Epidemiol. Infect. (2001), **126**, 129–133. Printed in the United Kingdom

SHORT PAPER

Survival of Shiga toxin-producing *Escherichia coli* O157 in marine water and frequent detection of the Shiga toxin gene in marine water samples from an estuary port

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(*Accepted 17 October 2000*)

SUMMARY

Shiga toxin-producing *Escherichia coli* (STEC) O157 was investigated with respect to its halotolerance and whether it can survive in marine water. STEC O157 could multiply in a medium containing 5% NaCl and in sterilized marine water, and could survive in unsterilized marine water for at least 15 days. On the basis of these results, we postulated that STEC O157 may survive in natural marine water, and attempted to isolate the bacterium and Shiga toxin gene (*stx*) from marine water in Japan. The *stx*, comprising stx_1 and stx_2, was detected from marine water samples by PCR. STEC and other *stx*-positive bacteria, however, could not be isolated from these samples in this study. These results indicate that *stx*-positive bacteria may survive in marine water and suggest the necessity of a survey.

Shiga toxin-producing *Escherichia coli* (STEC; synonyms: verotoxin-producing *E. coli* (VTEC) and enterohaemorrhagic *E. coli* (EHEC)) is the causative agent of a dysentery-like diarrhoea and, rarely, also causes haemolytic uraemic syndrome (HUS) [1]. STEC is characterized by the production of Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2). The transmission route of the bacterium is through food, such as contaminated beef [2], apple juice [3], vegetables [4], salmon roe in soy [5], and water [6, 7]. The natural hosts of the bacterium are domestic animals [8, 9] and the bacteria have also been found in water samples from lakes [10–12], pools [13, 14], and rivers [15]. Since faecally contaminated coastal water might pose a major threat to the public health, Lang et al. [16] intensively investigated the presence of enteropathogenic *E. coli* in marine water in the United States. They obtained 63 *E. coli* isolates from marine water from an outfall in California and 117 isolates from marine water from a closed estuary in North Carolina, and identified one enterotoxigenic *E. coli* strain and one STEC strain. However, they suggested that the infrequent detection of toxigenic *E. coli* indicates a low public health risk from the bacteria in coastal waters in the United States. In Japan, such survey of marine water has not yet been conducted. The O157 antigen-positive bacteria were confirmed by the fluorescent antibody technique from five rivers in and near metropolitan Tokyo in Japan [17]. We experienced a food-borne outbreak of STEC O157:H7 infection in Osaka prefecture, Japan, in 1996 [18, 19]. STEC were also isolated from river waters in Osaka near the place of the outbreak [15]. Since the river drains into the Port of Osaka, we hypothesized that the bacterium can be found in marine water from the estuary Port of Osaka. To verify the hypothesis, we examined whether STEC can survive in marine water and whether it can be isolated from natural marine water samples.

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https://doi.org/10.1017/S0950268801005003 Published online by Cambridge University Press

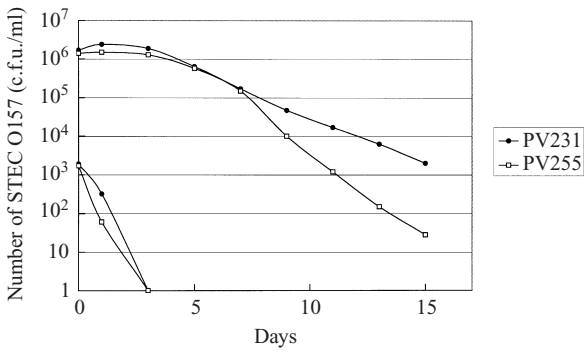

Fig. 1. Survival of STEC 157 in natural marine water. Two clinical strains of STEC O157, PV231 (●) and PV255 (□), were inoculated in freshly collected natural marine water and incubated at 27 °C for 1–15 days.

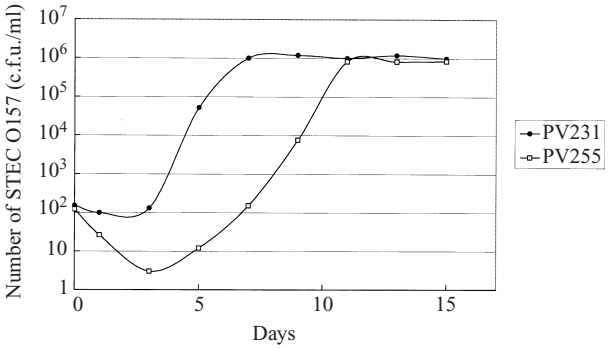

Fig. 2. Survival of STEC O157 in sterilized marine water. Two strains of STEC O157, PV231 (●) and PV255 (□), were inoculated in sterilized marine water and incubated at 27 °C for 1–15 days.

Halotolerance and survival of STEC O157. Five strains of STEC O157 were examined for halotolerance. Clinical strains of STEC O157:H7, namely, PV231, PV255, PV216, PV354 and PV435, were cultured overnight at 37 °C in 1% peptone water containing 0–8% NaCl.

To investigate the survival of STEC in natural marine water, marine water samples were obtained at 1-m depth, approximately 5 km offshore the Port of Osaka, kept at room temperature, and processed within 3 h. These marine water samples have a pH of 8·1 and contained 3% NaCl. Approximately 10^3 and 10^6 c.f.u./ml each of strains PV231 and PV255 were incubated in these marine water samples at 27 °C, which is the average natural temperature of marine water in summer, for 1–15 days. An aliquot of the marine water samples was inoculated onto plates of modified sorbitol MacConkey agar (CT-SMAC; Oxoid, Hampshire, England) containing 0·05 mg/l cefixim and 2·5 mg/l tellurous acid, incubated overnight at 37 °C; the colonies formed were then counted. Stx production by the cultured bacteria was determined using a reverse passive latex agglutination (RPLA) test (VTEC-RPLA; Denka Seiken Co. Ltd, Tokyo, Japan). PCR assay for the Shiga toxin gene (*stx*) was performed using the marine water samples in which no viable STEC was detected by culture.

In order to eliminate the influence of other organisms inherent in marine water, this was autoclaved and approximately 10^2 c.f.u./ml of the bacteria were inoculated in this water and incubated at 27 °C for 1–15 days. The culturable bacterial colonies were counted as described above.

Direct detection of the stx. We performed PCR for the *stx* and attempted to cultivate STEC in marine water samples collected from an estuary near the three rivers Aji, Kizu and Yamato, which drain into the Port of Osaka, from July to September in 1998 and 1999. A 1-l water sample was collected in a sterile bottle monthly at each sampling site (2–3 km offshore each estuary). The sample was obtained at 1 m depth. These water samples were kept at room temperature and processed within 5 h. The samples had a pH of 7·2–8·3 and contained approximately 2% NaCl. The bacteria from the 1-l water sample were trapped in 0·45 μm membrane filters and were cultured in 250 ml of mEC broth containing novobiocin (Kyokuto, Tokyo, Japan) for 15 h at 37 °C. The bacteria were concentrated in three different tubes using immunomagnetic beads coated with a O157-specific antibody (Dynabeads anti-*E. coli* O157; Dynal, AS, Oslo, Norway) [20] and the beads were resuspended in 0·1 ml of buffer containing 10 mM K_2HPO_4, 10 mM KH_2PO_4, 100 mM NaCl and 0·05% (w/v) Tween-20. The *stx* in the suspension was amplified by PCR. A commercially available primer set (EVC-1 and EVC-2; TaKaRa Biomedicals, Shiga, Japan), which recognized both stx_1 and stx_2, was used for screening. To differentiate between stx_1 and stx_2, commercially available primer sets (EVT-1 and EVT-2, EVS-1 and EVS-2; TaKaRa Biomedicals, Shiga, Japan) were used for amplification. The bacterial suspension was heated at 94 °C for 5 min. Five microlitres of the suspension was mixed with 0·5 μl each of the 20 mM dNTPs (Wako, Osaka, Japan), 0·5 μl each of the 19 pmol/μl primers, 1 μl of 2 U/μl Tth DNA polymerase (Toyobo, Osaka, Japan), 37·5 μl of distilled water and 5 μl of 10 × reaction mixture containing 15 mM $MgCl_2$, 800 mM KCl, 5 g/ml bovine serum albumin, 1% sodium cholate and 1% Triton X-100 in 100 mM Tris–HCl buffer (pH 8·9). Each PCR tube was heated as a predenaturation step at 94 °C for

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Table 1. *Detection of* stx_1 *and* stx_2 *from marine water from the Port of Osaka in the last 2 years*

	Detection of stx_1 and stx_2 by PCR*					
	River Aji estuary		River Kizu estuary		River Yamato estuary	
Year	stx_1	stx_2	stx_1	stx_2	stx_1	stx_2
1998						
July	—	—	—	—	—	—
August	2/3	3/3	—	3/3	—	—
September	—	—	—	—	—	—
1999						
July	—	—	—	1/3	—	—
August	—	—	—	—	—	—
September	—	—	—	—	—	—

* Samples originated from three points in the port area were examined for the *stx* by PCR with common primer pair in triplicates. (—) indicates to be negative for *stx* in the assay. The *stx*-positive samples were further examined for the stx_1 and stx_2 genes by the use of specific primer pairs to the respective genes. Numeral indicates positivity among triplicates.

1 min in a thermal cycler (Thermo processor TR-100; Taitec Co., Tokyo, Japan). Specific DNA was amplified in the cycler for 35 thermal cycles at 94, 55 and 75 °C for 30 sec each. The PCR products were analysed on a 1·5% agarose gel. A single band at 171 bp was determined to be positive for the *stx*. The *stx*-positive samples were further examined for stx_1 and stx_2 in the same manner. Single bands at 349 bp and 404 bp were determined to be positive for the stx_1 and stx_2, respectively.

Isolation of STEC O157. The isolation of STEC O157 from a 25 μl suspension of immunomagnetic beads in buffer was performed on CT-SMAC agar and sorbitol IPA bile agar (SIB; Kyokuto, Tokyo, Japan) plates. Approximately 50 colonies which do not ferment or slowly ferment sorbitol were confirmed by slide agglutination test using an O157 antiserum (Denka Seiken Co. Ltd, Tokyo, Japan).

In order to clarify whether STEC O157 is halotolerant, we examined the growth of the bacteria in the presence of NaCl. Three strains, PV216, PV255 and PV354, showed halotolerant growth in 6% NaCl in peptone water and two strains, PV231 and PV435, in 5% NaCl after overnight incubation at 37 °C. Since the concentrations of NaCl in the medium used for the halotolerance test are higher than those in marine water, STEC O157 may survive in natural marine water. To investigate whether STEC O157 can survive for a long term in the natural marine environment, two strains, PV231 and PV255, were inoculated at approximate 10^6 c.f.u./ml in natural marine water and incubated at 27 °C. When approximately 10^6 c.f.u./ml of the bacteria were inoculated, the number of culturable bacteria decreased, however, approximately 10–10^3 c.f.u./ml of STEC O157 were still culturable by the 15th day of incubation (Fig. 1). The bacterial production of Stx was confirmed by RPLA test (data not shown). Furthermore, we examined the bacterial growth in marine water in the absence of natural biological competitors, such as protozoae, using sterilized marine water. Both bacterial strains, PV231 and PV255, increased in number up to 10^6 c.f.u./ml 7–10 days after incubation (Fig. 2). The results above suggest that STEC may survive long-term exposure to marine water in the absence of biological competitors.

We attempted the detection of STEC O157 in natural marine water. Since we could detect the *stx* by PCR in the water samples collected from the estuary of the Port of Osaka near the rivers Aji and Kizu in August 1998, and River Kizu in July 1999, we further analysed the same positive samples for the presence of stx_1 and stx_2. The stx_1 was detected in samples collected from the River Aji estuary in 1998, and the stx_2 was detected in samples collected from the estuaries of rivers Aji and Kizu in 1998 and in samples collected from the River Kizu estuary in 1999 (Table 1, Fig. 3). We could not confirm the presence of culturable STEC O157 among the isolated colonies by the slide agglutination test (data not shown). This discrepancy between the presence of the *stx* and absence of culturable STEC is explained as follows: (1) The sensitivity of PCR assay (10^4 c.f.u./ml; data not shown) was sufficient to detect the *stx*, but the sensitivity of the culture method (10^5–10^6 c.f.u./ml; data not shown) was insufficient to isolate the

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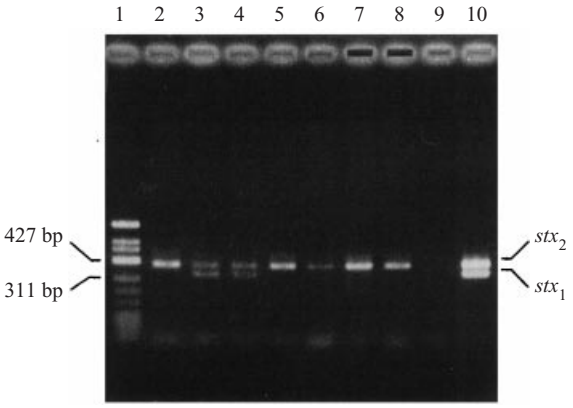

Fig. 3. Representative electrophoretograph of PCR products amplified using primer pairs for stx_1 and stx_2. To obtain the DNA bands corresponding to stx_1 and stx_2 in an agarose gel, 5 μl each of the PCR products of stx_1 and stx_2 were mixed with 4 μl of loading buffer. Ten microlitres of the final mixture was loaded on a 1·5% agarose gel and electrophoresed for 35 min at 100 V. Lane 1 contains molecular size markers (ø × 174RF I/*Hinf* I). Lanes 2–10 are specific PCR products amplified by primer pairs for stx_1 and stx_2; lanes 2–4 are samples from the River Aji estuary collected in August 1998. Lanes 5–7 are samples from the River Kizu estuary collected in August 1998 and lane 8 is the sample from the same place collected in July 1999. Lanes 9 and 10 are the negative and positive controls, respectively.

bacterium; (2) The beads may capture the sorbitol-fermenting STEC O157 [21] or viable but non-culturable (VNC) state STEC O157 [22]; and (3) The beads may also capture other O157 antigen-positive bacteria, such as the *stx*-positive *Citrobacter freundii* [23]. A small number, approximately 10^3 c.f.u./ml, of bacteria that were experimentally incubated in natural marine water could not be recovered as culturable bacteria (Fig. 1), and also the *stx* could not be detected from the water by PCR (data not shown) in this study. These results may indicate that the *stx*-positive marine water contained viable *stx*-positive bacteria.

We were not able to isolate STEC, however, the genes of *stx* are frequently detected in Japan. In order to clarify the origin of the *stx* in marine water, another survey of marine water for STEC and/or other *stx*-positive bacteria should be conducted. Since we did not perform the examination of VNC state bacteria in this study, the other study for VNC may be required in the survey. If STEC is isolated in the survey, we should determine whether the bacterium is present in routes of transmission to humans. When other bacteria bearing the genes of *stx* are isolated, they should be studied to determine if they are pathogenic to humans.

ACKNOWLEDGEMENTS

We thank Dr Y. Kusui, Mr N. Tanoda, Mr M. Matsuo, Mr K. Toguchi, Mr T. Yagi and Ms K. Mori of the Osaka Quarantine Station for their technical help, and Drs T. Kohno, T. Nakano, T. Goto and C. Morita of the Department of Microbiology, Osaka Medical College for their technical advice.

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