HEMOLYSINS AND PLASMID PROFILES OF *VIBRIO PARAHAEMOLYTICUS*

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Abstract. Forty clinical isolates of *Vibrio parahaemolyticus* were studied for the production of the thermostable direct hemolysin (TDH), and the TDH-related hemolysin (TRH) including the respective encoding genes, *tdh* and *trh*. The presence of TDH and its encoding genes were found amongst 95% of the strains, whereas the TRH was absent amongst these isolates. Thirty-two isolates were found to be plasmid-free, whereas eight isolates possessed plasmids with sizes ranging from 2.4 - > 23 kb. Using a DNA probe coding for the homologous region of the *tdh* and *trh*, it was found that the *tdh* genes were present on the chromosomal DNA.

INTRODUCTION

Vibrio parahaemolyticus is a well recognised etiological agent of gastroenteritis caused by the consumption of improperly cooked or raw seafood (Baker and Gangarosa, 1974) and an occasional cause of extraintestinal infections (Blake *et al*, 1980). It has been well documented that the production of an extracellular hemolysin known as the thermostable direct hemolysin (TDH) is the major virulence factor of *V. parahaemolyticus* (Shirai *et al*, 1990). Originally the TDH was demonstrated by the production of β-hemolysis around colonies of clinical isolates of *V. parahaemolyticus* on Wagatsuma agar, known as the Kanagawa phenomenon (Sakazaki *et al*, 1968). More recently several isolates from patients with diarrhea were found not to produce TDH but a TDH-related hemolysin (TRH). Comparative analysis indicated a 68.6% nucleotide sequence homology between the *tdh* and *trh* genes, suggesting a common ancestral origin, having evolved by random drift (Nishibuchi *et al*, 1989). Shirai *et al* (1990) found that 90% of clinical isolates from diarrheal cases carried the *tdh* or the *trh* genes singly or in combination thus providing evidence for the role of TDH and/or TRH in the pathogenesis of diarrhea caused by *V. parahaemolyticus*. To date, there have been no reports on the occurrence and distribution of TDH and/or TRH amongst *V. parahaemolyticus* isolates from diarrheal cases in Malaysia.

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In this study, clinical strains of *V. parahaemolyticus* were reconfirmed using routine biochemical tests under modified halophilic conditions. Further to this, the aim of this study was to investigate the distribution of TDH and TRH using various assays and to determine whether the *tdh/trh* genes were situated within the chromosomal or plasmid DNA.

MATERIALS AND METHODS

Bacterial strains

Forty strains of *V. parahaemolyticus* isolated from patients with acute gastroenteritis, at the University Hospital, Kuala Lumpur, and confirmed using routine biochemical tests were studied. These lyophilized strains were cultured on thiosulphate-citrate bile salts sucrose agar (TCBS) (Oxoid, UK) at 37°C overnight and stored on nutrient agar slopes at room temperature during the course of the study. *V. parahaemolyticus* 89-850 as a positive control, *Vibrio mimicus* ATCC 33653 and *Vibrio vulnifucus* TE 103 (Tokyo Metropolitan Hospital, Japan) were used as negative controls.

Biochemical characterization

Biochemical characterization of *V. parahaemolyticus* was performed using routine tests (Cowan, 1981; Tison and Kelly, 1984) supplemented with 5% sodium chloride (wt/vol). The determining tests for classifying *V. parahaemolyticus* included

cytochrome oxidase test, hydrogen sulphide production, Voges-Proskauer test, sucrose and mannitol utilization in which the media was supplemented with 5% sodium chloride (Table 1) (Honda *et al*, 1988). In addition *V. parahaemolyticus* was further differentiated from the other vibrios on the basis of sodium chloride requirement for growth.

Kanagawa phenomenon (KP)

The KP was performed using Wagatsuma agar (Nishibuchi *et al*, 1989) supplemented with fresh rabbit erythrocytes at a final concentration of 2.5% (vol/vol). Each bacterial strain was grown overnight at 37°C with shaking in trypticase soy broth (Oxoid, UK) supplemented with 3% sodium chloride (wt/vol) after which 25 ml of the culture was spot-inoculated onto Wagatsuma blood agar. Following overnight incubation at 37°C, the plate was observed for a zone of β-hemolysis (clear halo) around the colony as positive for the Kanagawa phenomenon.

Reverse passive latex agglutination test for TDH (RPLA)

The test was performed using a commercial KAP-RPLA kit (Denka Seiken Co, Japan). Briefly 25 µl of culture supernatants of bacterial strains grown overnight at 37°C with shaking in mannitol peptone broth supplemented with 5% sodium chloride (wt/vol) were diluted two-fold (1 : 2 - 1 : 8) with dilution buffer in V-bottom microtiter plates. Anti-TDH sensitized (25 µl) latex particles were added to each well, the solution was mixed by tapping the plate and incubated at room temperature overnight. Positive results for agglutination were recorded as (+++), (++) or (+) for each dilution.

Colony blot DNA-DNA hybridization assay for *tdh* and *trh* genes

The *tdh* and *trh* genes were detected using a rapid prototype commercial kit donated by Dr T Honda (Osaka University, Japan) enabling results within 3-4 hours. Briefly, colony blots of the bacterial strains were prepared on nylon membranes and hybridized using an enzyme-labeled *tdh/trh* DNA probe. Following hybridization the blots were developed at 37°C for 45 - 90 minutes using a substrate solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). A positive result was observed by the

Table 1

Biochemical tests to differentiate *V. parahaemolyticus* from other halophilic vibrios (Adapted from Hollis *et al*, 1976).

Tests	*V. parahaemolyticus*	Other halophilic vibrios
Cytochrome oxidase	+	+
Voges - Proskauer	-	-
* H_2S (KIA)[1]	-	-
* Lysine decarboxylase	+	+
* Ornithine decarboxylase	+	+
* Arginine decarboxylase	-	-
Sucrose utilization	-	-
Mannitol utilization	+	d
Na+ requirement for growth		
0% NaCl	-	-
3% NaCl	+	+
7% NaCl	+	d
9% NaCl	+	-
11% NaCl	-	-

* Media supplemented with 3% NaCl (wt/vol)
[1] KIA - Kliger's Iron Agar
d different results obtained for different strains.

development of purple colour of the substrate concurrent to the colony on the filter.

Plasmid and chromosomal DNA extraction and DNA hybridisation assay

Bacterial strains were cultured overnight at 37°C in 10 ml Luria Bertani (LB) broth supplemented with 3% NaCl of which 5 ml was used for plasmid DNA extraction and the remaining 5 ml for chromosomal DNA extraction. Plasmid DNA were extracted using the alkaline lysis method of Sambrooke *et al* (1989) with modifications. The cell wall and the outer membrane were disintegrated with lysing solution (50 mM glucose, 25 mM Tris-Cl, pH7.8 and 10 mM EDTA, pH8) following which the spheroplasts were lysed (0.2N NaOH, 1% SDS). Nucleic acids were extracted from the supernatant using an equal volume of 7.5 M ammonium acetate and chloroform : isoamyl alcohol (24 : 1) solutions. The precipated nucleic acids were collected using polyethylene glycol (PEG), (30% PEG6000, 1.5 M NaCl) followed by centrifugation at 13,000 rpm for 30 seconds. Traces of PEG liquid were removed and the nucleic acid pellet was resuspended in 25 µl of TE (10 mM Tris, 1 mM EDTA) buffer, pH8 after which 1 µl of RNaseA (10 mg/ml) was added to digest cellular RNA at 37°C for 1 hour.

Plasmid DNA profiles were studied on 1% agarose gel electrophoresis at 90 mA for 1.5 hours in 1X TBE buffer (89 mM Tris-borate, 25 mM EDTA) and stained using ethidium bromide for visualization under UV light.

The method for extracting the chromosomal DNA was modified from David *et al* (1986). Cells were collected by centrifugation at 10,000 rpm for 10 minutes after which the pellet was resuspended in 0.4 ml of homogenization buffer (25% sucrose, 10 mM Tris pH8) followed by the adition of 0.5 mg of proteinase-K and 0.4 ml of lysis buffer (2% SDS, 100 mM EDTA). The suspension was mixed gently by inversion of the tubes and incubated at 37°C after which an equal volume of phenol-chloroform (1 : 1) solution was added and mixed gently for a further 10 minutes. Following centrifugation at 4,000 rpm for 2 minutes the aqueous phase was then transferred to new tubes, and the phenol-chloroform extraction step was repeated until the interphase was clear. The aqueous phase was then further subjected to chloroform extractions to remove residual bacterial proteins. Chromosomal DNA was then precipitated from the aqueous phase using 0.3 M sodium acetate, pH 8.0 and 2 volumes of absolute ethanol, followed by 30 minutes incubation at -20°C prior to centrifugation at 12,000 rpm for 5 minutes. The chromosomal DNA pellet was then resuspended in 1.5 ml of 70% ethanol and centrifuged again at 12,000 rpm for 5 minutes after which it was dried in a speedvac for 10 minutes. The dried pellet was resuspended in 50 µl of TE buffer pH 7.5 and subjected to RNA digestion using 4 µg of RNaseA at 37°C for 1 hour.

10 µl of the plasmid DNA suspension and 10 µl of the chromosomal DNA suspension were then spotted on separate nylon membranes (Amersham, UK) and probed for the presence of *tdh/trh* genes using the DNA-DNA hybridization assay as described above.

RESULTS

Using the modified halophilic medium all 40 strains were found to produce cytochrome oxidase, utilize mannitol and required sodium chloride for growth, whereas, production of hydrogen sulphide, sucrose utilization and the Voges-Proskauer reaction were found to be negative, which is in agreement with the results of Hollis *et al* (1976) (Table 1). In addition, these strains exhibited the ability to grow in the presence of up to 9% sodium chloride concentration. Thus all 40 strains were confirmed as *V. parahaemolyticus*.

The presence of TDH as demonstrated by both the Kanagawa phenomenon and the reverse passive latex agglutination assay was found amongst 38 strains (95%) (Table 2). In addition the colony blot DNA-DNA hybridization performed to detect the presence of *tdh/trh* genes which encode for the production of TDH/TRH were also found to be present amongst these same 38 strains. The remaining 2 strains did not indicate the presence of these hemolysins or their encoding genes in any of the assays (Table 2).

The agarose gel electrophoresis indicated the presence of plasmids in 8 (20%) strains with sizes ranging from 2.4 - > 23 kb, but the majority of the isolates 32 (80%) did not possess any plasmids. DNA-DNA hybridization using the dot blot method did not demonstrate the presence of *tdh/trh* genes on these plasmid DNAs. The dot blot on chromo-

Table 2

Detection of TDH/TRH and *tdh/trh*.

KP (TDH)	RPLA (TDH/TRH)	DNA-DNA hybridization (*tdh/trh*)	No. of strains
+	+	+	38
-	-	-	2
Total			40

KP = Kanagawa phenomenon
RPLA = Reverse passive latex agglutination

somal DNA and Southern blot on total DNA from the same eight plasmid bearing strains indicated that *tdh/trh* genes were located on the chromosome of four strains. In addition, the dot and Southern blots, performed on both chromosomal and total DNA on the rest of the 32 plasmid free strains, showed the presence of *tdh/trh* on 23 strains (see Table 3). These 27 (67.5%) of the 40 isolates were shown to possess the *tdh/trh* genes, being present on the chromosomal DNA since no *tdh/trh* were detected on plasmid DNA.

DISCUSSION

The virulence factor involved in the pathogenesis of gastroenteritis due to *Vibrio parahaemolyticus* has been identified as the thermostable direct hemolysin (TDH) (Nishibuchi *et al* 1989; Shirai *et al*, 1990) demonstrated by the Kanagawa phenomenon (KP). TRH, a TDH-related hemolysin was found to be produced by 4 - 12% of clinical strains that did not produce TDH (KP-negative) (Miyamoto *et al*, 1969) and so has been presumed to be another important virulence factor of *V. parahaemolyticus* (Nishibuchi *et al* 1989; Shirai *et al*, 1990). In this study of 40 strains, 38 (95%) were found to produce hemolysin as determined by the Kanagawa phenomenon and by the reverse passive latex agglutination test indicating the presence of TDH amongst these strains whilst the remaining 2 strains were negative by both assays. To circumvent the problem of detecting the presence of the TDH and TRH under *in vitro* cultural conditions which may affect the production of these factors, a prototype DNA probe to the common region of the *tdh* and *trh* genes was used. It was found that the same 38 (95%) strains positive by the other assays were also positive by the colony blot DNA-DNA hybridization assay suggesting the presence of *tdh/trh*. The remaining 2 KP-negative strains were found to be negative by all these assays suggesting that the *trh* was not expressed, thus the TDH may be more common than TRH, in agreement with previous studies (Nishibuchi *et al*, 1989; Shirai *et al*, 1990).

Analysis of the extrachromosomal DNA demonstrated that only 8 (20%) isolates possessed plasmids (Table 3). Four of these plasmid-bearing

Table 3

Distribution of plasmids and *tdh/trh* amongst clinical isolates of *V. parahaemolyticus*.

Isolates	Plasmid sizes	Presence of *tdh/trh*			
		Plasmid	Chromosome	Total DNA	No. of isolates
1	4.0; 4.1; 6.3; 17.6	-	+	+	1
2	2.49; 4.19; 4.28	-	+	+	1
3	2.49; 4.19; 4.28	-	-	-	1
4	3.3; 4.1; 13.1	-	+	+	1
5	2.4	-	+	+	1
6	6.3	-	-	-	1
7	6.3	-	-	-	1
8	> 23	-	-	-	1
9 - 31	-	NA	+	+	23
32 - 40	-	NA	-	-	9

NA: Not applicable

isolates, indicated positivity for the presence of *tdh/trh* on chromosomal DNA, and not on any of the plasmids which ranged from 2.4 - > 23 kb. In addition, the detection of *tdh/trh* on the chromosomal DNA of 23 plasmid-free isolates, strongly suggested that the *tdh/trh* genes are harbored on the chromosomal DNA. The role of the additional plasmids could not be discerned in the scope of this study, although they may encode for other unknown putative virulence factors such as antibiotic resistance. Some of these extra bands may not be significant since they could be present as doublets or multiples of a smaller band.

The colony blot hybridization assay detected the presence of *tdh/trh* on 38 (95%) KP-positive isolates, whereas the dot and Southern blots indicated only 27 (67.5%) positivity of *tdh/trh* among the KP-positives. Since chromosomal DNA was not digested prior to Southern blot analysis, *tdh/trh* loci on the chromosome might not be exposed to the labeled probe if the genomic DNA was in a supercoiled condition. Thus, detection would be hampered if the genes are considerably small. In addition, in the dot blot procedures, the chromosomal DNA might be sheared during chromosome isolation, which could lead to the loss of the genes. Furthermore, the 2 KP-negative isolates were also found to be negative in the hybridization assays confirming the absence of the *trh* genes on either the chromosome or plasmids. It may be possible that the *trh* genes if originally present amongst these KP-negative strains may have undergone further gene rearrangement or mutation due to storage conditions or subculture. However the significance of not detecting TDH or *trh* genes amongst our KP-negative isolates may be biased due to the small number of strains studied.

The preponderance of KP-positive strains amongst our collection of *V. parahaemolyticus* isolates suggests that the TDH is the more important virulence factor of the 2 which is in agreement with reports by other workers (Sakazaki *et al*, 1968; Shirai *et al*, 1990). As reported by Miyamoto *et al* (1969) the *trh* genes are related to the *tdh* genes indicating a 68.6% homology suggesting a common ancestral origin. Therefore the TRH may only be detected perhaps as a result of gene selection or rearrangement of the *tdh* genes, sufficient to affect hemolytic activity (KP-negative) whilst still maintaining significant nucleotide homology to cause virulence.

In many developing countries, *V. parahaemolyticus* is not considered a major enteropathogen. The findings of this study indicate that in Malaysia, *V. parahaemolyticus* should be considered as one of the important etiological agents of diarrheal disease.

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