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Restriction Fragment Length Polymorphism of the *tdh* and *trh* Genes in Clinical *Vibrio parahaemolyticus* Strains

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The restriction fragment length polymorphism of the genes encoding thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) was analyzed for 137 strains of *Vibrio parahaemolyticus* isolated from specimens from diarrheal patients in Thailand. The *Hin*dIII restriction fragment patterns of *tdh* and *trh* were grouped into five and four types, respectively. A strong association between the restriction fragment patterns of *tdh* and *trh* was observed with *V. parahaemolyticus* strains.

Pathogenic *Vibrio parahaemolyticus*, which causes acute gastroenteritis after the consumption of raw or partially cooked seafood, has been known to produce either thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) or both TDH and TRH (4). TDH has been found in almost all of the strains of *V. parahaemolyticus* isolated from clinical sources but has rarely been found in strains isolated from environmental sources (9). Another hemolysin, TRH, has recently been recognized to help cause gastroenteritis in a manner similar to that of TDH-producing isolates (1, 5, 11, 12). At present, TDH and TRH are considered to be important virulence factors in the pathogenesis of *V. parahaemolyticus* (2, 4, 11–13).

Recently, we examined the presence or the absence of the genes encoding TDH and TRH (*tdh* and *trh*, respectively) in *V. parahaemolyticus* strains isolated from specimens of diarrheal patients in Thailand (12). The results indicated that 90% of the strains tested positive for *tdh* and/or *trh*, of which 6% were positive for both *tdh* and *trh*. In this study, the restriction fragment length polymorphism of *tdh* and *trh* in those isolates was examined to analyze the clonal relationships among clinical *V. parahaemolyticus* strains.

A total of 137 *V. parahaemolyticus* strains containing *tdh* and/or *trh* were selected from 489 clinical strains that had been isolated in Thailand and tested by PCR for the presence or absence of *tdh* and *trh* (signified here by the gene name with a superscript plus or minus, respectively) (12). From the 396 tdh^+ trh^- strains (12), 100 strains were selected randomly for this study. All of the tdh^- trh^+ (10 strains) and tdh^+ trh^+ (27 strains) strains found in the previous study (12) were also included. The 359-bp *Eco*RI fragment from pKY199 (3) for the *tdh* probe and the 334-bp *Eco*RI fragment from pKY298 (16) for the *trh* probe were labeled with digoxigenin-11-dUTP by the random primer extension method with a DNA labeling kit (Boehringer, Mannheim, Germany). Labeling was conducted according to the manufacturer's protocol. The labeled DNA fragments were separated from unincorporated nucleotides by ethanol precipitation and redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and then stored at −20°C until used. By following the method of Wilson (15), the genomic DNA of *V. parahaemolyticus* strains was extracted from overnight cultures in Luria-Bertani broth with 3% NaCl. For Southern hybridization, total genomic DNA was digested overnight with *Hin*dIII at 37°C, and then the *Hin*dIII digests were separated by electrophoresis on a 1% agarose gel. After electrophoresis, the DNA fragments were transferred with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]) onto a nylon membrane (GeneScreen; Dupont, Boston, Mass.) as described previously (10). They were then hybridized with the digoxigenin-labeled *tdh* or *trh* probe in hybridization solution containing 5× SSPE, 50% formamide, 0.1% sodium–laurylsarcosine, 0.02% sodium dodecyl sulfate, 0.1 mg of denatured single-stranded salmon sperm DNA per ml, and 1% blocking reagent of a DIG DNA labeling and detection kit (Boehringer). The hybridization temperature for the *tdh* probe was 37°C, and the hybridization temperatures for the *trh* probe were 30 and 42°C. Immunological detection of DNA fragments hybridized with the probes was performed with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer). The DNA targets were then detected with a chemiluminescent substrate for alkaline phosphatase, CSPD (Tropix, Bedford, Md.), as recommended by the kit manufacturer.

After Southern hybridization, the *Hin*dIII restriction fragment patterns (RFP) of the *tdh* genes among the tdh^+ strains (a total of 127 strains) were grouped by type, types D1 to D5 (Fig. 1; Table 1). Type D1 showed two bands, one of which was 1.3 kb and the other was 2.5 kb in size, and type D4 also had two bands, which were 2.8 and 12.7 kb. The single-band types had the following band sizes: type D2, 2.8 kb; type D3, 12.7 kb; and type D5, 5.0 kb. With the hybridization conditions used, all of the tdh^+ strains showed a positive reaction to the *tdh* probe. By contrast, the *trh* genes in trh^+ strains had heterogeneous reactions to the *trh* probe. All of the trh^+ strains gave a single band of *trh* when the hybridization temperature was 30°C (Fig. 2B). The trh^+ strains were divided into four groups according to band size, as determined from their *Hin*dIII RFP of *trh*, as follows: type R1 had a band size of 3.0 kb; type R2 had a band size of 6.7 kb; type R3 had a band size of 4.5 kb; and type R4 had a band size of 6.2 kb. None of these bands hybridized with

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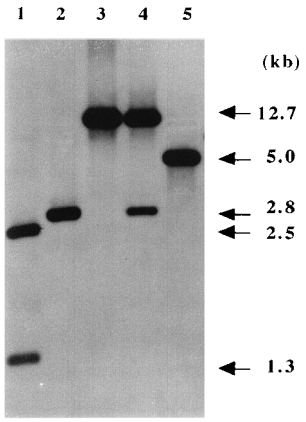

FIG. 1. Five types of the *Hin*dIII restriction fragment patterns of *tdh*. The lanes contain the following types: lane 1, D1; lane 2, D2; lane 3, D3; lane 4, D4; lane 5, D5.

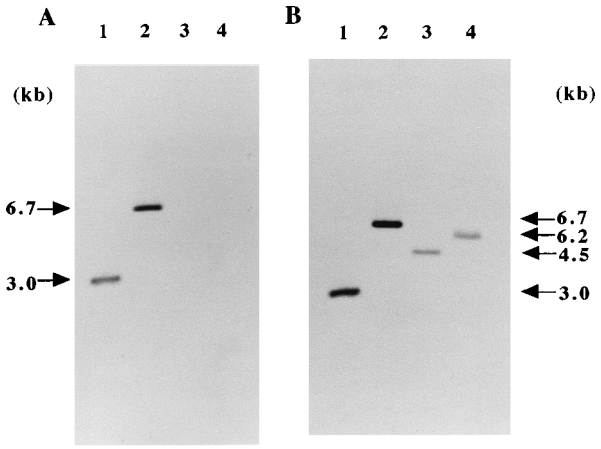

FIG. 2. Four types of the *Hin*dIII restriction fragment patterns of *trh*. The hybridization temperatures were 42°C (more stringent) for the gel in panel A and 30°C (less stringent) for that in panel B. The lanes contain the following types: lane 1, R1; lane 2, R2; lane 3, R3; lane 4, R4.

the *tdh* probe. When hybridization was done at 42°C, types R1 and R2 were still detected while types R3 and R4 were no longer detected (Fig. 2A). These results suggest that, compared with the originally reported *trh* gene (8, 16) from which the probe was prepared, there are dissimilarities in the sequences of the *trh* genes in R3 and R4. An example of such a variant *trh* has previously been reported (6).

Although, in total, over 100 strains were examined in this study, relatively few types of RFP were observed for both *tdh* (five types) and *trh* (four types). Among tdh^+ strains, type D1 was predominant (79%), followed by type D2 (11%) and type D3 (8%). Thus, these three types accounted for 98% of the tdh^+ *V. parahaemolyticus* isolates. As shown in Table 1, all of the tdh^+ trh^- strains showed type D1 *tdh*. Considering that we selected 100 strains randomly from the 396 tdh^+ trh^- strains present in the original 489 strains isolated (12), and because we used all of the tdh^+ trh^+ and tdh^- trh^+ strains in the population, type D1 strains must be predominant among all of the clinical *V. parahaemolyticus* isolates.

The most interesting observation of this study is the strong association between RFP of *tdh* and *trh* in clinical isolates. All of the type D1 strains were trh^-. D2 strains always had type R2 *trh*. Type D3 strains possessed type R4 *trh*. Type R1 and R3 strains were all tdh^-. Thus, a certain RFP of *tdh* always associates with a certain RFP of *trh*. As a previous study has demonstrated, only a small proportion of the isolates of environmental *V. parahaemolyticus* contains *tdh* and/or *trh* (11). It is believed that, sometime in the past, a certain group of *V. parahaemolyticus* organisms might have acquired *tdh* and/or *trh* through a mechanism involving plasmids and/or insertion sequence-like elements (7, 14). The association between the RFP of *tdh* and *trh* observed in our study might be somehow related to the mode of the ancient transfer of the genes into *V. parahaemolyticus*. However, to get an insight into this association, further studies, including detailed analyses of the locations of *tdh* and *trh* on genomic DNA and the structures of the flanking DNA regions of the genes, must be done.

TABLE 1. Relations among the types of *Hin*dIII RFP of *tdh* and *trh* in clinical *V. parahaemolyticus* isolates from Thailand

RFP of *trh* type:	No. of *V. parahaemolyticus* strains whose RFP of *tdh* were type:						Total
	None[a]	D1	D2	D3	D4	D5	
None[b]	NT[c]	100	0	0	0	0	100
R1	6	0	0	0	0	0	6
R2	0	0	14	0	1	0	15
R3	4	0	0	0	0	0	4
R4	0	0	0	11	0	1	12
Total	10	100	14	11	1	1	137

[a] None, a type that produced no *tdh* bands.
[b] None, a type that produced no *trh* bands.
[c] NT, not tested.

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