Geographical Features of Estuaries for Neritid Gastropods Including *Clithon retropictus* to Preserve Thermostable Direct Hemolysin-Producing *Vibrio parahaemolyticus*

Norichika H. KUMAZAWA, Kumiko HORI, Ken FUJIMORI¹⁾, Yoshito IWADA²⁾ and Akira SUGIYAMA²⁾

Tropical Biosphere Research Center, University of The Ryukyus, Nishihara, Okinawa 903-0213 and ¹⁾Taketomi, Okinawa 907-1541, and ²⁾Mie Prefectural Science and Technology Promotion Center, Institute of Public Health and Environment, Tsu, Mie 514-0003, Japan (Received 14 October 1998/Accepted 22 February 1999)

ABSTRACT. Thermostable direct hemolysin-producing strain of *Vibrio parahaemolyticus* was not detected from the alimentary tract of 7 neritid gastropods including *Clithon retropictus* at 9 estuaries of Southwest Islands in Japan in the present study. The strain has been detected from *C. retropictus* at 2 estuaries facing The Sea of Japan but not at 2 estuaries facing The Seto Inland Sea and The Pacific Ocean in Western Japan in our previous studies. In comparison with geographical features of the estuaries where the strain was detected and not, thick accumulation of muddy sediments at the riverbed and stagnation of brackish water at low tide seem to be essential for the strain to survive in neritid gastropods including *C. retropictus*.—**KEY WORDS:** geographical feature, neritid gastropod, *Vibrio parahaemolyticus*.

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Thermostable direct hemolysin(TDH)-producing strain of *Vibrio parahaemolyticus* is an organism which can cause acute gastroenteritis in human after consumption of various seafoods [2]. The strain was detected from muddy sediments and a neritid gastropod, *Clithon retropictus*, at Hashizu and Sada estuaries facing The Sea of Japan [4, 8]. The strain could attach to the surface pectin layer of estuarine diatom algae, *Navicula* spp. [3], so that the gastropod seems to ingest the strain with the microalgae. The strain was preserved in the alimentary tract of juvenile and adult *C. retropictus* at a level of 10^4 – 10^5 /g and 10^1 – 10^2 /g, respectively, and eliminated from marine neritid gastropods, *Nerita albicilla* and *Heminerita japonica*, within 4 days after ingestion [7, 9, 10]. Juvenile *C. retropictus* has an immature defense system [5, 11, 12]. From these evidences, the strain was suspected to overwinter in muddy sediments at estuaries, grow there with increasing water temperature, release to water, attach to the microalgae, enter the alimentary tract of juvenile *C. retropictus* with the microalgae and colonize there. The strain, however, was not detected from *C. retropictus* at Chigusa and Koso estuaries facing The Seto Inland Sea and The Pacific Ocean, respectively, in Western Japan [4]. Thus the estuaries where the gastropod could preserve the strain were suspected to be different from those where it could not preserve the strain in the geographical features.

C. retropictus inhabits estuaries south of the 38th parallel of latitude at the main land of Japan [16]. Several neritid gastropods including *C. retropictus*, *C. brevispina* and *C. sowerbianus* inhabit estuaries at the tropical and sub-tropical zones of East Asia including Southwest Islands in Japan [1] so that these gastropods were suspected to preserve TDH-producing strain there. The present study was carried out to detect the strain from the estuarine neritid gastropods in Southwest Islands using polymerase chain reaction (PCR) for *tdh* gene-specific nucleotide sequence. From our serial studies, geographical features of estuaries where neritid gastropods including *C. retropictus* could preserve the strain

are summarized here.

Seven neritid gastropods, *C. retropictus*, *C. brevispina*, *C. sowerbianus*, *Nerita squamulata*, *N. undata*, *Septaria porcellana* and *Neritina plumbea*, were collected at estuaries of 8 rivers in Okinawa and Iriomote Islands, Okinawa Prefecture, and at an estuary of Nagata river in Yakujima island, Kagoshima Prefecture, Japan. Samplings were done at low or falling tides. Salinity and temperature of water were measured at a depth of 10 cm with Salinometer S-28 (Shibuya, Japan) and thermometer, respectively. Fifty to 100 individuals of the gastropods were collected at each sampling site. Alimentary tracts were removed from the gastropods, pooled, homogenized in sterile artificial seawater with a salinity of 35‰ [9] using Polytron PT3000 (Kinematica, Switzerland) and examined the level of TDH-producing strains using a most-probable-number(MPN) method combined with PCR [15]. One ml aliquots from appropriate decimal dilutions of the homogenized samples in the artificial seawater were inoculated into three MPN tubes containing salt polymyxin broth (Nissui, Japan) and incubated at 37°C for 18 hr. MPN tubes showing visible growth were examined for the presence of *tdh* gene of *V. parahaemolyticus* by PCR [15] using two commercial primers, the forward primer VPD-1 and the reverse primer VPD-2 (Takara, Japan). The MPN broth culture was centrifuged at 3,500 rpm for 20 min. The precipitate was added by the distilled water to obtain the bacterial lysate. One ml of the lysate was heated at 90°C for 10 min and centrifuged at 12,000 rpm for 10 min to obtain the supernatant (bacterial lysate). The PCR assay (100 µl) contained amplification buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM MgCl₂), 200 µM of each deoxynucleotide, 0.19 pM of each primer, 0.025 U *Taq* DNA polymerase (Takara) and 10 µl of the bacterial lysate. The PCR involved an initial denaturation step of 2 min at 94°C followed by 35 amplification cycles, 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, in a thermal cycler, GeneAmp PCR System 9600 (Perkin Elmer). The PCR products were fractionated

on 1.5% agarose gels.

MPN broth cultures from which *tdh* gene was detected were plated on bromothymol blue lactose agar (Nissui, Japan) supplemented with 5% NaCl and 0.5% sucrose (pH 7.4) [8] and incubated at 37°C for 18 hr. Large green colonies grown on the agar plates were purified for single colonies and identified with *V. parahaemolyticus* as described previously [8].

Results of the surveys of TDH-producing *V. parahaemolyticus* in neritid gastropods at estuaries of Southwest Islands in Japan are shown in Table 1. TDH-producing strain was not detected from the gastropods, while control samples inoculated with TDH-producing strain D3 [8] revealed a clear band of the PCR product (251 bp). Water temperature was 18–32°C at estuaries in Okinawa Island and 26°C at estuaries in Iriomote Island. Salinity of water was 8‰ or less at some sampling sites, while it was high when the samplings were done at a falling tide. Some sampling sites were small pools appeared on the riverbed at low tide, where the salinity was sometimes high.

Geographical features of estuaries where TDH-producing strain was detected from *C. retropictus* in our previous studies [6, 8] were compared with those where it was not detected from neritid gastropods including *C. retropictus* in our previous and present studies (Table 2). Geographical features of estuaries where the strain was detected from *C. retropictus* were common in the presence of rocks, pebbles

or stakes in water for the gastropod to attach to eat microalgae there [3, 4], accumulation of muddy sediments at the bottom of the river and stagnation of brackish water with salinities of less than 20‰ at low tide. In contrast, at estuaries where the strain was not detected from neritid gastropods, riverbed was covered with sand or gravel and a little muddy sediment and exposed to fresh water at low tide.

TDH-producing strains have been detected from *C. retropictus* at Hashizu and Sada estuaries in our previous surveys [4, 8]. At Hashizu estuary, *C. retropictus* inhabits all over the estuary while the strain was detected from the gastropod only at the middle reaches of the estuary [8]. *C. retropictus* spends the veliger stage, settles and metamorphoses at the middle reaches of the estuary and then the juvenile ascends the river as it grows [1, 6, 16]. In fact, most of the gastropod found at the middle reaches of Hashizu and Sada estuaries were juveniles. TDH-producing strain could survive at a level of 10^4 – 10^5 /g and 10^0 /g in the alimentary tracts of juvenile and adult *C. retropictus*, respectively, maintained in ultraviolet-irradiated recirculating 20‰ artificial seawater [10]. Thus juvenile *C. retropictus* seems to become an important reservoir of the strain at estuaries in the adequate geographical conditions.

C. brevispina and *C. sowerbianus* are predominant neritid species at estuaries of Southwest Islands [1]. Juvenile *C. retropictus* is found a little in the mixed communities of

Table 1. Detection of TDH-producing *V. parahaemolyticus* from neritid gastropods at estuaries in Southwest Islands of Japan, using Polymerase chain reaction in 1996 to 1998

Sample	River of sampling ^{a)}	Date of sampling	Water temperature(°C)	Salinity of water (‰) ^{b)}	Number of TDH-producing strain/100g
<i>C. retropictus</i>	Benoki(O)	28 May '98	26	17	— ^{c)}
	Nagata(Y)	4 Aug.'97	24	0	—
<i>C. brevispina</i>	Ohkawa(O)	7 Nov.'96	26	30	—
	Ohura (O)	27 Nov.'96	23	7	—
<i>C. sowerbianus</i>	Benoki(O)	1 July'96	30	20	—
		7 Nov.'96	23	6	—
		6 Dec.'96	18	5	—
	Taiho (O)	7 Nov.'96	26	30	—
		6 Dec.'96	26	20	—
	Manna (O)	7 Nov.'96	30	0	—
		6 Dec.'96	18	5	—
	Ohura (O)	11 July'96	29	0	—
		29 July'96	31	15	—
	Tima (O)	29 July'96	32	7	—
		27 Nov.'96	24	2	—
	Okkubi(O)	27 Nov.'96	24	1	—
		11 Dec.'96	21	17	—
<i>N. squamulata</i>	Kuira (I)	4 Oct.'96	26	28	—
<i>N. undata</i>	Kuira (I)	4 Oct.'96	26	28	—
<i>S. porcellana</i>	Ohkawa(O)	7 Nov.'96	24	0	—
<i>N. plumbea</i>	Ohura (O)	11 Dec.'96	23	8	—

a) O, I and Y in parentheses represent Okinawa and Iriomote Islands, Okinawa Prefecture and Yakushima Island in Kagoshima Prefecture, respectively.

b) Samplings were done at low or falling tides.

c) Not detected.

Table 2. Geographical features of estuaries related with the detection of TDH-producing *V. parahaemolyticus* from neritid gastropods

	River (Prefecture)					
	Seven Rivers ^{a)} (Okinawa)	Kuira (Okinawa)	Hashizu ^{b)} (Tottori)	Sada ^{c)} (Shimane)	Chigusa ^{c)} (Hyogo)	Koso ^{c)} (Kochi)
Geographical feature						
Presence of rocks, pebbles or stakes in water	+	+	+	+	+	+
Thick accumulation of muddy sediment at the riverbed	—	+	+	+	—	—
Stagnation of brackish water at low tide	—~+	+ ^{d)}	+	+	—	—
Presence of <i>C. retropictus</i>	+ ^{e)}	—	+	+	+	+
Detection of TDH-producing <i>V. parahaemolyticus</i> from neritid gastropods	—	—	+	+	—	—

a) Ohkawa, Ohura, Benoki, Taiho, Manna, Tima and Okkubi rivers at Okinawa Island.

b) Reference[8].

c) Reference[4].

d) Salinity of water at the sampling site of *N. squamulata* and *N. undata* was high. Sampling was done on the ebb tide so that the sampling site seems to be located at the mouth of Kuira River facing Funauki Bay.

e) Two neritid gastropods, *C. brevispina* and *C. sowerbianus*, were seen more densely than *C. retropictus* at these estuaries.

these gastropods or hardly found out there. Adult *C. retropictus* is found at the freshwater areas above the estuaries. Thus, in the present study, juveniles of 7 neritid species were collected at estuaries of Southwest Islands. *C. retropictus* was collected only at Benoki and Nagata estuaries because it was not enough to be collected for our examinations at other estuaries. However, TDH-producing strain was not detected from these gastropods (Table 1).

Estuaries where TDH-producing strains were detected from *C. retropictus* [4, 6, 8] were characterized by the accumulation of muddy sediments and the stagnation of brackish water at low tide (Table 2). In fact, *V. parahaemolyticus* strains were detected at high levels from muddy sediments at Hashizu estuary even in winter [8]. Thus the sediments seemed to protect *V. parahaemolyticus* from cold stress. TDH-producing strain would overwinter in muddy sediments, grow there with increasing water temperature, release to water, attach to the microalgae, enter the alimentary tract of the gastropod with the microalgae and colonize there.

Estuaries where TDH-producing strain was not detected from the gastropods were shallow and based on rocky or pebbly riverbed so that the gastropods, microalgae and muddy sediments were exposed to fresh water at low tide (Table 2). Under the inconvenient geographical conditions, the halophilic TDH-producing strains would hardly survive on the microalgae, in muddy sediments or in the alimentary

tracts of the gastropods [9]. Thus the accumulation of muddy sediments and the stagnation of brackish water at low tide seem to be essential for TDH-producing strains to survive in *C. retropictus* and probably some other neritid gastropods.

Salinity of water was considerably high at some sampling sites (Table 1). Samplings were done sometimes at a falling tide, when the salinity was rather high. Some of the sites at Okinawa Island were small pools appeared on the riverbeds only at low tide. Sampling sites of Kuira River at Iriomote Island were the lower estuary facing Funauki Bay where seawater seemed to be scarcely diluted with river water even at low tide. Presence of predatory organisms including *Bdellovibrio* has been reported in seawater [13, 14]. Thus TDH-producing strains would be killed by the marine predatory organisms in the slightly diluted seawater.

TDH-non-producing strains were detected from *C. sowerbianus* at Benoki and Ohura estuaries of Okinawa Island (data not shown) and from *C. retropictus* at all estuaries examined at the main land of Japan [4, 8]. TDH-non-producing strains were detected from *C. retropictus* at all area of Hashizu estuary while TDH-producing strains were detected from the gastropod only at the middle reaches of the estuary [8]. From these evidences, geographical conditions of estuaries for the neritid gastropods to preserve TDH-producing strains seem to be more restricted than those to preserve TDH-non-producing strains.

Marine and estuarine samples are usually contaminated with high levels of TDH-non-producing strain so that many *V. parahaemolyticus* strains have to be isolated in order to detect TDH-producing strain quantitatively from these samples with the conventional MPN or colony counting methods. PCR could find out MPN broth cultures containing *tdh* gene-positive organisms from many MPN tubes contaminated with TDH-non-producing strains using rather simple operations. If one or more cultivable cells of TDH-producing strain would exist in each MPN tube, they would grow during the incubation. In fact, control samples contaminated with TDH-producing strain D3 showed a clear band of the PCR product (251 bp). TDH-producing strains could be isolated from the *tdh* gene-positive cultures effectively. Though the presence of *tdh* gene-positive organism other than *V. parahaemolyticus* in the aquatic environment is undeniable, the MPN method combined with PCR following the colony isolation and the test of TDH producibility of the isolated strains could estimate the level of TDH-producing *V. parahaemolyticus* in the environmental samples more effectively than the conventional quantifications.

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