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Surveillance and Molecular Characterization of *Vibrio parahaemolyticus* from Different Sources in Zhoushan Islands, China

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Abstract: *V. parahaemolyticus* is one of the most important foodborne pathogens in China and other countries. In this study, researchers investigated the contamination of *V. parahaemolyticus* in shellfish during different seasons in Zhoushan islands. Meanwhile, researchers reported the prevalence, molecular characterization and virulence genes of *V. parahaemolyticus* from shellfish and clinical patients. In shellfish, the positive rate was 85, 80, 75 and 10%, respectively in different seasons from Spring to Winter. Among the shellfish isolates, there was no tdh^+ strains and only 4% trh^+ strains. The prevalence of *V. parahaemolyticus* in clinical samples was 36 and 77.78% of clinical strains were tdh^+, $toxRS/new^+$, $orf8^+$ and 5.56% clinical strains were trh^+. In 80% similarity, the total of 53 strains could be divided into twenty nine types. Most of the shellfish strains present various patterns with far relationship while all the pandemic strains from clinical samples belonged to same clone group with 82.8% similarity. The tdh^+, $toxRS/new^+$ and $orf8^+$ strains were the main epidemic strains in Zhoushan islands.

Key words: *V. parahaemolyticus*, virulence gene, molecular typing, island, shellfish, strains

INTRODUCTION

V. parahaemolyticus is a water and foodborne pathogen, commonly present in sea water and marine product all over the world. Research has suggested that the shellfish were seriously polluted by *V. parahaemolyticus* and humans often get infection by consuming raw or undercooked shellfish. In the USA, raw oysters are the most important vehicles for transmission of *V. parahaemolyticus*. Zhoushan city is a island city with abundant shellfish and the infection caused by this bacterium is the most important cause of foodborne disease. In this study, researchers investigated the contamination of *V. parahaemolyticus* in the shellfish in Zhoushan. In addition, researchers analyzed the serovar distrubution, the presence of virulence genes and molecular typing of all strains isolated from shellfish and clinical samples in Zhoushan city.

MATERIALS AND METHODS

Shellfish samples: Shellfish food samples (n = 80) were collected from aquatic food markets (n = 5) in Zhoushan city. Of the 80 samples, shellfish food normally included *Scapharca subcrenata*, razor clam, clam, *Moerella iridescens*, mussels, oyster, Whelk, etc. which were bred with artificial seawater. Considering the influence of seawater temperature, samples were collected in different season in 2009.

Bacterial isolates from clinical patients: There were 18 strains from clinical patients, in which 7(VP51-VP57) isolates from patients of outbreaks and 11 (VP58-VP68) were from sporadic diarrhea outpatients of foodborne diseases from hospitals. All strains were isolated from May to September in 2009.

Identification of *V. parahaemolyticus*: About 25 g of food samples was added to 225 mL sodium chloride violet purple enrichment broth (or 1 g feces samples added to 9 mL of the broth) and incubated for 9 h. A loop of the enriched broth was streaked onto Vibrio Chromogrnic medium (CHROMagar, Paris, France) and TCBS. Typical colonies were confirmed as *V. parahaemolyticus* by ATB ID32 E kits.

Serogrouping: About 11 of Serum O and some common Serum K was bought from Denka Seiken, Tokyo, Japan. Following manufacturer's instructions, an aliquot of the cell suspension in normal saline was boiled for 2 h and

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Table 1: Oligonucletide primers used for polymerase chain reaction

Primers	Target gene	Sequence (5'-3')	Amplicon size (bp)	References
tdh-F	*tdh*	ATATCCATGTTGGCTGCATTC	531	Chao *et al.* (2009)
tdh-R		TTATTGTTGATGTTTACATTCAAAA		
trh-F	*trh*	ATGAAACTAAAACTCTACTTTGC	553	Chao *et al.* (2009)
trh-R		TTAATTTTGTGACATACATTCAT		
orf8-F	*orf8*	GTTCGCATACAGTTGAGG	700	Nasu *et al.* (2000)
orf8-R		AAGTACAGCAGGAGTGAG		
toxRS/new-F	*toxRS/new*	TAATGAGGTAGAAACA	651	Matsumoto *et al.* (2000)
toxRS/new-R		ACGTAACGGGCCTACA		

used for serotyping based on O antigen. The remaining cell suspension (not boiled) was used for serotyping based on K antigen.

PCR for *tdh*, *trh*, *orf8* and *toxRS/new* genes: Bacteria were grown overnight at 37°C on a tryptic soy agar plate. The genetic DNA was extracted from overnight cultures using TIANamp Bacteria DNA kit (Tiangen Biotech (Beijing) Co., Ltd. Beijing, China) following manufacturer's instructions.

Four sets of oligonucletide primers (tdh-F/tdh-R, trh-F/trh-R, orf8-F/ orf8-R and toxRS/new-F/ toxRS/new-R) (Table 1) were referred from document (Chao *et al*., 2009) to detect the presence of virulence genes in the *V. parahaemolyticus* isolates. PCR assays testing was performed as described earlier (Chao *et al*., 2009). Briefly, 25 ng purified total DNA, 25 pmol primer 4, 1.5U Taq polymerase, 10x PCR buffer containing 20 mM $MgCl_2$ and 0.125 mM each dNTP were used for each amplification reaction. The reactions were performed with a MyCyclerTM Thermal Cycler (BIO-RAD) as followings for tdh and trh amplification: the amplification conditions were one cycle of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 55 sec, annealing at 50°C for 50 sec and extension at 72°C for 2 min followed by a final extension at 72°C for 10 min. For amplification of orf8 and toxRS/new, the conditions were one cycle of 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR product was visualized by electrophoresis in a 1.5% agarose gel and documented using a GelDoc System (Bio-Rad Laboratories).

PFGE: Pulsed-Field Gel Electrophoresis (PFGE) of NotI-digested DNA of *V. parahaemolyticus* was performed using a standardized protocol as described earlier (Kam *et al*., 2008). XbaI-digested Salmonella Braenderup reference standard strain, H9812 was used as a molecular size marker. Following electrophoresis, gels were stained with ethidium bromide (10 mg mL^{-1}) and photographed under UV transillumination. The clonal relatedness among the strains was determined using the Dice coefficient and cluster analysis was carried out using Unweighted-Pair Grouping with Mathematical Averaging (UPGMA) and PFGE profiles of *V. parahaemolyticus* strains were drawed by using Quantity One™ Software.

RESULTS AND DISCUSSION

***V. parahaemolyticus* obtained from shellfish:** The prevalence of *V. parahaemolyticus* in shellfish was 85% (17/20) in Spring, 80% (16/20) in Summer, 75% (15/20) in Autumn and 10% (2/20) in Winter. There was no significantly difference between the Spring, Summer and Autumn. While the positive rate in Winter (10%) was significantly different from foregoing seasons (Table 2).

Servars of all 68 *V. parahaemolyticus* isolates: About 50 strains from shellfish (except 1 isolate cannot be recognized) belong to 7 serogroups. The O1, O2, O3, O4, O5, O10 and O11 accounted for 22.0% (11/50), 10.0% (5/50), 2.0% (1/50), 14.0% (7/50), 6.0% (3/50), 22.0% (11/50) and 22.0% (11/50) of all foodborn isolates, respectively. The O1, O4, O10 and O11 accounted for 80% (40/50) of food isolates. About 72.2% (13/18) of clinical isolates belong to O3 serogroup (O3K6); 22.2% (4/18) of them belong to O1 serogroup; 5.6% (1/18) belong to O4 serogroup (Table 3).

PCR for virulent genes: Only 2 strains of all 50 isolates detected from shellfish were *trh* gene positive and other 18 strains were negative of all 4 virulent genes. About 14 strains of 18 clinical isolates carry the virulent genes (tdh, toxRS/new and orf8); 1 strain of them was *trh* gene positive; 3 strains of them were negative of all 4 virulent genes (Table 4).

PFGE: The electrophoresis migration pattern of NotI-digested fragments of the chromosomal DNA of 53 strains of *V. parahaemolyticus* was obtained by PFGE. The other 15 strains were degraded including 7 strains from shellfish isolates and 8 strains from clinical isolates.

Analysis of PFGE patterns revealed that various types have a major difference, the same serotype strains could obtain different PFGE draft while different serotype strains could obtain same PFGE draft. In 80% similarity, the 53 strains could be divided into twenty nine types

(P1-P29), of which the 43 shellfish strains could be divided into twenty six types and 10 clinical strains could be divided into four types (Fig. 1, Table 5).

V. parahaemolyticus is widely distributed in marine environments and is associated with gastroenteritis cases caused by consumption of seafood. Because shellfish usually be consumed by raw and undercooked in Zhoushan island and is often associated with food poisoning caused by *V. parahaemolyticus*, researchers surveyed the contamination of *V. parahaemolyticus* in Zhoushan island. Several studies showed that the contamination level of *V. parahaemolyticus* in seafood products is associated to the water temperatures: it is more likely to isolate *V. parahaemolyticus* in seafood products in the Summer than in the Winter (Kaneko and Colwell, 1973; Depaola *et al*., 1990; Cook *et al*., 2002). In this study, results showed that the contamination of *V. parahaemolyticus* on shellfish in Zhoushan island was seriously, especially in Spring, Summer and Autumn, the positive rate was high to 80% while the positive rate in winter was 10%, significantly lower than other seasons. Therefore, the first three seasons were pandemic time of diarrhea infected by *V. parahaemolyticus*. Consequently, health supervisinon department should strengthen the supervision on seafood snacks and restaurants serving with aquatic products during the pandemic time.

Table 2: Positive rate of *V. parahaemolyticus* detected from shellfish in different seasons

Seasons (month)	Water Temp. (°C)	No. of samples	No. of strains	Positive rate (%)
Spring (Apr.)	14	20	17	85.0
Summer (Aug.)	22	20	16	80.0
Autumn (Nov.)	12	20	15	75.0
Winter (Jan.)	4	20	2	10.0
Total	88	80	50	62.5

Table 3: Serotyping for *V. parahaemolyticus* in 68 isolates

	Serotype (No. of isolates (%))							
Source of isolates	O1	O2	O3	O4	O5	O10	O11	KUT
Shellfish isolates	11 (22.0)	5 (10.0)	1 (2.0)	7 (14.0)	3 (6.0)	11 (22.0)	11 (22.0)	1 (2.0)
Clinical isolates	4 (22.2)	0 (0.0)	13 (72.2)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	15 (22.1)	5 (7.4)	14 (20.6)	8 (11.8)	3 (4.4)	11 (16.2)	11 (16.2)	1 (1.5)

Table 4: Virulent gene of *V. parahaemolyticus* in 68 isolates

		Virulent genes			
Source of isolates	No. of isolates	*tdh*	*trh*	*orf8*	*toxRS/new*
Shellfish isolates	48	-	-	-	-
	2	-	+	-	-
Clinical isolates	14	+	-	+	+
	1	-	+	-	-
	3	-	-	-	-

Table 5: Virulent, pandemic and molecular subtype characteristics of 50 shellfish isolates and 18 clinical isolates

		Virulent genes					
Serotype	No. of isolates	*tdh*	*trh*	*orf8*	*toxRS/new*	Isolates number (PFGE types)	Source of isolates (No. of isolates)
O1	11	-	-	-	-	2(P15), 7(P12), 12(P20), 13(P04), 21(P15), 29(P12), 38(P28), 43(P13), 45(P01), 58(P10), 67(P21)	Shellfish (9), Clinical (2)
	3	-	+	-	-	49(P23), 50(P23), 64(P19)	Shellfish (2), clinical (1)
	1	+	-	+	+	65(P17)	Clinical (1)
O2	5	-	-	-	-	8(*), 19(P04), 28(P08), 33(P08), 37(P27)	Shellfish (5)
O3	1	-	-	-	-	1(P14)	Shellfish (1)
	13	+	-		+	51(P17), 52(P17), 53(P17), 54(*), 56(*), 57(*), 59(P17), 60(P17), 61(*), 62(P17), 63(*), 66(*), 68(*)	Clinical (13)
O4	8	-	-	-	-	3(P18), 17(P25), 26(P14), 36(P17), 39(P05), 47(*), 48(P22), 55(*)	Shellfish (7) Clinical (1)
O5	3	-	-	-	-	27(P12), 41(P02), 44(P07)	Shellfish (3)
O10	11	-	-	-	-	4(*), 6(P04), 9(P10), 10(P06), 15(P05), 16(P18), 20(P09), 24(P26), 25(P04), 34(P24), 35(P03)	Shellfish (11)
O11	11	-	-	-	-	5(*), 11(P25), 14(P16), 18(P14), 22(*), 23(P01), 30(P12), 32(P11), 40(P29), 42(*), 46(P13)	Shellfish (11)
KUT	1	-	-	-	-	31(*)	Shellfish (1)

*Degraded strains by PFGE

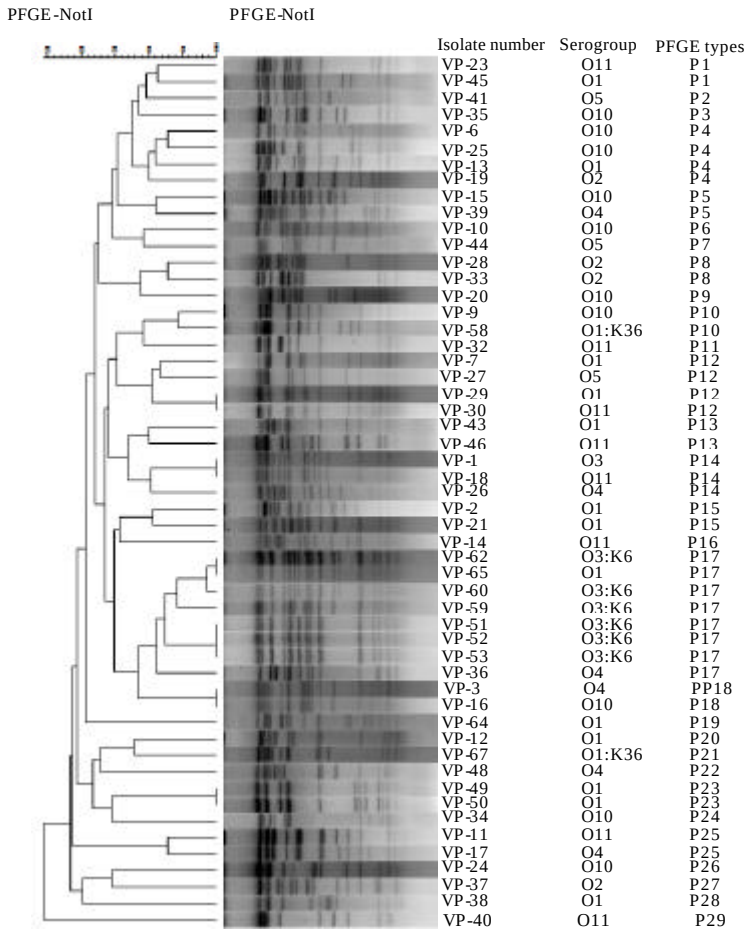

Fig. 1: Dendrogram derived from PFGE showing 53 *V. parahaemolyticus* strains of different serotypes using Salmonella Braenderup as the standard

Meanwhile, CDC Department should strengthen the education to improve public's health consciousness and warn the high-risk foods earlier. Additionally, consumers also should strengthen self-consciousness by eatting less or forbidding to eat raw or undercooked seafood.

The 50 strains from shellfish belong to 7 serogroups and O1, O4, O10 and O11 were the main serogroups, accounting for 80%. About 18 clinical isolates belonged to O3, O1 and O4 serogroups, accounting for 72.2, 22.2 and 5.6%, respectively. Results showed that *V. parahaemolyticus* isolates from shellfish present extensive diversity in Zhoushan islands while the clinical isolates present comparatively concentrative distributing.

Pathogenic *V. parahaemolyticus* has been known to produce either Thermostable Direct Hemolysin (TDH), TDH-Related Hemolysin (TRH) or both (Miyamoto *et al*., 1969; Honda and Iida, 1993). Since, 1996 with the emergence of a new pandemic O3:K6 clone, the number of cases with *V. parahaemolyticus* infection has increased considerably in many countries (Okuda *et al*., 1997; Matsumoto *et al*., 2000). Studies showed that pandemic strains contain the *orf8* gene which encodes an adherence protein that increases the ability of *V. parahaemolyticus* to adhere to host intestinal cells or the surfaces of marine plankton (Nasu *et al*., 2000). Several studies reported that the toxRS operon of the pandemic strains contains a unique (toxRS/new) encoding transmembrane proteins involved in the regulation of virulence associated genes (Chowdhury *et al*., 2000; Matsumoto *et al*., 2000). The presence of toxRS/new is necessary but not always sufficient whereas orf8 is sufficient but not always necessary for detection of pandemic strains (Chowdhury *et al*., 2000; Chao *et al*., 2009). In this study, the detection of tdh, toxRS/new and orf8 were all negative in 50 shellfish isolates and only 2 strains isolated in the winter contained the *trh* gene suggesting no pandemic

strain in shellfish isolates in this study. However, among 18 clinical strains, 14 isolates were pandemic accouting for 77.8%, of which 13 isolates belong to O3:K6, 1 isolates belong to O1:KUT suggesting that a group of pandemic O3:K6 clone with tdh, toxRS/new and orf8 positive spread in zhoushan islands. In addition, researchers identified 16.7% (3/18) non-pathogenic isolates with both tdh and trh negative in clinical isolates. As for how can these strains invade and cause human illness is still unclear. Although, pathogenic *V. parahaemolyticus* were rarely detected in environment or foodborn isolates, it doesn't mean that they are not the important epidemiological factors of food-borne diseases but just a reflection of the complexity of the *V. parahaemolyticus* in environment. Probably because the non-pathogens are more dominant than pathogens in environment, traditional method is difficult to detect the pathogens. How to improve the methods to detect the pathogens in the environment still need further research.

CONCLUSION

Pulsed-Field Gel Electrophoresis (PFGE) has been widely used as a molecular typing method to subspiecies differentiation of *V. parahaemolyticus* (Wong *et al*., 1996). In this study, results showed that much difference of the PFGE fingerprint map exist in the shellfish isolates suggesting they belong to several different clones with far genetic relationship. Most of the isolates display different map which demonstrate that the *V. parahaemolyticus* isolates from shellfish present extensive genetic diversity in Zhoushan islands. Two shellfish strains (VP-49, VP-50) isolated in Winter with trh positive present the same PFGE patterns, different from other's, possibly indicating that the two strains mutated to cold-resistant strains with the acquisition of cold-resistant gene (Boyd *et al*., 2008).

In 10 clinical strains, all pandemic strains (VP-51, VP-52, VP-53, VP-59, VP-60, VP-62 and VP-65) belong to the same clone group with 82.8% similarity of which 3 strains (VP-51, VP-52, VP-53) isolated from patients of an outbreak, 4 strains (VP-59, VP-60, VP-62 and VP-65) isolated from dissemination cases of diarrhea. In these pandemic strains, a O1:KUT strain was closely related to other O3:K6 strains suggesting that the O1:KUT strain maybe the derivative of pandemic O3:K6 clone. Three non-pandemic strains (VP-58, VP-62 and VP-67) from clinical isolates however, showed significantly different genetic map from those of the universal clone of the pandemic serotype.

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