Research Article

Phylogenetic and In Silico Functional Analyses of Thermostable-Direct Hemolysin and tdh-Related Encoding Genes in Vibrio parahaemolyticus and Other Gram-Negative Bacteria

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Emergence and spread of pandemic strains of Vibrio parahaemolyticus have drawn attention to make detailed study on their genomes. The pathogenicity of V. parahaemolyticus has been associated with thermostable-direct hemolysin (TDH) and/or TDH-related hemolysin (TRH). The present study evaluated characteristics of tdh and trh genes, considering the phylogenetic and in silico functional features of V. parahaemolyticus and other bacteria. Fifty-two tdh and trh genes submitted to the GenBank were analyzed for sequence similarity. The promoter sequences of these genes were also analyzed from transcription start point to −35 regions and correlated with amino acid substitution within the coding regions. The phylogenetic analysis revealed that tdh and trh are highly distinct and also differ within the V. parahaemolyticus strains that were isolated from different geographical regions. Promoter sequence analysis revealed nucleotide substitutions and deletions at −18 and −19 positions among the pandemic, prepandemic, and nonpandemic tdh sequences. Many amino acid substitutions were also found within the signal peptide and also in the matured protein region of several TDH proteins as compared to TDH-S protein of pandemic V. parahaemolyticus. Experimental evidences are needed to recognize the importance of substitutions and deletions in the tdh and trh genes.

1. Introduction

Vibrio parahaemolyticus is a Gram-negative bacterium, which is a part of the normal flora of marine and estuarine waters. Despite its halophilic nature, this pathogen has also been isolated from fresh water and freshwater fishes. Genetically and by serology, V. parahaemolyticus strains are very diverse. During February 1995, an unusual incidence of V. parahaemolyticus belonging to serovar O3:K6 was recorded among acute diarrheal cases in the Infectious Diseases Hospital, Kolkata [1]. Since 1996, this O3:K6 serovar has been associated with several outbreaks in different countries and hence designated as the pandemic strain [1]. The O3:K6 and its genetically related serovars of V. parahaemolyticus are now documented as a pandemic clonal complex and have been related to its global spread [1].

The pandemic serovars of V. parahaemolyticus are now considered as an emerging pathogen in Asia and coastal regions of the United States [2] due to several episodes of large seafood-associated infections. This pathogen has been frequently detected in shellfish than in sediment or water samples [3]. Apart from gastroenteritis, wound infections and septicemia are the other major clinical manifestations caused by pathogenic strains of V. parahaemolyticus. This Vibrio causes infections in human due to consumption of raw or undercooked seafood or the wounds exposed to warm seawater. Patients with chronic liver diseases and leukemia are predisposed to septicemia caused by V. parahaemolyticus, which is sometimes fatal [4]. Gastroenteritis is caused by diverse serovars of V. parahaemolyticus; however, strains of O3:K6 with unique toxRS gene sequence are distributed throughout the world as a pandemic serovar. The O3:K6 serovars that lacked the toxRS sequence isolated before 1996 are known as prepandemic strains of V. parahaemolyticus. Serovar O3:K6 continued to exist in the environment, con-
fronting several ecological and immunological changes in the population resulting in progression of several other new pandemic serovars.

Enterotoxicity of this pathogen is attributed to the extracellular production of a putative virulence factor, the thermostable-direct hemolysin (TDH). TDH has been phenotypically shown as the β-type hemolysin on Wagatsuma agar, which is also known as the Kanagawa phenomenon (KP). Apart from the KP-test, the purified TDH has been tested in myocardial cells [5], rabbit ileal loops [6], and enzyme-linked immunosorbent assay. The purified TDH caused a dose-dependent increase in intracellular free calcium in both Caco-2 and IEC-6 as detected by microspectrofluorimetry [7]. Significant lethal activity of TDH was also demonstrated in the murine infection model [8]. Sometimes, the KP-negative strains of V. parahaemolyticus produce a TDH-related hemolysin (TRH). The TRH has similar physicochemical properties like TDH, but it is liable at temperature 60°C [9]. The pathogenic strains of V. parahaemolyticus that harbor only the tdh and express KP were found to be associated with acute diarrheal infection and epidemics [10]. The environmental strains that cause extraintestinal infections may differ in this virulence profile [11]. Generally, the detection rate of trh in clinical strains is very less but comparatively more in environmental strains. However, high frequencies of tdh and trh genes positive strains have been detected recently in a pristine estuary of US [12]. Considering their importance, detection of these virulence marker genes is important to differentiate pathogenic strains from nonpathogenic V. parahaemolyticus.

TDH is associated with type-three secretion systems (T3SSs) [13, 14]. V. parahaemolyticus has two sets of T3SS genes on chromosomes 1 and 2 (T3SS1 and T3SS2, resp.). The T3SS1 can induce cytotoxicity [14], whereas the T3SS2 can induce cytotoxicity in Caco-2 cells and also plays an important role in fluid secretion in the ileal loops [15]. Comparative genomic analysis confirmed that the T3SS2-containing PAI was conserved in KP-positive strains [16].

V. parahaemolyticus that lacks typical tdh and trh may phenotypically express hemolytic activity due to the presence of its variant forms. These variants have considerable homology with established prototypes of tdh/trh. In this study, we assessed molecular diversity of tdh and trh gene sequences in order to understand the phylogenetic relationship and in silico functionality among V. parahaemolyticus and other Gram-negative strains reported from different geographical areas. In V. parahaemolyticus, five tdh alleles have been identified, namely, tdh1 to tdh5, with similar biological activities [17]. These alleles have >96.7% sequence identity. However, expression of these alleles varied due to the defect in their promoter activities [18].

3. Results and Discussion

Hemolysin is a potential virulence factor in many bacterial pathogens. It is well known that the TDH has a combination of biological actions including hemolysin, cardiotoxicity, and enterotoxicity. The severity of diarrheal illness caused by this bacterium is closely related to the presence of two types of tdh and tdh-related genes [20]. Depending on the environmental conditions, these virulence genes also play an important role in the stress tolerance in V. parahaemolyticus [21]. The results of phylogenetic analysis of tdh and trh genes are shown in Figure 1. In the phylogenetic tree, three distinct clades (A to C) were identified. In clade A, tdh gene from diverse serogroup of Vibrio spp. had 85 to 100% sequence similarity within the coding region. Clade A contained more of V. parahaemolyticus nonpandemic strains (91%) than pandemic strains (8%). Clade B had the trh sequences of V. parahaemolyticus and L. anguillarum. Clade C contained mostly the nonpandemic strains of V. parahaemolyticus.

So far, five tdh genes have been identified in plasmids and chromosomes of Vibrio spp. [22] and their sequence displayed >96.7% identity with similar biological activity [18]. These tdh genes not only are restricted to V. parahaemolyticus alone but also have been documented in other Vibrio species such as V. cholerae, V. mimicus, and V. parahaemolyticus [22]. Typical hemolysin-producing V. parahaemolyticus strains carry two copies of tdh genes (tdh1 and tdh2) in their chromosomes [22]. Strains that harbor any one of these genes have been associated with weak or negative hemolytic activity. The gene tdh2 holds 97.2% homology with tdh1 and was found primarily responsible for the phenotypic expression of hemolytic activity [22]. These two genes are designated as tdhA and tdhS [23] and detected in a gene cluster known as tdh pathogenicity island (tdh-PAIs) of pandemic serovars [24]. These tdh-PAIs are very similar in many epidemic strains of V. parahaemolyticus but are absent in a prepandemic strain AQ4037 [24]. Although this PAI has been detected in another prepandemic strain of AQ3810, the tdhS gene orientation was reversed [24]. The difference in the presence of tdh-PAIs in the pandemic strains and positioning of tdh genes among prepandemic strains indicated that these genes have been acquired by lateral gene transfer in V. parahaemolyticus. This hypothesis was supported by differences in the G+C content of the tdh-PAI and the rest of the genome [25].

2. Materials and Methods

A total of 5 diverse bacteria with fully sequenced hemolysin genes (tdh, trh, and other hemolysin genes of V. parahaemolyticus) were selected and aligned for phylogenetic analyses (maximum parsimony and neighbor-joining methods) using MEGA software version 5.2 [19]. Nucleotide sequence length of 570 bp and alignment score of 13 were considered for the phylogenetic analysis of tdh and trh. The resulting phylogenetic tree (Figure 1) shows three distinct clades (A to C) identified, with similar biological activity [18]. These tdh genes not only are restricted to V. parahaemolyticus alone but also have been documented in other Vibrio species such as V. cholerae, V. mimicus, and V. parahaemolyticus [22]. Typical hemolysin-producing V. parahaemolyticus strains carry two copies of tdh genes (tdh1 and tdh2) in their chromosomes [22]. Strains that harbor any one of these genes have been associated with weak or negative hemolytic activity. The gene tdh2 holds 97.2% homology with tdh1 and was found primarily responsible for the phenotypic expression of hemolytic activity [22]. These two genes are designated as tdhA and tdhS [23] and detected in a gene cluster known as tdh pathogenicity island (tdh-PAIs) of pandemic serovars [24]. These tdh-PAIs are very similar in many epidemic strains of V. parahaemolyticus but are absent in a prepandemic strain AQ4037 [24]. Although this PAI has been detected in another prepandemic strain of AQ3810, the tdhS gene orientation was reversed [24]. The difference in the presence of tdh-PAIs in the pandemic strains and positioning of tdh genes among prepandemic strains indicated that these genes have been acquired by lateral gene transfer in V. parahaemolyticus. This hypothesis was supported by differences in the G+C content of the tdh-PAI and the rest of the genome [25].
Figure 1: Neighbor-joining phylogenetic tree obtained by the analysis of tdh and trh genes. Bootstrap values are presented next to the tree nodes. The branch of the tree is not proportional to evolutionary distance. The bar represents 0.02 nucleotide substitution per site.
In the phylogenetic analysis, the pandemic and pre-pandemic strains were placed in A and C clades. The size of the typical tdh coding sequence was 570 bp. However, in this analysis, we have included only the published full length sequences. The trh gene from Aeromonas veronii biovar Veronii sequences has also been analyzed for this study. Since all the three trh sequences are identical, we have considered one to examine its relation to the trh of V. parahaemolyticus. The trh sequences of Aeromonas spp. are highly diverse and their bootstrap values remained less than 50%. Clades A and C are the two clusters in which diverse hemolysin encoding genes have been grouped. Clade A contains tdh of pandemic and nonpandemic strains. The tdh sequence of pandemic serovars exhibited 86–99% bootstrap homology with nonpandemic serovars and trh gene of the V. parahaemolyticus ATCC strain 17802 (serovar O1:K1) [26]. In addition, the tdh also showed 86% homology with trh of Listonella anguillarum, which is a member in the family Vibrionaceae. In clade A, tdhA of RIMD2210633 had 86% sequence homology with a Peruvian pandemic strain Peru-466 and the tdhS had 64% homology with tdh of Indian pandemic strain K5030 [24]. This genetic comparison demonstrates that pandemic strains isolated from several geographical areas displayed sequence dissimilarity within the tdh coding region. However, clade A contained tdh genes of V. parahaemolyticus from US, Bangladesh, and Russia. The pandemic serovars from US and Bangladesh had 93% sequence homology [27] but the information on the types of Russian serovars is not available.

In this study, tdh of V. cholerae non-O1 and non-O139, V. mimicus, and V. hollisae showed sequence homology with tdh of V. parahaemolyticus. However, the bootstrap similarities are distinct (Figure 1). Although these organisms had some sequence similarities within the coding regions of hemolysin encoding genes, a comparative analysis showed that they had different flanking regions as compared to V. parahaemolyticus [22]. Honda et al. [28] reported the presence of plasmid-encoded TDH in some of the environmental strains of V. cholerae non-O1 and non-O139 (also called nonagglutinable (NAG) vibrio) strains. Type-III secretion system (T3SS) located in ∼49.7 kb genomic island has been identified in NAG strains, which has a strong homology with T3SS2 of V. parahaemolyticus. The TDH and TRH encoding genes in NAG strains have been identified either within [29] or outside [30] the T3SS genomic island. Although V. hollisae strains had T3SS island, TDH/TRH was not reported as a part of this island [31].

It has already been established that the expression of tdh and trh genesis different due to defect in the promoter regions [18, 27]. In V. parahaemolyticus, changes in the promoter sequences of different tdh genes have shown considerable variation in the expression of KP [18]. It was shown that the nucleotide sequence positions from −35 to −10 of tdh gene promoter act as a hotspot and nucleotide substitution at −34 from A to G affects the expression of hemolytic activity [18]. This −34 position corresponds to −35 in our realigned sequence comparison (Figure 2). In a recent finding, it was revealed that, in the absence of any substitution or an additional mutation at position −3 (substitution of G to A) relative to −10, sequence of promoter region could change the expression of hemolysin [32]. This information facilitated analyzing the nucleotide sequences of promoter regions from transcription start point to −35 position of tdhS of RIMD2210633 with other available 23 promoter sequences of tdh from the GenBank (except tdhS, which was not available in the database). The gene tdhS is highly transcribable under the influence of the promoter region, which was associated with stronger KP [18]. However, in the comparative analysis,
instead of substitution at −3 position, we have detected nucleotide changes at −2 (C for T), −4 (T for C), −5 (A for G), −6 (C for A), −8 (A for G), −15 (T for C/A/G), and −17 (A for G) in *tdh*S of RIMD2210633, which is a pandemic serovar O3:K6. Site-directed mutagenesis experiments are required to address the importance of these substitutions. In addition to nucleotides, positions −18 and −19 relative to the −10 were found to be altered among nine *tdh* genes, which are intact, mostly in pandemic serovars such as O3:K6, O4:K68, and O1:KUT (K antigen untypable) (Figure 2). However, these changes were absent in four *tdh* genes sequenced from strains of ATCC 17803 (gi|30171234), T4750 (gi|217196), and Bangladesh-1980 (serovar O3:K6) (gi|21326607) and in sequence gi|2326595 (from serovar O4:K13) (Figure 2). Among the 10 *tdh* genes, a nucleotide deletion at position −18 was found among pandemic, prepandemic, and one of each of *V. cholerae* non-O1 and non-O139 and *V. mimicus* strains. We also analyzed protein sequences in the promoter region of all the strains. The TDH consists of 189 amino acids, of which first 24 amino acid residues belonged to signal peptide. A site-directed mutagenesis study on the remaining 165 amino acids residues has shown that Trp<sup>65</sup> and Leu<sup>66</sup> are very important in the hemolytic activity of TDH and any change in these residues could reduce its activity [33]. In addition to these residues, Arg<sup>68</sup>, Gly<sup>62</sup>, Thr<sup>67</sup>, Gly<sup>86</sup>, Glu<sup>116</sup>, and Glu<sup>138</sup> were also shown to be vital for the hemolysis [33, 34]. TDH has one intramolecular disulphide bond between Cys<sup>151</sup> in β10 and Cys<sup>161</sup> in the 310 helix [35]. This contiguous positioning of Cys<sup>151</sup> and Cys<sup>161</sup> suggests the formation of side channels and influences the hemolytic activity of TDH. These two Cys residues were also found to be highly conserved in all the TDH. However, mutations in other positions were detected when comparing TDH sequences of RIMD2210633 with others (Table 1). TDH-A of RIMD2210633, TDH3, TDH4, *V. mimicus* TDH, *V. cholerae* non-O1 and non-O139-TDH, and other prepandemic strains of *V. parahaemolyticus* had amino acid substitutions within the signal peptide at positions 3 (tyrosine for histidine), 4 (glutamine for arginine), and 23 (phenylalanine for serine) as compared to RIMD2210633. Interestingly, these groups of TDH amino acids do not have histidine in the signal peptide, which is essential in the protein active or binding sites. In a *V. mimicus* (VmTDH),

### Table 1: Comparison of the deduced amino acid sequences of the products of the *tdh* genes taken from GenBank.

| Particulars of *tdh* genes | Positions of signal peptide | Positions of mature protein sequence |
|-----------------------------|----------------------------|-------------------------------------|
| **Pandemic strains**        |                            |                                     |
| gi|21326604|1998 (O3:K6)       | H Q S T Q K N E S G N N E G N I K H Q |
| gi|21326606|1998 (O3:K6)       | H Q S T Q K N E S G N N E G N I K H Q |
| gi|21326609|2002 (O4:K68)      | H Q S T Q K N E S G N N E G N I K H Q |
| gi|21326602|2002 (O1:KUT)      | H Q S T Q K N E S G N N E G N I K H Q |
| gi|38044806|2011              | H Q S T Q K N E S G N N E G N I K H Q |
| gi|28901233|RIMD, TDH S, 1996 (O3:K6) | Y R F T N E N K H D D N E D N I K H Q |
| gi|28901169|RIMD, TDH A, 1996 (O3:K6) | Y R F T N E N K H D D N E D N I K H Q |
| **Nonpandemic strains**     |                            |                                     |
| gi|48478|1992              | H Q S T Q K N E S G N N E G N I K H Q |
| gi|15229|1993              | H Q S T Q K N E S G N N E G N I K H Q |
| gi|21326608|1980 (O3:K6)       | H Q S T Q K N E S D N N E G N I K H Q |
| gi|217197|1985              | H Q S T Q K N E S D N N E G N I K H Q |
| gi|30171235|ATCC 17803, 2003   | H Q S T Q K N E S D N N E G N I K H Q |
| gi|21326594|2001 (O4:K8)      | H Q S T Q K N E S D N N E G N I K H Q |
| gi|37580917|2011              | H Q S T Q K N E S D N N E G N I K H Q |
| gi|21326598|2001 (O5:K15)     | H Q S T Q K N E S D N N E G N I K H Q |
| gi|21326596|2001 (O4:K13)     | H Q S T Q K N E S D N N E G N V — — — |
| gi|155295|Vm-TDH, 1991      | Y R F T K N K D E S D N E D N V E H R |
| gi|39748669|2003              | Y R F T N E N K H D D N E D N I K H Q |
| gi|217193|1990              | Y R F T N E N K H D D S E D N I K H Q |
| gi|38044805|2011              | Y R F T N E N K H D D N E D N I K H Q |
| gi|48483|1990              | Y R F T N K K N D N N E D D I K Y N |
| gi|155240|VcNAG-TDH, 1991   | Y R F T N K K N D N N E D D I K Y N |
| gi|48481|1990              | Y R F T N K K N D N N E D D I K Y N |

*Truncated *tdh* sequence; Vm, *V. mimicus*; VcNAG, *V. cholerae* non-O1 and non-O139; Bang, Bangladesh; RIMD, RIMD2210633. Known serovars are mentioned in parentheses.
substitution at position 4 was absent. Except in one, all tdh sequences that contained double deletion in the promoter sequence at −18 and −19 gained Gly\(^9\) in the place of aspartic acid (Asp\(^9\)). The significance of this mutation needs to be evaluated.

It has been reported that trh gene has two alleles, namely, trh1 and trh2. The sequences of trh1 and trh2 share 84% and 68% similarity with tdh2, respectively [36]. In the initial studies, it was thought that downstream inverted repeat sequence (IRS) from −35 to −10 of trh1 and trh2 may have some association with low expression of TRH [37, 38]. In the subsequent finding, it was reported that the promoter-bearing region was responsible for the low expression trh transcription rather than the role of IRS [36].

The trh harboring V. parahaemolyticus strains universally carries a urease gene (ureR); V. parahaemolyticus strains isolated from Asian countries always exhibit a strong correlation between the ureR gene and trh positivity [39]. However, the association between these two genes is not related in the transcription of trh [36]. In a clinical perspective, urease-positive phenotype is considered for elevated virulence in V. parahaemolyticus [40]. We did not find any differences in the promoter sequence between trh and tdhS of RIMD2210633, as reported before [36]. Recently, the whole-genome of Oceana monas (strain GKI) belonging to the family Aeromonadaceae has been sequenced and a tdh gene has been detected in the chromosome [41]. The protein sequence of TDH matched with a TDH of Aeromonas spp., but not with the TDH of V. parahaemolyticus. Among the Vibrio species, only V. alginolyticus carried tdh and trh genes. The trh of gene of V. alginolyticus also shared considerable homology with trh of V. parahaemolyticus (data not shown). trh genes of V. parahaemolyticus and Listonella anguillarum have been placed in clade B (Figure 1).

Phylogenetic analysis suggested that there is a high level of sequence diversity in tdh and trh among V. parahaemolyticus strains and in other vibrios. Since these genes are carried by the transposon, they have been detected in many Vibrio spp. [42]. The reason for selective uptake of these genes only in Vibrio species needs to be investigated. Using this in silico approach, differences in promoter sequences were identified among the pandemic and nonpandemic strains of V. parahaemolyticus. Such differences are probably associated with differential transcription in V. parahaemolyticus strains. More experimental evidences may prove the importance of mutations detected in this study.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] G. B. Nair, T. Ramamurthy, S. K. Bhattacharya, B. Dutta, Y. Takeda, and D. A. Sack, “Global dissemination of Vibrio parahaemolyticus serotype O3:K6 and its serovariants,” Clinical Microbiology Reviews, vol. 20, no. 1, pp. 39–48, 2007.
[2] J. W. Turner, R. N. Paranjpye, E. D. Landis et al., “Population structure of clinical and environmental Vibrio parahaemolyticus from the pacific northwest coast of the United States,” PloS ONE, vol. 8, no. 2, Article ID e55726, 2013.
[3] W. T. Yu, K. J. Jong, Y. R. Lin, S. E. Tsai, Y. H. Tey, and H. C. Wong, “Prevalence of Vibrio parahaemolyticus in oyster and clam culturing environments in Taiwan,” International Journal of Food Microbiology, vol. 160, no. 3, pp. 185–192, 2013.
[4] G. J. Hsu, T. Young, M. Y. Peng, F. Y. Chang, and M. Y. Chou, “Septicemia caused by Vibrio parahaemolyticus: a case report,” Zhonghua Yi Xue Za Zhi, vol. 52, no. 5, pp. 351–354, 1993.
[5] T. Honda, K. Goshima, Y. Takeda, Y. Sugino, and T. Miwatani, “Demonstration of the cardiotoxicity of the thermostable direct hemolysin (lethal toxin) produced by Vibrio parahaemolyticus,” Infection and Immunity, vol. 13, no. 1, pp. 163–171, 1976.
[6] Y. Miyamoto, Y. Obara, and T. Nikiwawa, “Simplified purification and biophysical characteristics of kanagawa phenomenon-associated hemolysin of Vibrio parahaemolyticus,” Infection and Immunity, vol. 28, no. 2, pp. 567–576, 1980.
[7] F. Raimondi, J. P. Y. Kao, C. Fiorentini et al., “Enterotoxigenicity of Vibrio parahaemolyticus: thermostable direct hemolysin in vitro systems,” Infection and Immunity, vol. 68, no. 6, pp. 3180–3185, 2000.
[8] S. Bechlers, D. A. Wüstenhagen, K. Drägert, R. Dieckmann, E. Strauch, and S. Kubick, “Cell-free synthesis of functional thermostable direct hemolysins of Vibrio parahaemolyticus,” Toxicon, vol. 76, pp. 132–142, 2013.
[9] T. Honda, Y. Ni, and T. Miwatani, “Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative Vibrio parahaemolyticus and related to the thermostable direct hemolysin,” Infection and Immunity, vol. 56, no. 4, pp. 961–965, 1988.
[10] M. Alam, W. B. Chowdhury, N. A. Bhuiyan et al., “Serogroup, virulence, and genetic traits of Vibrio parahaemolyticus in the estuarine ecosystem of Bangladesh,” Applied and Environmental Microbiology, vol. 75, no. 19, pp. 6268–6274, 2009.
[11] M. E. Cabrera-García, C. Vázquez-Salinas, and E. I. Quiñones-Ramirez, “Serologic and molecular characterization of Vibrio parahaemolyticus strains isolated from seawater and fish products of the gulf of Mexico,” Applied and Environmental Microbiology, vol. 70, no. 11, pp. 6401–6406, 2004.
[12] C. K. Gutierrez West, S. L. Klein, and H. R. Lovell, “High frequency of virulence factor genes tdh, trh, and thl in Vibrio parahaemolyticus strains isolated from a pristine estuary,” Applied and Environmental Microbiology, vol. 79, no. 7, pp. 2247–2252, 2013.
[13] H. Wang, M. M. L. Wong, D. O’Toole, M. M. H. Mak, R. S. S. Wu, and R. Y. C. Kong, “Identification of a DNA methyltransferase gene carried on a pathogenicity island-like element (VPAI) in Vibrio parahaemolyticus and its prevalence among clinical and environmental isolates,” Applied and Environmental Microbiology, vol. 72, no. 6, pp. 4455–4460, 2006.
[14] X. Zhou, M. E. Konkel, and D. R. Call, “Type III secretion system 1 of Vibrio parahaemolyticus induces oncisis in both epithelial and monocytic cell lines,” Microbiology, vol. 155, no. 3, pp. 837–851, 2009.
[15] K. Park, T. Ono, M. Rokuda et al., “Functional characterization of two type III secretion systems of Vibrio parahaemolyticus,” Infection and Immunity, vol. 72, no. 11, pp. 6659–6665, 2004.
[16] T. Sugiyama, T. Iida, K. Izutsu, K. Park, and T. Honda, “Precise region and the character of the pathogenicity island in clinical
Vibrio parahaemolyticus strains,” *Journal of Bacteriology*, vol. 190, no. 5, pp. 1835–1837, 2008.

[17] M. Nishibuchi and J. B. Kaper, “Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium,” *Infection and Immunity*, vol. 63, no. 6, pp. 2093–2099, 1995.

[18] J. Okuda and M. Nishibuchi, “Manifestation of the Kanagawa phenomenon, the virulence-associated phenotype, of *Vibrio parahaemolyticus* depends on a particular single base change in the promoter of the thermostable direct haemolysin gene,” *Molecular Microbiology*, vol. 30, no. 3, pp. 499–511, 1998.

[19] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, and J. Okuda and M. Nishibuchi, “Duplication and variation of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus* required for its hemolytic activity,” *Microbial Pathogenesis*, vol. 12, no. 4, pp. 279–287, 1992.

[20] M. C. Chifiriu, C. Bleotu, G. Pircărăbiu et al., “Cytokine profiles of HeLa and human diploid cells induced by different fractions of *Vibrio parahaemolyticus* cultures exposed to stress conditions,” *Romanian Archives of Microbiology and Immunology*, vol. 69, no. 3, pp. 164–172, 2010.

[21] A. Hasegawa, Y. Haraka-Kudo, K. Ogata, S. Saito, Y. Sugita-Konishi, and S. Kumagai, “Differences in the stress tolerances of *Vibrio parahaemolyticus* strains due to their source and harboring of virulence genes,” *Journal of Food Protection*, vol. 76, no. 8, pp. 1456–1462, 2013.

[22] M. Nishibuchi and J. B. Kaper, “Duplication and variation of the thermostable direct haemolysin (*tdh*) gene in *Vibrio parahaemolyticus*,” *Molecular Microbiology*, vol. 4, no. 1, pp. 87–99, 1990.

[23] T. Iida and K. Yamamoto, “Cloning and expression of two genes encoding highly homologous hemolysis from a Kanagawa-phenomenon-positive *Vibrio parahaemolyticus* T4750 strain,” *Gene*, vol. 93, no. 1, pp. 9–15, 1990.

[24] Y. Chen, O. C. Stine, J. H. Badger et al., “Comparative genomic analysis of *Vibrio parahaemolyticus*: serotype conversion and virulence,” *MBMC Genomics*, vol. 12, article 294, 2011.

[25] K. Makino, K. Oshima, K. Kurokawa et al., “Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*,” *The Lancet*, vol. 361, no. 9359, pp. 743–749, 2003.

[26] J. Martinez-Urtaza, A. Lozano-Leon, A. DePaola et al., “Characterization of pathogenic *Vibrio parahaemolyticus* isolates from clinical sources in Spain and comparison with Asian and North American pandemic isolates,” *Journal of Clinical Microbiology*, vol. 42, no. 10, pp. 4672–4678, 2004.

[27] J. Okuda, M. Ishibashi, S. L. Abbott, J. M. Janda, and M. Nishibuchi, “Analysis of the thermostable direct hemolysin (*tdh*) gene and the *tdh*-related hemolysin (*trh*) genes in urease-positive strains of *Vibrio parahaemolyticus* isolated on the west coast of the United States,” *Journal of Clinical Microbiology*, vol. 35, no. 8, pp. 1965–1971, 1997.

[28] T. Honda, M. Nishibuchi, T. Miwatani, and J. B. Kaper, “Demonstration of a plasmid-borne gene encoding a thermostable direct hemolysin in *Vibrio cholerae* non-O1 strains,” *Applied and Environmental Microbiology*, vol. 52, no. 5, pp. 1218–1220, 1986.

[29] K. A. Miller, E. Hamilton, and M. Dziejman, “The *Vibrio cholerae* *trh* gene is coordinately regulated in vitro with type III secretion system genes by VtrA/VtrB but does not contribute to caco2-bbe cell cytotoxicity,” *Infection and Immunity*, vol. 80, no. 12, pp. 4444–4455, 2012.

[30] A. Alam, K. A. Miller, M. Chaand, J. S. Butler, and M. Dziejman, “Identification of *Vibrio cholerae* type III secretion system effector proteins,” *Infection and Immunity*, vol. 79, no. 4, pp. 1728–1740, 2011.

[31] N. Okada, S. Matsuda, J. Matsuyama et al., “Presence of genes for type III secretion system 2 in *Vibrio mimicus* strains,” *BMC Microbiology*, vol. 10, article 302, 2010.

[32] O. A. Shalu, R. V. Pisanov, and E. V. Monakhova, “Effectiveness of expression of *tdh* gene of *Vibrio parahaemolyticus* depends on two point mutations in promoter region,” *GeneTika*, vol. 48, no. 12, pp. 1364–1371, 2012.

[33] K. Baba, S. Yamasaki, M. Nishibuchi, and Y. Takeda, “Examination by site-directed mutagenesis of the amino acid residues of the thermostable direct hemolysin of *Vibrio parahaemolyticus* required for its hemolytic activity,” *Microbial Pathogenesis*, vol. 12, no. 4, pp. 297–287, 1992.

[34] T. Iida, G. Tang, S. Suttikulpitug, K. Yamamoto, T. Miwatani, and T. Honda, “Isolation of mutant toxins of *Vibrio parahaemolyticus* hemolysin by in vitro mutagenesis,” *Toxicon*, vol. 33, no. 2, pp. 209–216, 1995.

[35] Y. Yanagihara, K. Nakahira, T. Yamane et al., “Structure and functional characterization of *Vibrio parahaemolyticus* thermostable direct hemolysin,” *The Journal of Biological Chemistry*, vol. 285, no. 21, pp. 16267–16274, 2010.

[36] Y. Nakaguchi and M. Nishibuchi, “The promoter region rather than its downstream inverted repeat sequence is responsible for low-level transcription of the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*,” *Journal of Bacteriology*, vol. 187, no. 5, pp. 1849–1855, 2005.

[37] M. Nishibuchi, T. Taniguchi, T. Misawa, V. Khaeomanee-Lam, T. Honda, and T. Miwatani, “Cloning and nucleotide sequence of the gene (*trh*) encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*,” *Infection and Immunity*, vol. 57, no. 9, pp. 2691–2697, 1989.

[38] M. Kishishita, N. Matsuoka, K. Kumagai, S. Yamasaki, Y. Takeda, and M. Nishibuchi, “Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*,” *Applied and Environmental Microbiology*, vol. 58, no. 8, pp. 2449–2457, 1992.

[39] A. Parvathi, H. S. Kumar, A. Bhanumathi, M. Ishibashi, M. Nishibuchi, and I. Karunasagar, “Molecular characterization of thermostable direct haemolysin-related haemolysin (TRH)-positive *Vibrio parahaemolyticus* from oysters in Mangalore, India,” *Environmental Microbiology*, vol. 8, no. 6, pp. 997–1004, 2006.

[40] O. Suthienkul, M. Ishibashi, T. Iida et al., “Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand,” *Journal of Infectious Diseases*, vol. 172, no. 5, pp. 1405–1408, 1995.

[41] L. P. Yeganeh, R. Azarbaijani, S. Sarikhani et al., “Complete genome sequence of *Oceanimonas* sp. GKI, a halotolerant bacterium from gavkhouni wetland in Iran,” *Journal of Bacteriology*, vol. 194, no. 8, pp. 2123–2124, 2012.

[42] A. Terai, K. Baba, H. Shirai, O. Yoshiida, Y. Takeda, and M. Nishibuchi, “Evidence for insertion sequence-mediated spread of the thermostable direct hemolysin gene among *Vibrio* species,” *Journal of Bacteriology*, vol. 173, no. 16, pp. 5036–5046, 1991.