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High prevalence of thermostable direct hemolysin (TDH)-like toxin in *Vibrio mimicus* strains isolated from diarrhoeal patients

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SUMMARY

A total of 17 isolates of *Vibrio mimicus* from patients, 29 from environment and 2 from food was examined for toxigenicity. Sixteen (94%) clinical isolates and one (50%) from food produced TDH-like toxin, whereas none of the environmental isolates did so. The food from which *V. mimicus* with TDH-like toxin production was isolated, was one which had caused food poisoning. Only one environmental strain produced CT-like toxin, whilst ST-like toxin was not detected from any strains tested.

INTRODUCTION

Vibrio mimicus is distributed widely in marine and estuarine environments and in seafoods. The organisms are recognized as a causative agent of human diarrhoea [1, 2]. Clinical studies on *V. mimicus* infection revealed that diarrhoea was accompanied in the majority of patients by vomiting and abdominal cramps, some with bloody diarrhoea [2]. Gastroenteritis due to *V. mimicus* has occurred following ingestion of seafoods [1, 2].

Some *V. mimicus* strains have been reported to produce toxins similar to cholera enterotoxin (CT) [3] and heat-stable enterotoxin (ST) [4]. However, the frequency of such toxin-producing strains was estimated to be less than 10% of both clinical and environmental isolates [1, 5]. Recently, the organisms were found to produce hemolysin which has some similarity to thermostable direct hemolysin (TDH) in *V. parahaemolyticus* [6]. The sequence structure of the genetic locus for producing hemolysin in *V. mimicus* is homologous to that in TDH-gene of *V. parahaemolyticus* [7]. The primers to detect the TDH-gene of *V. parahaemolyticus* by polymerase chain reaction (PCR) have been established [8].

In this report, the frequency of toxin production in *V. mimicus* strains of clinical and environmental origins was compared.

MATERIALS AND METHODS

Strains of V. mimicus

The *V. mimicus* strains used in this study were isolated from patients with diarrhoea including 5 sporadic cases, 4 cases of food poisoning and 4 overseas travellers, those from environment sources included 15 water samples and 14 live

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fish, and those from purchased sea-foods. The *V. mimicus* isolates were identified by standard biochemical tests [1]. The *V. parahaemolyticus* strains used were isolated in this laboratory from diarrhoeal patients.

Bacterial growth conditions

For toxin assay, bacteria were cultured in brain heart infusion broth (Difco Laboratories) supplemented with 0·5% NaCl for 20 h at 37 °C with shaking [9]. Cultures were centrifuged, and the supernatant was filtered through a sterile membrane at 0·22 mμ porosity (Toyo Roshi Kaisha, Ltd.). For PCR, bacteria were cultured in L-broth for 20 h at 37 °C.

Toxin assay

For the ST-like toxin assay, three mice of 3–5 days of age were used for each test [10]. The milk-filled stomach of each mouse was administered with 0·1 ml of the culture filtrate containing Evans blue (0·01%). Inoculated mice were kept for 4 h at 25 °C then sacrificed. The whole intestine was removed, and the ratio of the weight of intestines to the remaining body weight was measured to calculate the fluid accumulation ratio. Samples with a ratio greater than 0·09 were defined as ST positive. For CT-like toxin, culture filtrates were assayed by using the bead ELISA method (Nissui Pharmaceutical Co.) described previously [11]. Technical procedures were briefly as follows; 25 μl of each culture filtrate was mixed with an equal volume of the appropriate buffer, and the anti-CT IgG coated polystyrene beads added into the mixture. After incubation for 1 h at 37 °C, the beads were washed three times with distilled water and subsequently incubated for 1 h at 37 °C with 0·5 ml of Fab'-horseradish peroxidase conjugate. After the incubation, the beads were washed three times with distilled water. Peroxidase activity was determined by incubating the beads for 1 h at 30 °C with 0·6 ml of 0·56 mM-3-3′,5-5′-tetramethylbenzidine in 0·1 M sodium acetate buffer (pH 5·5) containing 2 mM-EDTA followed by the addition of 0·02% of H_2O_2. Finally, the reaction was stopped by adding 0·2 ml of 4N H_2SO_4 and the intensity of the resulting yellow colour was measured at 450 nm using a spectrophotometer. For TDH-like toxin, culture filtrate was assayed by using the beads ELISA method (Nissui Pharmaceutical Co.) reported previously [12]. The procedures for ELISA was the same as the beads method for CT detection, except that the polystyrene beads were coated with anti-TDH IgG. The heat stability of TDH-like toxin was confirmed after heating them for 15 min at 100 °C.

PCR amplification

For PCR amplification, 3 μl of the bacterial culture was heated initially to 100 °C for 5 min to disrupt bacteria and denature DNA, and was put into a total volume of 30 μl reaction mixture composed of 10 mM-Tris-HCl (pH 9·0), 50 mM-KCl, 1·5 mM-$MgCl_2$, 0·01% gelatin, 0·6 μM each of the primers [8], 0·2 mM each of the four deoxynucleoside triphosphates (Wako Junyaku Kogyo Co. Ltd.), 0·05% Tween 20, 0·05% Nonidet P-40 and 0·75 U of Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). A total of 35 PCR cycles was run in a DNA thermal cycler; one cycle included denaturation for 1 min at 94 °C, primer annealing for 1 min at 55 °C, and extension for 1 min at 72 °C. Four μl of each PCR-mixture was

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Table 1. *Toxin production of various* V. mimicus *strains isolated*

Origine of strains	Strains examined	Positive no. (%) of strains: TDH-like toxin	CT-like toxin	ST-like toxin
Human diarrhea	17	16 (94·1)	0	0
Purchased sea food	2	1 (50·0)	0	0
Environment water	15	0	0	0
Live marine fishes	14	0	1 (7·1)	0

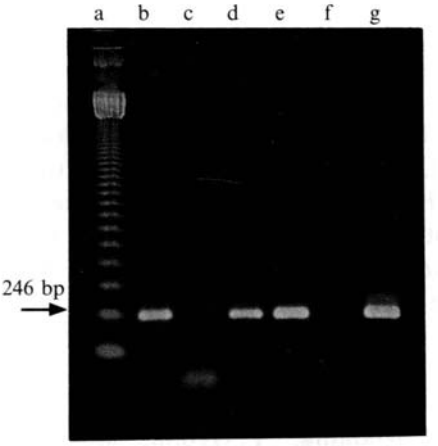

Fig. 1. Electrophoretic analysis of PCR amplified DNA from *V. mimicus* strains. Lane a, 123-bp ladder (Bethesda Research Laboratories, Ins., Gaithersburg, Md.); lane b, TDH positive *V. parahaemolyticus*; lane c, TDH negative *V. parahaemolyticus*; lanes d, e, g. TDH-like toxin positive *V. mimicus* isolated from a sporadic patient of food poisoning, an overseas traveler and a food causing food poisoning, respectively; lane f, *V. mimicus* negative for TDH-like toxin.

electrophoresed on a 1·5% agarose gel. After electrophoresis, the gel was stained in 0·5 μg/ml ethidium bromide solution and photographed under UV light.

RESULTS

The activities of CT-like toxin, ST-like toxin and TDH related toxin were determined in 48 *V. mimicus* strains isolated from human diarrhoea cases, seafood and environment. As shown in Table 1, TDH-like toxin was detected in 16 of 17 human diarrheal strains (94%) and in 1 of 2 strains from purchased seafood (50%). The seafood from which a TDH-like toxin positive strain was detected, was one which had previously caused food poisoning. CT-like toxin was detected in one strain from environment, whereas ST-like toxin was not detected in any strains examined. Presence of TDH-gene was examined in isolated *V. mimicus* strains by PCR using the DNA primer specific to the TDH-gene of *V. parahaemolyticus*. The expected DNA fragment at 251 bp was produced by the strains positive for TDH-like toxin but not by the strains negative for TDH-like toxin (Fig. 1, Table 2). Also, the presence of CT-gene was revealed by PCR-amplification test in a *V. mimicus* strain producing CT-like toxin.

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Table 2. *Detection of toxin genes by PCR from 48 strains of* V. mimicus

TDH-like toxin production	No. of strains examined	No. of TDH-gene positive strains
Positive	17	17
Negative	31	0

DISCUSSION

In this paper, it was demonstrated that production of TDH-like toxin together with the presence of TDH producing gene were detected frequently in *V. mimicus* isolated from diarrheal patients. However, strains isolated from environmental sources examined in this study, did not produce the toxin nor possess the toxin gene. As the TDH in *V. parahaemolyticus* is closely related to its enteropathogenicity [13, 14], TDH-like toxin is estimated to be the enteropathogenicity factor in *V. mimicus*. Recently, Nishibuchi and colleagues [15] demonstrated that the TDH had enterotoxigenic activity and might be implicated in the watery diarrhoea by *V. parahaemolyticus*. Further study will be required for understanding the more actual role of TDH-like toxin in human diarrhea.

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