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Identification of VPA1327 (*vopT*) as a Novel Genetic Marker for Detecting Pathogenic *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus **is a Gram-negative bacterium that is ubiquitous in marine and estuarine environments. The pathogenic strains can cause foodborne gastroenteritis especially when raw or undercooked seafood contaminated with pathogenic *V. parahaemolyticus* are consumed. This study aims to obtain a novel species-specific genetic marker using comparative genomics and to investigate their prevalence in both clinical and seafood isolates of *V. parahaemolyticus*. VPA1327 (*vopT*) was identified as a unique gene of this organism, but it was reported present in only 33.3% of *V. parahaemolyticus* (n=72 isolates). Interestingly, *vopT* was found exclusively in clinical isolates. The combination PCR analysis was used to determine the presence of three genes (*tdh*, *trh*, and *vopT*) in 36 clinical isolates. Four genotypes were classified namely, $tdh^{+}trh^{-}vopT^{+}$ (24 isolates, 66.7%), $tdh^{+}trh^{+}vopT^{-}$ (2 isolates, 5.6%), $tdh^{-}trh^{+}vopT^{-}$ (2 isolates, 5.6%) and $tdh^{-}trh^{-}vopT^{-}$ (8 isolates, 22.2%). Furthermore, all $vopT^{+}$ isolates were tdh^{+} isolates, and none of them coexist with trh^{+} isolates. This study has provided strong evidence to support *vopT* as an additional virulence marker for the detection of *V. parahaemolyticus* with the pathogenic potential to cause human disease.**

Keywords: *Vibrio parahaemolyticus*, comparative genomic, VPA1327, VopT, T3SS, TDH.

Vibrio parahaemolyticus is a Gram-negative halophilic marine and estuarine bacterium, which is an important pathogen causing seafood-borne gastroenteritis and occasional outbreaks[1]. This microorganism is generally detected in various seafood including oyster, clam, shrimp, and fish. Thus, consumption of raw or undercooked seafood contaminated with a virulent strain may put the individual at risk of getting the infection and causing disease. Detection and identification of *V. parahaemolyticus* strains in clinical and food samples are essential for diagnosing the cause of disease, as well as for surveillance and risk assessment of the contaminated food. High specificity and sensitivity detection assay are needed. For this purpose, polymerase chain reaction (PCR), a molecular DNA-based method has been developed and widely used since it is a rapid, convenient and applicable assay. The specificity of the test usually depends on the uniqueness of the target DNA (*V. parahaemolyticus)*. Till date, *toxR*[2] and *tl*[3] are widely used for specific detection of *V. parahaemolyticus*[4–7]. However, some strains revealed false positive or false negative results to these genes in PCR assay[5] due to high diversities and its close relation with *Vibrio* species. Therefore, a novel genetic marker with higher specificity is required for reliable

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diagnosis. Besides accurately identifying the organisms, their pathogenic potential also need to be evaluated. Most of *V. parahaemolyticus* strains isolated from diarrheal patients carry genes encoding thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH). TDH, but not TRH is responsible for the Kanagawa phenomenon (KP) showing beta hemolysis on a particular blood agar plate (Wagatsuma agar)[8,9]. However, it was demonstrated that pathogenicity of *V. parahaemolyticus* is not exclusively the responsibility of TDH[10] and TRH[11].

KP-positive *V. parahaemolyticus* strain, RIMD2210633, has two chromosomes, chromosome 1 (large) and chromosome 2 (small). The large chromosome contains most of the essential genes required for growth and viability, whereas, the small chromosome play a role in adaptation to environmental changes[12]. Besides being smaller in size, the chromosome 2 is more diverse in genetic organization and content. Genome sequencing data also revealed the presence of two sets of type III secretion system (T3SS) genes located in chromosome 1 (T3SS1) and 2 (T3SS2). The T3SS2 which is more divergent from the T3SS in gram-negative bacteria is present only in pathogenic KP-positive strains[12]. T3SS1 is responsible for cytotoxicity, whereas, T3SS2 is responsible for enterotoxicity and intestinal fluid accumulation[13]. T3SS2 was further characterized and two distinct lineages, T3SS2α and T3SS2β were further classified[13–15]. T3SS2α is associated with *tdh* (*tdh*$^+$ *trh*$^-$) isolates, while T3SS2β is associated with *trh* (*tdh*$^-$ *trh*$^+$) isolates.

As earlier stated, the success of genome sequencing has contributed widely to related genomic studies especially the comparative genomic studies. By using *in silico* comparison, specific targets or genetic markers for the detection and identification of pathogenic bacteria can be identified[7,16–18]. This study primarily aimed to explore a novel genetic marker for exclusively detected *V. parahaemolyticus* by comparison analysis between chromosome 2 of *V. parahaemolyticus* and *V. alginolyticus*.

MATERIALS AND METHODS

Bacterial strains

A total of 125 bacterial strains used in this study were collected and kept in our laboratory. There were 106 and 19 stains of *Vibrionaceae* and non-*Vibrionaceae,* respectively. Among the *Vibrionaceae,* 72 strains were *V. parahaemolyticus* including ATCC17802 (1), clinical (36) and seafood (35) strains and 34 strains were non-*V. parahaemolyticus*. All bacterial strains and their sources were listed in Table 1.

Comparison between chromosome II genome sequences of *V. parahaemolyticus* and *V. alginolyticus*

Closely related chromosome II DNA sequences of *V. parahaemolyticus* RIMD2210633 (Accession No. NC_004605.1) and *V. alginolyticus* ATCC 17749 (Accession no. NC_022359.1) were retrieved from the GeneBank database. Firstly, the two genome sequences were aligned using Microbial Nucleotide BLAST and BLASTn from Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/) optimized to produce highly similar sequences. The uniquely identified regions on *V. parahaemolyticus* chromosome II were then selected and analyzed for their gene identifications (ID) and corresponding coding sequences (CDSs) were obtained from the database (https://www.ncbi.nlm.nih.gov/nuccore). To confirm the absence of the archive *V. parahaemolyticus*-specific sequence, these CDSs were individually aligned against non-redundant protein sequence (nr) using BLASTx with the exclusion of *V. parahaemolyticus* sequences. The CDSs revealed no similarity/identity with other *Vibrio* spp. ORFs or low identity to the proteins of other genus was considered as unique genes of *V. parahaemolyticus*.

The primers of the selected unique CDS were designed using DNAMAN (Lynnon BioSoft, Canada). All primers used in this study were commercially synthesized (Bioneer, South Korea).

Preparation of DNA template

A colony of the bacterial strains cultured overnight was picked, suspended in sterile distilled water (DW) and the OD_{600nm} was adjusted to one. After the adjusted bacterial cell was boiled and centrifuged at 8,100 x*g* for 5 min, the supernatant was transferred to a new tube and kept at 4°C for use as a template for PCR amplification.

PCR amplification of *vpa1327*

The VPA1327 primers were shown in Table 2. The reaction mixture contained 1.0 U of Taq polymerase (Fermentas, USA), 1x of reaction

buffer containing KCl, 2.0 mM $MgCl_2$, 0.4 µM of each primer *vpa1327*F and *vpa1327*R, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 µl of bacterial DNA template and the reaction volume which was made up to 20 µl using sterile DW. The PCR cycling conditions were one cycle of 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C followed by a final extension time of 5 min at 72°C. The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and then visualized by a gel documentation system (Syngene, UK).

Multiplex PCR

The multiplex PCR was designed to determine *toxR*, *tdh* and *trh* of *V. parahaemolyticus* strains within one reaction tube only. The reaction mixture volume of 25 µl contained 1.0 µl of DNA template, 1.0 U *Taq* DNA polymerase, 1x *Taq* buffer containing KCl, 2.0 mM $MgCl_2$, 0.2 mM for each dNTPs, and 0.4, 0.8, 0.4 and 0. 2 mM of *tdh*, *trh*, *toxR* and *16SrRNA* primer pair respectively (Table 2). The thermal cycling was set similarly for *vpa1327* amplification. The PCR products were examined by agarose gel electrophoresis as described in the previous section.

RESULTS AND DISCUSSION

This study primarily aimed to identify a unique gene encoding for a protein that is unique to *V. parahaemolyticus* by genomic comparison. To identify the unique gene, the *'in silico'* genomic comparison between chromosome II DNA sequences of *V. parahaemolyticus* (1,877,212 bp) and closely related species, as well as that of *V. alginolyticus* (1,812,170 bp) was done. The reason behind the usage of chromosome II for comparison is that it is more diverse in structure and gene content than chromosome I. Generally, 56.8% unique genes of *V. parahaemolyticus* were carried from chromosome II, whereas, only 29.5% were carried from chromosome I[12]. Moreover, genes encoding type III secretion system (T3SS2) located on this chromosome was previously considered as being related to the pathogenicity of *V. parahaemolyticus* to humans[15]. Chromosomes II from both species were compared based on nucleotide sequence homology using BLASTn, and the BLASTn analysis showed 94% homology over 64% coverage of chromosome II nucleotide sequence. Approximately 200 discrete regions located on the small chromosome of *V. parahaemolyticus* were predicted as the region of differences. Among these, 106 regions were more extended than 1,000 bp.

The three large regions spanning over 20,000 bp were located, and their predicted CDSs were retrieved from *V. parahaemolyticus* genome sequence (Accession No. NC_004605.1) in the NCBI database as shown and summarized in Fig. 1. A total of 121 CDSs were further analyzed by BLASTx, and the results indicated that only two designated genes, VPA1327 and VPA1331, revealing low identity to the proteins of other genus were selected. VPA1327 encoding an exoenzyme T revealed 64% (over 233 amino acids) and 46 % (over 173 amino acids) with exoenzyme T of *Providencia alcalifaciens* and *Pseudomonas aeruginosa*, respectively. Interestingly, this orthologous exoenzyme T (VPA1327) was not found in *Vibrio* spp. other than *V. parahaemolyticus*. In contrast, VPA1331 exhibited one matched sequence with 41% identity to the putative OspC2 of *Vibrio* spp. strain FF_286. These results indicated that VPA1327 is unique to *V. parahaemolyticus* CDS.

Since VPA1327 gene encoding for an exoenzyme T, also known as *Vibrio* outer protein (Vop) T was found to be unique to *V. parahaemolyticus,* we hypothesized the use of *vopT* as a genetic marker for specific detection. Although, there are several genes used for diagnosis of *V. parahaemolyticus* such as *toxR*[2], *tl*[3] and *atpA*[19], they were not uniquely designed for *V. parahaemolyticus*. These gene sequences, 86, 97, and 85% respectively, showed a high similarity with those of *V. alginolyticus*[7]. As previously described in our study that apart from *V. parahaemolyticus, vopT* (VPA1327) was not found in vibrios, therefore, exclusive specificity should be obtained from *V. parahaemolyticus* detection based on a *vopT* gene. To detect the *vopT*, the specific primers were designed. The PCR assay was developed, and the optimal annealing temperature was determined to be 58°C. The PCR reaction yielded an amplified fragment of 687 bp. The specificity of the designed primer pair was evaluated by the optimized single PCR using various *Vibrio* species and other representative pathogenic bacteria, as listed in Table 1. The

PCR results showed that *vopT* was specific to *V. parahaemolyticus*, deprived of false positive submitting to 100% specificity as expected. Nevertheless, only 24 of 72 (33.3%) tested *V. parahaemolyticus* strains revealed positive results reflecting vastly low sensitivity to accept detection application. Interestingly, these all positive strains were from clinical samples. This observation prompted us to determine whether *vopT* could be used to distinguish between pathogenic strains from nonpathogenic ones. Therefore, all *V. parahaemolyticus* strains used in our study were

Table 1. Bacterial strains used in this study

Bacterial species	No. of strains	Source (number of isolates)
V. parahaemolyticus	72	ATCC17802 (1), clin (36), sf (35)
V. alginolyticus	10	ATCC17749 (1), clin (5), sf (4)
V. cholerae	5	Ref. (3 *i.e*., N16961, 569B, O17SR.), clin (1), sf (1)
V. fluvialis,	5	Ref.V26/36 (1),),
V. furnisii	3	clin (3) clin (3), sf (1)
V. vulnificus	2	ATCC27526 (1), sf (1)
V. mimicus	3	clin (1), sf (2)
V. harveyi	2	ATCC14126 (1), sf (1)
V. campbellii	1	ATCC25920 (1)
Grimontia hollisae	1	ATCC33564 (1)
Photobacterium damselae,	2	sf (2)
Providencia rettigeri	1	unk
Escherichia coli	1	ATCC25933
Pseudomonas aeruginosa	4	ATCC27857, Ref. (3 *i.e*., DMS00554, L165NC, P137)
Shigella flexneri	3	clin (3)
Salmonella Typhimurium	1	ATCC13311 (1)
S. Enteritidis	1	clin
S. Paratyphi A	1	clin
S. Oslo	1	unk
S. Orion	1	unk
Klebsiella pneumoniae	1	unk
Citrobacter fruendii	1	unk
Enterobacter cloacae	1	unk
Enterococcus faecalis	1	unk
Edwardsiella tarda	1	unk

ATCC, American Type Culture Collection; clin, clinical isolate; sf, seafood isolate; unk, unknown origin

Table 2. Sequences of primers used in this study

Gene	Primer sequences	Size (bp)	Reference
toxR	F: 5' -GTCTTCTGACGCAATCGTTG-3' R: 5' -ATACGAGTGGTTGCTGTCATG-3'	368	Kim *et al*.[2]
tdh	F: 5' -GTAAAGGTCTCTGACTTTTGGAC-3' R: 5' -TGGAATAGAACCTTCATCTTCACC-3'	269	Bej *et al*.[3]
trh	F: 5' -CGCTCTCATATGCTTCGAC-3' R: 5' -TTCGCGATTGATCTACCA-3'	433	this study
vpa1327	F: 5' -ACATACGGAAAATATAGGTAGTG-3' R: 5' -AAATCTAGCGCATCAAGT -3'	687	this study
16SrRNA	F: 5' -CGGTGAAATGCGTAGAGAT -3' R: 5' -TTACTAGCGATTCCGAGTTC -3'	663	Tarr *et al*.[33]

F, Forward primer; R, Reverse primer.

further used to determine the presence of *toxR*, *tdh,* and *trh* using optimized multiplex PCR.

All 106 *Vibrionaceae* were positive for *16SrRNA* of which 72 isolates were *toxR*$^+$, species-specific marker confirmed the culture-based identification of *V. parahaemolyticus* used in this study. A total of 35 seafood isolates were negative for both *tdh* and *trh* indicating nonpathogenic *V. parahaemolyticus,* of which *vopT* was also absent. In contrast, 26 (72.2%), 4 (11.1%) and 24 (66.7%) of 36 clinical isolates besides ATCC17802 were positive for *tdh*, *trh*, and *vopT*, respectively (Table 3). These results are consistent with several previous studies as summarized in Table 4. These studies showed quite high frequency of *tdh* which is more than 50% of the clinical isolates. However, contradicting results reported by Jone *et al*.[20] showed a higher prevalence of *trh* (61.7%) but a

Table 3. Distribution of *tdh*, *trh,* and *vopT* in clinical isolates of *V. parahaemolyticus* by PCR

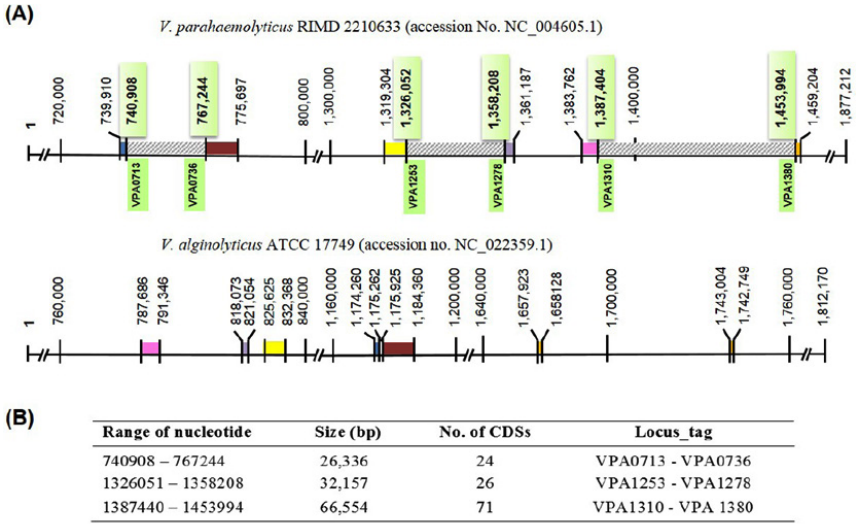
No.	*V. parahaemolyticus* isolate	PCR result				
		16SrRNA	*toxR*	*tdh*	*trh*	*vopT*
1	52605281	+	+	+	-	+
2	53607188	+	+	+	-	+
3	52605165	+	+	+	-	+
4	52605981	+	+	+	-	+
5	52604921	+	+	+	-	+
6	52605516	+	+	+	-	+
7	52604579	+	+	+	-	+
8	52604517	+	+	-	-	-
9	52605668	+	+	-	-	-
10	52605556	+	+	+	-	+
11	52605517	+	+	-	-	-
12	52605675	+	+	-	-	-
13	52605774	+	+	+	-	+
14	52605822	+	+	+	-	+
15	53-01	+	+	+	-	+
16	53-02	+	+	+	-	+
17	53-04	+	+	+	-	+
18	53-05	+	+	+	-	+
19	53-06	+	+	+	-	+
20	53-07	+	+	-	-	-
21	53603951	+	+	+	-	+
22	53603910	+	+	+	-	+
23	53604316	+	+	+	-	+
24	53604513	+	+	-	-	-
25	53604518	+	+	+	-	+
26	53605975	+	+	+	+	-
27	53606206	+	+	-	-	-
28	53606637	+	+	+	-	+
29	53607058	+	+	+	-	+
30	53608005	+	+	-	+	-
31	53609903	+	+	+	-	+
32	53610963	+	+	+	-	+
33	53611025	+	+	-	+	-
34	53611080	+	+	-	-	-
35	53611224	+	+	+	-	+
36	53607926	+	+	+	+	-
37	ATCC17802	+	+	-	+	-

quite low proportion of *vopT* (11.7%) in clinical isolates. The difference may be due to differences in geographic regions where the bacterium was isolated. The Jone's study was carried out in USA[20], while others were carried out in Asian Countries such as China[21–23] and Thailand[24], including this study. Additionally, the PCR results of the three genes of clinical isolates in this study could be classified into four groups (Table 4) *i.e.*, *tdh*$^+$*trh*$^-$*vopT*$^+$ (24 isolates), *tdh*$^+$*trh*$^+$*vopT*$^-$ (2 isolates), *tdh*$^-$*trh*$^+$*vopT*$^-$ (2 isolates) and *tdh*$^-$*trh*$^-$*vopT*$^-$ (8 isolates). These results showed that all *vopT*$^+$ isolates were *tdh*$^+$, accounted for 92.3% (24/26) and none of them coexist with *trh*. The *vopT* express exclusively with

Table 4. The occurrence of *tdh, trh,* and *vopT* in clinical *V. parahaemolyticus* isolates compared to data retrieved from several studies

Gene	This study % (no.)	Jones *et al.* [20] % (no.)	Tsai *et al.* [22] % (no.)	Mala *et al.* [24] % (no.)	Chen *et al.* [23] % (no.)	Li *et al.* [21] % (no.)
no. isolates	36	77	49	74	501	42
tdh	72.2% (26)	57.1% (44)	85.7% (42)	93.2% (69)	93.0% (466)	88.1% (37)
trh	11.1% (4)	61.0% (47)	2.0% (1)	10.8% (8)	1% (5)	16.7% (7)
vopT	66.7% (24)	11.7% (9)	85.7% (42)	98.6% (73)	93.0% (466)	90.5% (38)
vopB2	nd	11.7% (9)	85.7% (42)	89.2% (66)	93.0% (466)	95.2% (40)
Gene combination						
tdh$^+$*trh*$^-$*vopT*$^+$	66.7% (24)	11.7% (9)	na	70.8% (17/24*)	92.4% (463)	80.9% (34)
tdh$^+$*trh*$^+$*vopT*$^+$	0% (0)	0% (0)	na	20.8% (5/24*)	0.6% (3)	2.4% (1)
tdh$^-$*trh*$^+$*vopT*$^+$	0% (0)	0% (0)	na	4.2% (1/24*)	0% (0)	4.8% (2)
tdh$^-$*trh*$^-$*vopT*$^+$	0% (0)	0% (0)	na	4.2% (1/24*)	0% (0)	2.4% (1)
tdh$^-$*trh*$^-$*vopT*$^-$	22.2% (8)	27.3% (21)	na	0% (0/24*)	6.6% (33)	0% (0)

nd, not determined; na, not available; *, data provided for only 24 samples



Range of nucleotide	Size (bp)	No. of CDSs	Locus_tag
740908 – 767244	26,336	24	VPA0713 - VPA0736
1326051 – 1358208	32,157	26	VPA1253 - VPA1278
1387440 – 1453994	66,554	71	VPA1310 - VPA 1380

Fig. 1. Comparative chromosomal 2 genome *V. parahaemolyticus* RIMD 2210633 (accession No. NC_004605.1) and *V. alginolyticus* ATCC 17749 (accession no. NC_022359.1). (A), Diagram of *V. parahaemolyticus* chromosome 2 (upper panel) showed three selected regions of differences (RD, striped boxes) which were not found on the chromosome 2 of *V. alginolyticus* (lower panel). The flanking regions of each RD are homologous regions shown as identically colored blocks in each genome. (B), List of three selected RD and their CDSs carried on *V. parahaemolyticus* chromosome 2

tdh, as demonstrated in several studies[13,14,20,22,25] including this study. However, few isolates lack *tdh*, with or without *trh*, but express *vopT* could be found as reported recently[21,24].

TDH and TRH encoded by *tdh* and *trh* genes, respectively, are considered as major virulence factors in *V. parahaemolyticus*[26,27]. It was found that the majority of clinical cases were associated with *V. parahaemolyticus* strains carrying either *tdh* or *trh*[8,20], and they are therefore used as indicators for the pathogenic strains. Our study also revealed that most of the clinical isolates of *V. parahaemolyticus* (28/36, 77.8%) carried either *tdh* or *trh*. Among 28 isolates, almost all isolates (26, 72.2%) were tdh^+, but only 4 (11.1%) trh^+ were in good agreement with several previous studies reported. For example, the distribution of *tdh* and *trh* in clinical isolates were respectively 63.6% *vs*. 35.5%[8] and 84% *vs*. 12%[25]. These finding indicated that *trh* gene is less pronounced for pathogenic *V. parahaemolyticus* isolated from clinical samples. In contrast, *trh* positive strains were more frequently isolated from the environment or seafood than *tdh* positive ones, *e.g*., only 7% (5/71) of environmental strains carry *trh* gene, while *tdh* gene could not be detected[8]. Vongxay *et al*.[25] also reported that 1.6% and 3.7% of 191 seafood samples carried *tdh* and *trh*, respectively. Within the human host, the hostile environment in the intestine, *i.e*., high temperature as well as the presence of bile is a potent stimulator to upregulate the expression of genes encoding TDH and T3SS2 including *vopT*[28]. Therefore, *tdh* gene was detected more frequently in clinical than in seafood or environmental strains. In our study, none of *tdh* or *trh* was found in all 35 *V. parahaemolyticus* strains from seafood samples. Since the prevalence of pathogenic strains in the environment or seafood are very low, there is minimal opportunity for *tdh*/*trh* genes to recover unless the higher number of isolates are examined. Although, either *tdh* or *trh* is indicated as genetic markers for the pathogenic strains, 22.2% of clinical strains lacking both *tdh* and *trh* (tdh^-trh^-) were obtained in this study similar to those obtained in several previous studies[8,20]. Additionally, it was found that deletion of *tdh* from *trh*-negative strain merely partially inhibited cytotoxicity[10]. This evidence indicated that the two virulence factors, TDH and TRH, do not adequately account for *V. parahaemolyticus* pathogenicity and suggested the existence of other virulence factors besides TDH and TRH[20,29]. Owing to recent report that *vopT* could be detected in clinical isolates lacking *tdh*, *trh* or *both* genes[21,24], VopT may result in the pathogenicity of these strains.

Kodama and colleagues[30] had identified and characterized VopT and reported that it is one of the T3SS2 effector proteins which play a role in enterotoxicity. The amino acid sequence of VopT revealed 45% and 44% identity with the C-terminal ADP-ribosyltransferase (ADPRT) domain of two effector proteins secreted by T3SS of *P. aeruginosa*, exoenzyme T, and exoenzyme S, respectively. The ADPRT domain of exoenzyme T was shown to involve in the pathogenesis of *P. aeruginosa* infection[31]. Also, VopT was previously[30] shown to be secreted and injected through T3SS2 into the cytoplasm of the host cells in which Ras protein was ADP-ribosylation. Like exoenzyme T of *P. aeruginosa*, the ADPRT domain of VopT is required for cytotoxicity. The cytotoxic activity decreased when *vopT* was deleted. Hence, it was implied that VopT is one of the virulence factors produced by the organism and it plays an important role in inducing cytotoxicity. Moreover, our results showed that *vopT* was detected mainly from the majority of clinical isolates (66.7%) and coexisted consistently with *tdh* (tdh^+vopT^+, 92.3%). Therefore, the expression of *vopT* is *tdh* or *tdh*-dependent. However, it may function independently for the pathogenicity of a few isolates harboring *vopT* without either *tdh* or *trh*. From the study of *V. parahaemolyticus* pathogenicity, it was observed that the enteropathogenicity of *trh*-positive isolates seemed to fall between *tdh*-positive isolates and those without *tdh* and *trh*[25]. Likewise, the presence or absence of *vopT* may reflect the degree of virulence or pathogenicity of *V. parahaemolyticus*. VopT may function parallel to or in agreement with TDH which need further investigation.

The distribution of *vopT* among the clinical isolates revealed in this study was similar to that of *vopB2*, another effector gene of T3SS2α, reported by Noriea *et al*.[14]. Since *vopB2* was found only in the clinical isolates and co-existed with *tdh* gene but was not found in the environmental strains, Noriea *et al*. suggested *vopB2* to be a representative gene for identifying increased virulence among strains. However, the recent studies revealed that the three genes, *i.e*., *tdh*, *vopT* and *vopB2* were not

coexisting stringently and few *vopT/vopB2* gene could be found together with *trh*-positive or *tdh*-negative isolates[21,23,24]. By comparing the detection rate of *vopT* and *vopB2* genes among several studies, the positive rate of *vopT* is usually equal to *vopB2* since they coexist, but one study found the percentage of *vopT* over *vopB2* and *vice versa* in another study (Table 4). Moreover, VopT was found unique to *V. parahaemolyticus* in opposition to TDH which is a major virulence factor and is not restricted to only *V. parahaemolyticus,* but it has also been documented in other *Vibrionaceae* such as *G. hollisae*, *V. mimicus*, and *V. cholerae*[32]. Taken together, we considered that *vopT*, likewise *tdh*, is also a major virulence factor and the use of *vopT* was suggested, in addition to *tdh* and *vopB2*, as a genetic marker for more reliable identification of pathogenic *V. parahaemolyticus*.

CONCLUSION

The objective of the present study was primarily to search for a new genetic marker for the identification of *V. parahaemolyticus* by genomic analysis approach. Finally, our study has identified *vopT* (VPA1327), which is one of the T3SS2 effector genes specific to *V. parahaemolyticus*. The distribution of *vopT* is the same as *tdh*, and being mainly obtained from clinical isolates indicated that they were associated with pathogenicity. Moreover, *vopT* always coexist with *tdh,* but it is not dependent on *tdh*. Therefore, in this study, we proposed the screening of *vopT* parallel to *tdh* for more reliable identification of pathogenic *V. parahaemolyticus* strains.

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REFERENCES

1. Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. *Clin Microbiol Rev*. 2007; **20**(1):39-48. doi:10.1128/CMR.00025-06.
2. Kim YB, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. *J Clin Microbiol*. 1999; **37**(4):1173-1177.
3. Bej AK, Patterson DP, Brasher CW, Vickery MCL, Jones DD, Kaysner CA. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *J Microbiol Methods*. 1999; **36**(3):215-225. doi:10.1016/S0167-7012(99)00037-8.
4. Caburlotto G, Gennari M, Ghidini V, Tafi M, Lleo MM. Presence of T3SS2 and other virulence-related genes in *tdh*-negative *Vibrio parahaemolyticus* environmental strains isolated from marine samples in the area of the Venetian Lagoon, Italy. *FEMS Microbiol Ecol*. 2009; **70**(3):506-514. doi:10.1111/j.1574-6941.2009.00764.x.
5. Croci L, Suffredini E, Cozzi L, et al. Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *J Appl Microbiol*. 2007; **102**(1):229-237. doi:10.1111/j.1365-2672.2006.03046.x.
6. Jiang Y, He L, Wu P, et al. Simultaneous identification of ten bacterial pathogens using the multiplex ligation reaction based on the probe melting curve analysis. *Sci Rep*. 2017; **7**(1):1-9. doi:10.1038/s41598-017-06348-z.
7. Li R, Chiou J, Chan EWC, Chen S. A novel PCR-based approach for accurate identification of *Vibrio parahaemolyticus*. *Front Microbiol*. 2016; **7**(JAN):1-5. doi:10.3389/fmicb.2016.00044.
8. Shirai H, Ito H, Hirayama T, et al. Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. *Infect Immun*. 1990; **58**(11):3568-3573.
9. Honda T, Iida T. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. *Rev Med Microbiol*. 1993; **4**(2):106-113. doi:10.1097/00013542-199304000-00006.
10. Park KS, Ono T, Rokuda M, Jang MH, Iida T, Honda T. Cytotoxicity and enterotoxicity of the thermostable direct hemolysin-deletion mutants of *Vibrio parahaemolyticus*. *Microbiol Immunol*. 2004; **48**(4):313-318. doi:10.1111/j.1348-0421.2004.tb03512.x.
11. Ming X, Yamamoto K, Honda T. Construction and characterization of an isogenic mutant of *Vibrio parahaemolyticus* having a deletion in the thermostable direct hemolysin- related hemolysin gene (*trh*). *J Bacteriol*. 1994; **176**(15):4757-4760.
12. Makino K, Oshima K, Kurokawa K, et al.

Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet*. 2003; **361**(9359):743-749. doi:10.1016/S0140-6736(03)12659-1.

13. Park K-S, Ono T, Rokuda M, et al. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infect Immun*. 2004; **72**(11):6659-6665. doi:10.1128/IAI.72.11.6659-6665.2004.
14. Noriea NF, Johnson CN, Griffitt KJ, Grimes DJ. Distribution of type III secretion systems in *Vibrio parahaemolyticus* from the northern Gulf of Mexico. *J Appl Microbiol*. 2010; **109**(3):953-962. doi:10.1111/j.1365-2672.2010.04722.x.
15. Okada N, Iida T, Park KS, et al. Identification and characterization of a novel type III secretion system in trh-positive *Vibrio parahaemolyticus* strain TH3996 reveal genetic lineage and diversity of pathogenic machinery beyond the species level. *Infect Immun*. 2009; **77**(2):904-913. doi:10.1128/IAI.01184-08.
16. Amaral GRS, Dias GM, Wellington-Oguri M, et al. Genotype to phenotype: Identification of diagnostic *Vibrio* phenotypes using whole genome sequences. *Int J Syst Evol Microbiol*. 2014; **64**(PART 2):357-365. doi:10.1099/ijs.0.057927-0.
17. Kim H-J, Ryu J-O, Lee S-Y, Kim E-S, Kim H-Y. Multiplex PCR for detection of the *Vibrio* genus and five pathogenic *Vibrio* species with primer sets designed using comparative genomics. *BMC Microbiol*. 2015; **15**(1):239. doi:10.1186/s12866-015-0577-3.
18. Yu S, Chen W, Wang D, He X, Zhu X, Shi X. Species-specific PCR detection of the food-borne pathogen *Vibrio parahaemolyticus* using the *irgB* gene identified by comparative genomic analysis. *FEMS Microbiol Lett*. 2010; **307**(1):65-71. doi:10.1111/j.1574-6968.2010.01952.x.
19. Izumiya H, Matsumoto K, Yahiro S, et al. Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. *Mol Cell Probes*. 2011; **25**(4):174-176. doi:10.1016/j.mcp.2011.04.004.
20. Jones JL, Lüdeke CHM, Bowers JC, et al. Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. *J Clin Microbiol*. 2012; **50**(7):2343-2352. doi:10.1128/JCM.00196-12.
21. Li H, Tang R, Lou Y, Cui Z, Chen W, Hong Q. A Comprehensive epidemiological research for clinical *Vibrio parahaemolyticus* in Shanghai. 2017; **8**(June):1-10. doi:10.3389/fmicb.2017.01043.
22. Tsai S, Jong K-J, Tey YH, et al. Molecular characterization of clinical and environmental *Vibrio parahaemolyticus* isolates in Taiwan. *Int J Food Microbiol*. 2013; **165**(1):18-26. doi:10.1016/j.ijfoodmicro.2013.04.017.
23. Chen Y, Chen X, Yu F, et al. Serology, virulence, antimicrobial susceptibility and molecular characteristics of clinical *Vibrio parahaemolyticus* strains circulating in southeastern China from 2009 to 2013. *Clin Microbiol Infect*. 2016; **22**(3):258.e9-258.e16. doi:10.1016/j.cmi.2015.11.003.
24. Mala W, Alam M, Angkititrakul S, et al. Sero group, virulence , and molecular traits of *Vibrio parahaemolyticus* isolated from clinical and cockle sources in northeastern Thailand. *Infect Genet Evol*. 2016; **39**:212-218. doi:10.1016/j.meegid.2016.01.006.
25. Vongxay K, Wang S, Zhang X, et al. Pathogenetic characterization of *Vibrio parahaemolyticus* isolates from clinical and seafood sources. *Int J Food Microbiol*. 2008; **126**(1-2):71-75. doi:10.1016/j.ijfoodmicro.2008.04.032.
26. Honda T, Ni Y, Miwatani T. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infect Immun*. 1988; **56**(4):961-965.
27. Nishibuchi M, Fasano A, Russell RG, Kaper JB. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. *Infect Immun*. 1992; **60**(9):3539-3545.
28. Gotoh K, Kodama T, Hiyoshi H, et al. Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. *PLoS One*. 2010; **5**(10). doi:10.1371/journal.pone.0013365.
29. Lynch T, Livingstone S, Buenaventura E, et al. *Vibrio parahaemolyticus* disruption of epithelial cell tight junctions occurs independently of toxin production. 2005; **73**(3):1275-1283. doi:10.1128/IAI.73.3.1275.
30. Kodama T, Rokuda M, Park KS, et al. Identification and characterization of VopT, a novel ADP-ribosyltransferase effector protein secreted *via* the *Vibrio parahaemolyticus* type III secretion system 2. *Cell Microbiol*. 2007; **9**(11):2598-2609. doi:10.1111/j.1462-5822.2007.00980.x.
31. Garrity-Ryan L, Shafikhani S, Balachandran P, et al. The ADP Ribosyltransferase domain of *Pseudomonas aeruginosa* exoT cContributes to its biological activities. *Infect Immun*. 2004; **72**(1):546-558. doi:10.1128/IAI.72.1.546-

558.2004.

32. Bhowmik SK, Pazhani GP, Ramamurthy T. Phylogenetic and *in silico* functional analyses of thermostable-direct hemolysin and *tdh*-related encoding genes in *Vibrio parahaemolyticus* and other gram-negative bacteria. *Biomed Res Int*. 2014;2014. doi:10.1155/2014/576528.

33. Tarr CL, Patel JS, Puhr ND, Sowers EG, Bopp CA, Strockbine NA. Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *J Clin Microbiol*. 2007; **45**(1):134-140. doi:10.1128/JCM.01544-06.