than one isolate: ST-1755 (4), ST-114 (3), ST-1081 (3), ST-1318 (3), ST-1774 (3), ST-150 (2), ST-693 (2), ST-1757 (2), ST-1777 (2), ST-1781 (2) and ST-1793 (2).

3.5. Clonal complexes

To gain an overview of clonal relations within the analyzed isolates, a 'population snapshot' was obtained by eBURST analysis (Fig. 1). The 68 STs were separated into two doublets (D1 and D2 which contained SLVs) and 64 singletons. D1 corresponds to two isolates (ST-693) from shrimp and one isolate (ST-864) from shellfish in Shandong Province. D2 corresponds to one isolate (ST-1764) from sea cucumber in Shandong Province and one isolate (ST-1778) from shellfish in Liaoning Province. Among the singletons, ST-433 and ST-1781 were DLVs which shared five of the seven loci, and ST-3, ST-114, ST-155 belonged to clonal complexes CC3, CC114 and CC155. In this study, the value of I_A^S was 0.2586 ($p < 0.01$) when it was calculated for all of 90 isolates, indicating that the alleles of the MLST loci were in linkage disequilibrium. When the calculation was repeated using one isolate to represent each of 68 STs, the value of I_A^S was 0.0366 ($p < 0.01$).

3.6. Clustering and phylogenetic analysis

The UPGMA analysis based on the concatenated sequences revealed a high genetic diversity of 90 *V. parahaemolyticus* isolates (Fig. 2). ST-1754, ST-1761, ST-1765 and ST-1771 formed an individual cluster in the UPGMA tree. The two doublets (D1 and D2) and DLVs (ST-433 and ST-1781) found by eBURST also grouped together in the UPGMA tree. Many novel STs were clustered with the old STs recorded in pubMLST database and showed close relationship. Some isolates from different resources including samples, regions and years might closely related, while most of isolates from the same collection region or sample type were distantly related.

Table 1
Allele profiles, STs, virulence genes, antimicrobial resistance profiles and sources of 90 *V. parahaemolyticus* isolates.

Strain	Sources			Allele profiles							ST	Virulence gene		Antimicrobial resistance profiles
	Sample	Region	Year	*dnaE*	*gyrB*	*recA*	*dtdS*	*pntA*	*pyrC*	*tnaA*		*tdh*	*trh*	
Vp01	Sea cucumber	Liaoning	2009	98	4	112	107	77	97	23	433	−	−	AMP-CFZ
Vp02	Sea cucumber	Liaoning	2009	103	186	31	78	2	144	26	347	−	−	AMP-CFZ
Vp03	Sea cucumber	Liaoning	2009	31	106	135	74	37	212	54	564	−	−	AMP-CFZ-AMK-TET-SXT
Vp04	Sea cucumber	Liaoning	2009	11	75	64	67	26	7	50	155	−	−	AMP-CFZ-STR
Vp05	Sea cucumber	Liaoning	2009	31	**494**	107	153	128	160	33	**1754**	−	−	AMP-CFZ-AMK-TET
Vp06	Sea cucumber	Liaoning	2010	5	88	229	27	18	218	178	**1755**	−	−	AMP-CFZ-STR
Vp07	Sea cucumber	Liaoning	2010	5	88	229	27	18	218	178	**1755**	−	−	AMP-STR
Vp08	Sea cucumber	Liaoning	2010	5	88	229	27	18	218	178	**1755**	−	−	AMP-CFZ-STR
Vp09	Sea cucumber	Liaoning	2010	84	103	79	46	62	46	60	**1756**	−	−	AMP-CFZ
Vp10	Sea cucumber	Shandong	2010	31	106	135	74	37	212	54	564	−	−	AMP-CFZ-AMK-STR-OFX-CIP-NAL-TET-SXT
Vp11	Sea cucumber	Shandong	2010	31	106	135	74	37	212	54	564	−	−	AMP-CFZ-AMK-STR-OFX-CIP-NAL-TET-SXT
Vp12	Sea cucumber	Shandong	2010	5	88	229	27	18	218	178	**1755**	−	−	AMP-CFZ
Vp13	Sea cucumber	Liaoning	2010	28	310	35	270	154	37	33	**1757**	−	−	AMP-CFZ-STR
Vp14	Sea cucumber	Liaoning	2010	104	147	31	171	211	11	23	**1758**	−	−	AMP-CFZ-CXM
Vp15	Sea cucumber	Liaoning	2010	158	383	62	117	195	46	26	**1759**	−	−	AMP-CFZ
Vp16	Sea cucumber	Liaoning	2010	207	106	30	303	28	275	61	**1760**	−	+	AMP-CFZ-AMK
Vp17	Sea cucumber	Liaoning	2010	188	106	107	331	26	3	86	**1761**	−	+	AMP-CFZ-AMK
Vp18	Sea cucumber	Liaoning	2010	26	106	31	73	26	11	23	1092	−	−	AMP-CFZ
Vp19	Sea cucumber	Shandong	2010	176	241	61	190	50	**406**	20	**1762**	−	−	AMP-CFZ
Vp20	Sea cucumber	Shandong	2010	299	184	27	**428**	50	37	169	**1763**	−	−	AMP-CFZ
Vp21	Sea cucumber	Shandong	2010	98	4	30	19	77	10	82	**1764**	−	−	AMP-CFZ
Vp22	Sea cucumber	Shandong	2010	47	58	53	19	50	37	26	162	−	−	AMP-CFZ
Vp23	Sea cucumber	Shandong	2010	14	30	105	78	27	7	13	**1765**	−	−	AMP-CFZ
Vp24	Sea cucumber	Shandong	2010	183	493	61	269	128	3	26	**1766**	−	−	AMP-CFZ
Vp25	Sea cucumber	Shandong	2010	76	88	31	13	53	45	13	165	−	−	AMP-CFZ-AMK
Vp26	Sea cucumber	Shandong	2010	51	4	218	84	60	8	33	1318	−	−	AMP-CFZ
Vp27	Shrimp	Shandong	2012	12	146	117	29	28	10	54	659	−	−	AMP-CFZ
Vp28	Shrimp	Shandong	2012	31	106	135	74	37	212	54	564	−	−	AMP-CFZ-STR-SXT
Vp29	Shrimp	Shandong	2012	183	247	172	27	23	182	57	469	−	−	AMP-CFZ
Vp30	Shrimp	Shandong	2012	3	230	61	268	2	46	2	693	−	−	AMP-CFZ-AMK-STR-TET-SXT
Vp31	Shrimp	Shandong	2012	3	230	61	268	2	46	2	693	−	−	AMP-CFZ-CXM-AMK-STR-TET-SXT
Vp32	Shrimp	Shandong	2012	182	355	**358**	**429**	26	**402**	128	**1767**	−	−	AMP-CFZ-AMK
Vp33	Shrimp	Shandong	2012	31	106	135	74	37	212	54	564	−	−	CFZ-SXT
Vp34	Shrimp	Shandong	2012	35	20	356	29	23	186	27	**1768**	−	−	AMP-CFZ
Vp35	Shrimp	Shandong	2012	160	203	15	118	82	5	80	376	−	−	AMP-CFZ
Vp36	Shrimp	Shandong	2012	49	209	249	50	112	37	23	984	−	−	AMP-CFZ
Vp37	Shrimp	Shandong	2012	51	**495**	30	129	26	23	26	**1769**	−	+	AMP-CFZ
Vp38	Shrimp	Shandong	2012	155	29	141	73	26	100	26	**1770**	−	−	AMP-CFZ
Vp39	Shrimp	Shandong	2012	162	399	80	150	11	158	51	1357	−	+	AMP-CFZ
Vp40	Shrimp	Shandong	2012	71	345	246	167	177	69	47	815	−	−	CFZ
Vp41	Shrimp	Shandong	2012	270	371	105	342	23	238	26	**1771**	−	+	AMP-CFZ-AMK-STR
Vp42	Shrimp	Shandong	2012	55	15	31	55	18	58	46	114	−	−	AMP-CFZ
Vp43	Shrimp	Shandong	2012	55	15	31	55	18	58	46	114	−	−	AMP-CFZ
Vp44	Shrimp	Shandong	2012	4	13	220	38	18	9	23	1081	−	−	AMP-CFZ-AMK-STR
Vp45	Shrimp	Shandong	2012	4	13	220	38	18	9	23	1081	−	−	AMP-CFZ
Vp46	Shrimp	Shandong	2012	4	13	220	38	18	9	23	1081	−	−	AMP-CFZ
Vp47	Shrimp	Shandong	2012	47	287	19	252	**245**	18	217	**1772**	−	−	AMP-CFZ
Vp48	Shrimp	Shandong	2012	214	342	3	297	61	14	23	800	−	−	AMP-CFZ
Vp49	Shrimp	Shandong	2016	19	74	61	68	48	11	26	150	−	−	AMP-CFZ-AMK-TET-SXT
Vp50	Shrimp	Shandong	2016	19	74	61	68	48	11	26	150	−	−	AMP-CFZ-AMK-TET-SXT
Vp51	Shellfish	Shandong	2010	346	184	4	74	28	**403**	23	**1773**	−	−	AMP-CFZ-CXM-AMK-TET-SXT
Vp52	Shellfish	Liaoning	2014	98	4	136	243	26	11	99	**1774**	−	−	AMP
Vp53	Shellfish	Shandong	2014	55	15	31	55	18	58	46	114	−	−	AMP-CFZ
Vp54	Shellfish	Shandong	2014	**363**	246	19	75	**246**	10	26	**1775**	−	−	AMP
Vp55	Shellfish	Shandong	2014	28	310	35	270	154	37	33	**1757**	−	−	AMP-CFZ-AMK
Vp56	Shellfish	Liaoning	2014	98	4	136	243	26	11	99	**1774**	−	−	AMP-CFZ
Vp57	Shellfish	Liaoning	2014	113	145	134	70	23	11	26	**1776**	−	−	AMP
Vp58	Shellfish	Liaoning	2014	3	82	21	69	26	195	51	**1777**	−	−	CFZ
Vp59	Shellfish	Liaoning	2014	3	4	19	4	29	4	22	3	+	−	AMP-CFZ-AMK
Vp60	Shellfish	Liaoning	2014	98	4	30	19	77	11	82	**1778**	−	−	AMP
Vp61	Shellfish	Shandong	2014	36	372	**359**	19	1	11	26	**1779**	−	−	AMP
Vp62	Shellfish	Shandong	2014	103	490	31	169	77	401	79	**1780**	−	−	AMP-CFZ
Vp63	Shellfish	Liaoning	2014	31	106	135	74	37	212	54	564	−	−	CFZ-SXT
Vp64	Shellfish	Hebei	2015	51	4	218	84	60	8	33	1318	−	−	AMP
Vp65	Shellfish	Liaoning	2015	51	4	218	84	60	8	33	1318	−	−	AMP-CFZ-AMK
Vp66	Shellfish	Shandong	2015	60	106	31	72	66	62	65	676	−	−	AMP-CFZ
Vp67	Shellfish	Shandong	2015	98	4	136	107	77	11	23	**1781**	−	−	AMP-CXM
Vp68	Shellfish	Shandong	2015	167	147	109	19	145	219	12	1696	−	−	AMP-CFZ-CXM
Vp69	Shellfish	Shandong	2015	98	4	136	243	26	11	99	**1774**	−	−	AMP
Vp70	Shellfish	Shandong	2015	5	40	38	209	45	190	94	505	−	−	AMP-CFZ-CXM
Vp71	Shellfish	Shandong	2015	3	82	21	69	26	195	51	**1777**	−	−	AMP
Vp72	Shellfish	Hebei	2015	27	226	127	19	26	18	23	**1782**	−	−	AMP-CFZ-AMK-NIT

(*continued on next page*)

Table 1 (*continued*)

Strain	Sources			Allele profiles							ST	Virulence gene		Antimicrobial resistance profiles
	Sample	Region	Year	*dnaE*	*gyrB*	*recA*	*dtdS*	*pntA*	*pyrC*	*tnaA*		*tdh*	*trh*	
Vp73	Shellfish	Hebei	2015	2	198	72	94	26	7	23	**1783**	–	–	AMP-CFZ-CXM-AMK-TET-SXT
Vp74	Shellfish	Liaoning	2015	98	4	136	107	77	11	23	**1781**	–	–	AMP-CFZ-CXM
Vp75	Shellfish	Liaoning	2015	49	**496**	**360**	133	18	**404**	143	**1784**	–	–	AMP-CFZ-CXM-TET-SXT
Vp76	Shellfish	Liaoning	2015	3	29	2	33	18	5	17	**1785**	–	–	AMP-CXM
Vp77	Shellfish	Hebei	2015	98	49	45	49	4	50	23	**1786**	–	–	AMP-CFZ-CXM-SXT
Vp78	Shellfish	Shandong	2015	3	230	61	268	2	245	2	864	–	–	AMP-AMC-CFZ-CXM-AMK-STR-TET-SXT
Vp79	Shellfish	Liaoning	2015	**364**	**497**	99	74	26	11	33	**1787**	–	–	AMP-CFZ
Vp80	Shellfish	Liaoning	2015	47	8	166	19	28	46	121	413	–	–	AMP-CFZ-CXM
Vp81	Shellfish	Liaoning	2016	116	251	72	76	45	184	26	1107	–	–	AMP-CXM
Vp82	Shellfish	Liaoning	2016	35	103	286	224	50	190	73	**1788**	–	–	AMP-CFZ -CXM-AMK-TET-SXT
Vp83	Shellfish	Liaoning	2016	188	106	199	331	2	3	20	**1789**	–	–	AMP-CFZ
Vp84	Shellfish	Liaoning	2016	9	1	62	296	49	85	20	**1790**	–	–	AMP-CFZ -CXM-AMK
Vp85	Shellfish	Shandong	2016	31	25	44	**430**	31	133	57	**1791**	–	–	AMP-CXM-AMK
Vp86	Shellfish	Shandong	2016	36	168	102	215	4	285	62	**1792**	–	–	AMP-CXM
Vp87	Shellfish	Shandong	2016	28	106	82	204	18	7	26	491	–	–	AMP-CFZ-AMK-NIT
Vp88	Half-smooth tongue sole	Shandong	2011	98	25	3	206	23	**405**	33	**1793**	–	–	AMP-AMK-TET-CHL
Vp89	Half-smooth tongue sole	Shandong	2011	19	297	248	13	26	250	157	**1794**	–	–	AMP-CFZ-TET-NIT
Vp90	Half-smooth tongue sole	Shandong	2011	98	25	3	206	23	**405**	33	**1793**	–	–	AMP-CFZ-AMK-TET-CHL

Note: a bold-faced number refers a novel allele or ST.

Table 2
Nucleotide sequence variations of each MLST locus for 90 *V. parahaemolyticus* isolates.

Locus	Fragment size (bp)	No. of alleles	GC content (%)	Nucleotide diversity (SE)	No. of polymorphic site (%)	d_N/d_S ratio
dnaE	557	44	48.67	0.0113 (0.0007)	39 (7.00)	0.0279
gyrB	592	49	47.67	0.0147 (0.0006)	50 (8.45)	0.0000
recA	729	47	45.15	0.0358 (0.0093)	183 (25.1)	0.0252
dtdS	458	52	50.29	0.0224 (0.0011)	43 (9.39)	0.0022
pntA	430	32	43.81	0.0119 (0.0008)	31 (7.21)	0.0273
pyrC	493	44	48.29	0.0124 (0.0008)	39 (7.91)	0.0327
tnaA	423	34	48.81	0.0118 (0.0008)	25 (5.91)	0.0035

3.7. Association of antimicrobial resistance and virulence with genetic population of isolates

There were no relationship between ST and antimicrobial resistance as shown in Table 1. Except that all three isolates belonging to ST-114 were resistant to AMP and CFZ, other isolates which shared the same ST had the different antimicrobial resistance profiles. Interestingly, all six isolates belonging to ST-564 from different sources were resistant to SXT although their resistance profiles were not the same. There was only one *tdh*$^+$ pathogenic strain identified in this study, which corresponded to pandemic sequence type ST-3. Five *trh*$^+$ strains identified in this study were singletons belonging to ST-1357, ST-1760, ST-1761, ST-1769 and ST-1771, which were all novel STs.

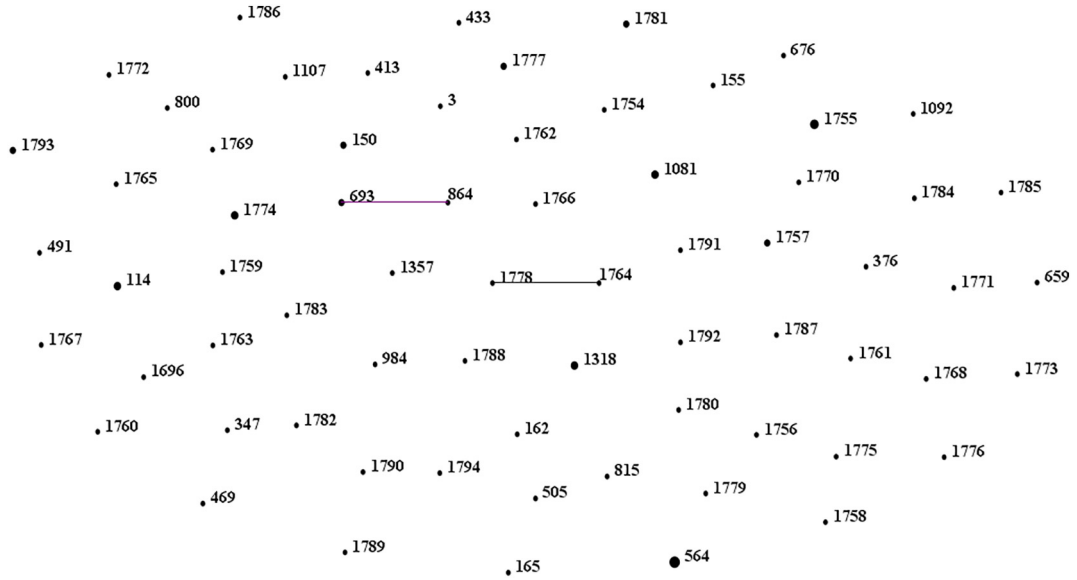

Fig. 1. "Population snapshot" of 90 *V. parahaemolyticus* isolates created by eBURST v 3.0. The two groups connected by black lines were defined using stringent criteria (6/7 shared alleles). The sizes of the circles are relative to the numbers of strains in the ST.

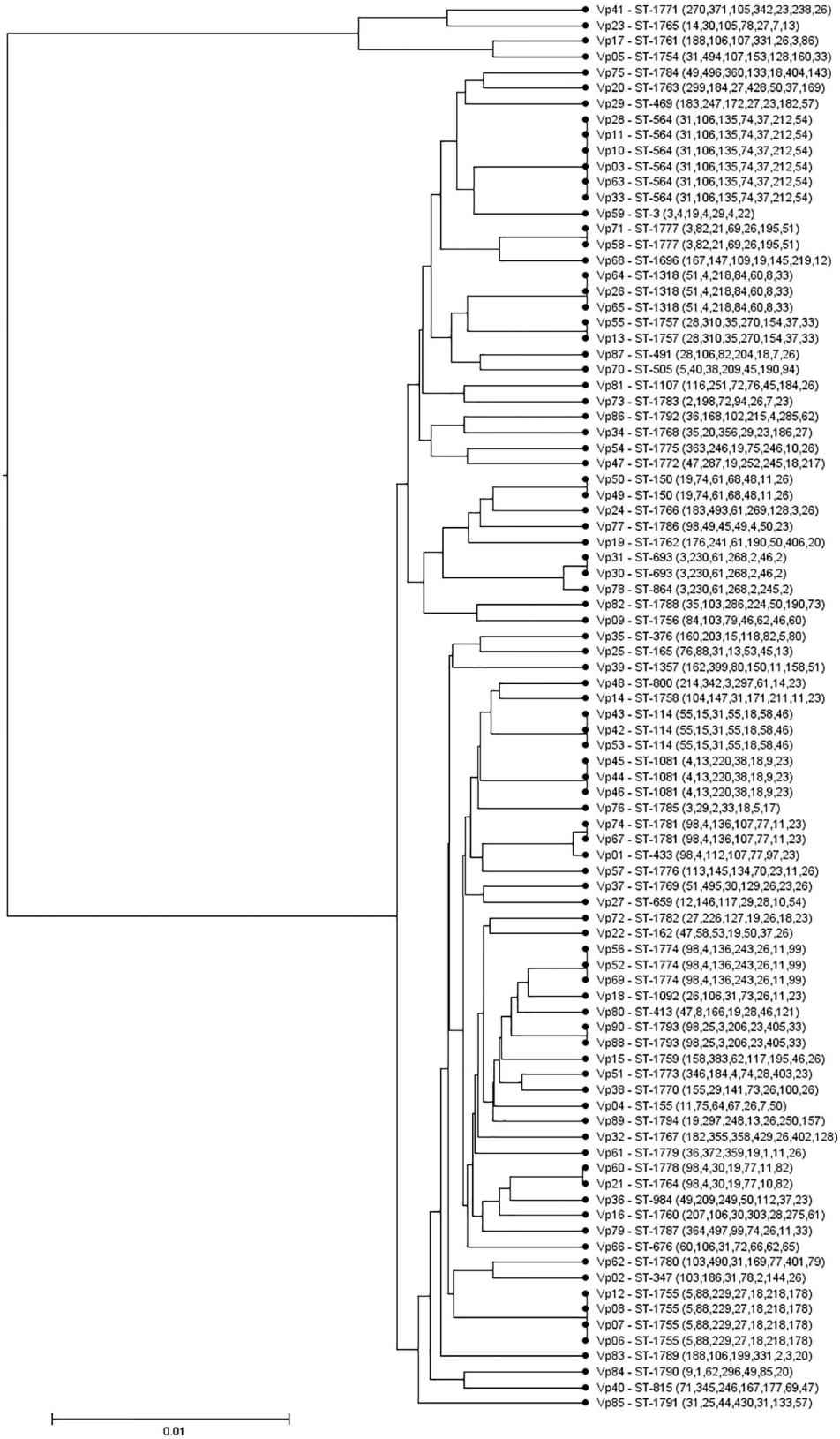

Fig. 2. Phylogenetic relationship of 90 *V. parahaemolyticus* isolates based on UPGMA generated from the concatenated sequences of housekeeping genes structured by START v 2.0.

4. Discussion

In the present study, a total of 90 *V. parahaemolyticus* isolates from different seafoods from coasts of Bohai Sea and Yellow Sea were analyzed. Most of *V. parahaemolyticus* isolates were resistant to AMP and CFZ which is in agreement with the previous study (Jun et al., 2012). A certain proportion of isolates were resistant to the second generation cephalosporins (CXM), aminoglycosides (AMK and STR),

tetracyclines (TET), and sulphonamides (SXT). Like most previous reports (Jun et al., 2012; Kitiyodom et al., 2010; Wong et al., 2012), the new front-line antimicrobials such as extended-spectrum cephalosporins, fluoroquinolones, carbapenems were highly effective on *V. parahaemolyticus* isolates. While in other reports, the antimicrobial resistance ratios could be different (Kitiyodom et al., 2010; Kang et al., 2016; Wong et al., 2012; Xie et al., 2017), for instance, 50.7% of *V. parahaemolyticus* isolates from oysters in Korea exhibited resistance to streptomycin in the report of Kang et al. (2016), and 90.53%, 33.68% and 30.53% of aquatic product isolates from Guangdong, China were resistant to streptomycin, ampicillin and cephalothin in the report of Xie et al. (2017). These differences in antimicrobial resistance among our study and other reports may be due to the different antimicrobial stress in different environments which affect the antimicrobial resistance of bacteria (Sommer and Dantas, 2011). Nearly half of *V. parahaemolyticus* isolates demonstrated MAR to at least three antimicrobials, some of which were even resistant to nine antimicrobials. Emergence of MAR of *V. parahaemolyticus*, which also occurred in many isolates from different environmental samples (Kitiyodom et al., 2010; Kang et al., 2016; Wong et al., 2012; Xie et al., 2017), is a serious clinical problem in the treatment and containment of vibriosis and poses a threat to public health and ecological diversity of marine microorganisms.

For investigating the pathogenicity of isolates, *tdh* and *trh* genes which encode the virulence factors thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH) were tested. Only one *tdh*$^+$ isolate and five *trh*$^+$ isolates were detected, which is in agreement with many previous reports suggesting low prevalence of pathogenic *V. parahaemolyticus* in environmental samples (Xie et al., 2016; Yang et al., 2017; Zhao et al., 2011). Among these six pathogenic isolates, four showed MAR to three or four antimicrobials, which was not necessarily more severe than the non-pathogenic isolates. The report of Xie et al. (2017) showed the extent of MAR of clinical isolates was commonly more severe than that of environmental isolates, however, the non-pathogenic isolates might show MAR to more antimicrobials than pathogenic isolates just like our study. These results suggest that there is no correlation between pathogenicity and antimicrobial resistance among environmental *V. parahaemolyticus* stains.

In the present study, the extent of genetic diversity of *V. parahaemolyticus* isolates was analyzed on nucleotide level. The low d_N/d_S ratio obtained for seven housekeeping genes indicated that purifying selection was dominant for these genes, which were consistent with the results of previous reports (González-Escalona et al., 2008; Han et al., 2014, 2015). A total of 68 different allelic combinations and an average of 43 alleles per locus were identified, indicating a high degree of genotypic diversity at slowly evolving loci. Among these seven housekeeping genes, *recA* represents the greatest number of polymorphic nucleotide sites (183) and the highest degree of nucleotide diversity (0.0358). The similar results were obtained by other studies that there were highly divergence and frequent combination occurred in *recA* alleles (Han et al., 2014, 2015; Theethakaew et al., 2013). These similar conclusions suggested that *recA* had rather a large influence over the population structure and phylogenetic relationships of *V. parahaemolyticus* strains. Theethakaew et al. (2013) have reported that *recA107* and *recA120* alleles are highly divergent and have been acquired by horizontal DNA transfer by isolates representing ST-265 and ST-251, which formed an individual cluster by Bayesian clustering analysis. In this study, four isolates containing the *recA105* and *recA107* were clustered in the individual branch in the UPGMA tree, which may be due to the genetic diversity of these isolates influenced by *recA105* and *recA107* alleles.

As many as 68 STs were identified among 90 *V. parahaemolyticus* isolates and a high proportion of novel STs were found, indicating that the high degree of environmental diversity and how poorly the current pubMLST data set represents this diversity within *V. parahaemolyticus*. In this study, ST-564 is the predominant ST which was seldom reported by other researchers (Li et al., 2016). Numerous singletons were observed, of which, ST-3, ST-114, ST-155 belonged to clonal complexes CC3, CC114 and CC155. Other isolates did not relate to any known clonal complexes. One isolate represented pandemic ST-3 which was identical to those of isolates recovered from clinical sources of worldwide distribution (González-Escalona et al., 2008; Han et al., 2014, 2015; Li et al., 2016; Theethakaew et al., 2013; Turner et al., 2013). With regard to the 27 STs which bad been recorded in the MLST database, except for ST-3, only ST-162, ST-433, ST-491 and ST-815 is respectively composed of one clinical isolate, and other STs are composed of environmental isolates. The clonal population structure is also supported by value of $\bar{I}_A^S$ which were significantly different to zero for all analyzed sets or for only one isolate per ST, suggesting that recombination was a major force driving diversification and speciation of *V. parahaemolyticus* isolates (González-Escalona et al., 2008; Theethakaew et al., 2013; Urmersbach et al., 2014).

From the results we can see that some *V. parahaemolyticus* isolates were shown to be the same ST, such as ST-564, even though they originated from different samples, regions and collection years. The UPGMA analysis further proved that there was no correlation between collection sources and phylogenetic relatedness of *V. parahaemolyticus* isolates, which was similar with a previous study (Ellis et al., 2012). Some isolates from different sources including samples, regions and collection years might be closely related. For example, the isolate Vp02 (ST-347) from sea cucumber in Liaoning Province at 2009 and the isolate Vp62 (ST-1780) from shellfish in Shandong Province at 2014 was closely related. While most of the isolates from the same sources were distantly related, for instance, the five isolates recovered from sea cucumber in Liaoning Province at 2009 were distributed in different phylogenetic clusters. The presence of the same ST or close relateness of *V. parahaemolyticus* isolates from different sources has no easy explanation, we speculated that this could be due to either ocean currents or interchange of seafoods among different coastal areas surrounding Bohai Sea and Yellow Sea.

In the present study, the probable relationship between ST and antimicrobial resistance were analyzed in order to find out the dissemination and prevalence of antimicrobial resistant isolates. Except that all three isolates belonging to ST-114 were resistant to AMP and CFZ, other isolates which shared the same ST had the different antimicrobial resistance profiles, indicating that there were no relationship between ST and antimicrobial resistance. However, we found that all six isolates corresponded to ST-564 were resistant to SXT although their resistance profiles were not the same. Among these isolates, Vp03 was firstly recovered from sea cucumber in Shandong Province at 2009, and then Vp10 and Vp11 were isolated from sea cucumber in Shandong Province at 2010, Vp28 and Vp33 were from shrimp in Shandong Province at 2012, and Vp63 was lastly found from shellfish in Liaoning Province at 2014. According to the sources and the antimicrobial resistance profile, it is speculated that these six isolates were evolved from the same clone which was already resistant to SXT and developed resistance to other antimicrobials during the process of reproduction and dissemination in different environments. The whole genome sequencing is recommended to be used for analysis of the evolution of these isolates.

The genetic relationships between ST and the virulence of *V. parahaemolyticus* isolates were also analyzed. There was only one *tdh*$^+$ pathogenic strain identified in this study, which corresponded to pandemic sequence type ST-3 (González-Escalona et al., 2008; Han et al., 2014, 2015; Li et al., 2016; Theethakaew et al., 2013; Turner et al., 2013). According to the pubMLST database, ST-3 is presently composed of 251 isolates recovered from all over the world, 33 of which were environmental strains. This study and the previous records provided the evidence of a genetic link between environment and clinical isolates and showed that seafood could become the source of infection by transmitting potentially pathogenic *V. parahaemolyticus* to human body (Theethakaew et al., 2013). Each of five *trh*$^+$ isolates identified in this

study belonged to different STs, all of which were novel STs. Ellingsen et al. (2013) has analyzed the genetic relationships among 20 *trh*$^+$ isolates from Norwegian mussels and seawater or clinical sources and USA environment, and found that all isolates did not appear to any pandemic STs. Similar result was obtained in the study of Bechlars et al. (2015). All these results indicate that *trh*$^+$ isolates are the non-pandemic strains although they are considered as pathogenic strains.

The present study is (to the author's knowledge) the first to indicate a genetic relationship of *V. parahaemolyticus* isolates from different seafoods from the coasts of Bohai Sea and Yellow Sea, China and the first to analysis of the probable association of antimicrobial resistance and virulence with genetic population of these isolates. The *V. parahaemolyticus* isolates in this study displayed a several MAR which were irrelevant to the genetic population. In addition, few of the isolates were pathogenic and the *trh* positive has no association with pandemic STs. The diversity of *V. parahaemolyticus* isolates as well as the independence on genetic population of antimicrobial resistant isolates or *trh*$^+$ isolates may increase the difficulty of prevention and control of this pathogen, and pose a potential risk to human health. More *V. parahaemolyticus* isolates from Chinese coastal environments are further needed to be analyzed in order to find the prevalent genetic populations.

Acknowledgements

The authors thank Dr. Narjol Gonzalez-Escalona, the curator of *V. parahaemolyticus* pubMLST database, for his kind processing of new allele sequences for novel alleles and sequence types. This work was jointly supported by Shandong Provincial Natural Science Foundation, China (No. ZR2014CQ054) and National Natural Science Foundation of China (No. 31601566).

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