An Estuarine Neritid Gastropod, *Clithon corona*, a Potential Reservoir of Thermostable Direct Hemolysin-Producing *Vibrio parahaemolyticus*

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ABSTRACT. An estuarine neritid gastropod, *Clithon corona*, maintained in UV-irradiated recirculating artificial seawater with a salinity of 15 per mil (‰) was found to retain thermostable direct hemolysin (TDH)-producing *Vibrio parahaemolyticus* in the gut at significantly higher levels than TDH-non-producing one for at least 14 days. Another estuarine neritid gastropod, *C. sowerbianus*, was not able to support the preferential survival of TDH-producing organisms. This evidence suggests that, if TDH-producing vibrios are brought to estuaries inhabited by *C. corona*, repeated ingestion of *V. parahaemolyticus* by this gastropod could lead to accumulation of TDH-producing vibrios in the estuaries.

KEY WORDS: *Clithon corona*, reservoir, *V. parahaemolyticus*.

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Vibrio parahaemolyticus is an autochthonous estuarine organism that can cause gastroenteritis in man after consumption of contaminated raw fish and shellfish [1, 5, 8]. *V. parahaemolyticus* was detected in its highest level, 10^4 – 10^5 colony forming units (cfu)/g, in *Clithon retropictus* (neritid gastropod), attached-microalgae and muddy sediments of Hashizu and Sada Estuaries in Japan [3, 5]. Thermostable direct hemolysin (TDH)-producing (enteropathogenic) vibrios were found to be approximately 20% of *V. parahaemolyticus* organisms isolated from *C. retropictus* at Hashizu Estuary, though the occurrence in muddy sediments and water there was 12% and 1%, respectively [5]. Another field survey at Sada Estuary found that TDH-producing vibrios grew in the gut of juvenile *C. retropictus* from a non-detectable level to 2.0×10^3 cfu/g (approximately 15% of *V. parahaemolyticus* organisms) in a two-month period between July and September 1991 [3]. TDH-producing vibrios survived at a level of 10^4 – 10^5 cfu/g maintained in UV-irradiated recirculating artificial seawater with a salinity of 20‰, while TDH-non-producing (non-pathogenic) vibrios survived there at a level of only 10^1 – 10^2 cfu/g [7]. *V. parahaemolyticus* can attach to estuarine diatom algae, *Navicula* spp. regardless of TDH-productivity [2]. This evidence suggests that *V. parahaemolyticus* would overwinter in muddy sediments at estuaries, grow there with rising water temperature, be released into the water column and attach to diatom algae, where it would be ingested by juvenile *C. retropictus*, thereby supporting selective growth of TDH-producing vibrios in the gut.

The range of *C. retropictus* includes many estuaries of the main land of Southern Japan [6], though *V. parahaemolyticus*-gastroenteritis has been reported throughout the world including Southeast Asia [8]. Two neritid gastropods, *C.*

corona and *C. sowerbianus*, predominate in the estuaries of the Southwest Islands (subtropical zone) of Japan, where *C. retropictus* seems to take refuge in freshwater areas above estuaries in competition with these gastropods [10]. A halophile, *V. parahaemolyticus*, could not survive in the gut of *C. retropictus* which inhabits freshwater areas. This raises the possibility that *C. corona* and *C. sowerbianus* are the reservoirs for TDH-producing strains of *V. parahaemolyticus* in the estuaries of tropical and sub-tropical zones.

In the present study, two TDH-producing and two TDH-non-producing strains of *V. parahaemolyticus* were investigated. The TDH-producing strains, D5 (O3:K6) and D12 (O3:K6), are nalidixic acid-resistant derivatives of clinical strains, while TDH-non-producing strains, R15 (O8:K41) and R13 (untypable), are rifampicin-resistant derivatives of environmental strains. The bacteria were grown overnight in Heart Infusion Broth (Eiken) supplemented with 3% NaCl at 37°C, washed with artificial seawater (ASW) [7] with a salinity of 35‰, and resuspended at 10^6 cfu/ml in ASW.

The two neritid gastropods, *C. corona* and *C. sowerbianus*, were collected from Benoki and Ohura rivers in Okinawa, Japan. Adult and juvenile specimens, 9–13 mm and 5–7 mm in shell height, respectively, were cleansed of halophilic organisms of the gut by placement in recirculating freshwater at room temperature for one week. They were then kept in ASW with test salinities of 15 and 25‰ for *C. corona* and *C. sowerbianus*, respectively, for one week to acclimatize them to the salinities of their natural habitats.

Fifty adults or 120 juvenile gastropods were incubated in ASW containing 10^6 cfu/ml of four *V. parahaemolyticus* strains. After 24 hr, the gastropods were washed with distilled water and maintained in UV-irradiated recirculating ASW at 28°C. They were fed on diatom algae grown on concrete blocks or stones. Five adults and 15 juveniles were sacrificed at appropriate time intervals and the guts attached

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Fig. 1. Survival of *V. parahaemolyticus* strains D5 (●), D12 (■), R13 (○) and R15 (□) in juvenile (a) and adult (b) *C. corona* with a salinity of 15‰ at 28°C.

Fig. 2. Survival of *V. parahaemolyticus* strains D5 (●), D12 (■), R13 (○) and R15 (□) in juvenile (a) and adult (b) *C. sowerbianus* with a salinity of 25‰ at 28°C.

to mid-gut glands were homogenized in sterilized 35‰ ASW with a Polytron PT3000 (Kinematica, Switzerland). Viable counts of the homogenates were estimated on Bromothymol Blue Lactose Agar (Eiken) plates supplemented with 5% NaCl and 5 µg/ml of appropriate selective drugs. Experiments were repeated three times and the results are presented as a geometric mean. Statistical significance was determined by T-test (SAS system for Windows V8).

TDH-producing strains, D5 and D12, survived at a level of 10^2 cfu/g or higher for at least 14 days in the gut of juvenile and adult *C. corona*, while TDH-non-producing strains R13 and R15 exhibited early decrease in survival (Figs. 1a and 1b). Levels of the TDH-producing strains were significantly higher than those of TDH-non-producing strains both in juveniles ($P < 0.01$) and adults ($P < 0.01$), upon removal of day 0 data and low-reliability data of 10^0 cfu/g or less. The results show that TDH-producing strains, via repeated passages through the gut of *C. corona*, could accumulate in estuaries containing this gastropod.

Levels of the test strains decreased gradually and reached 10^1 cfu/g or lower on day 14 in the gut of juvenile and adult *C. sowerbianus* (Figs. 2a and 2b), indicating the lack of selective survival of TDH-producing strains in the gut of *C. sowerbianus*.

V. parahaemolyticus strains were not detected from tank water from the second day of the experiments onward (data not shown), indicating that UV-irradiation eliminated the possibility of the gastropods to re-ingest *V. parahaemolyticus*.

The variable levels of the survival of these strains in gas-

Fig. 3. Survival of *V. parahaemolyticus* strains D5 (●), D12 (■), R13 (○) and R15 (□) in ASW with a salinity of 15‰ at 28°C. Levels of the organism are shown as relative number in order to compare the viability of the four strains in ASW.

tropods were concluded to be the results of interaction between *V. parahaemolyticus* strains and these gastropods, since in non-UV-irradiated 15‰ ASW (28°C) *V. parahaemolyticus* strains were confirmed to survive in a similar numbers for at least 21 days (Fig. 3).

TDH-producing strains were found to survive in the gut of *C. corona* at significantly higher levels than TDH-non-producing strains (Fig. 1), suggesting that TDH-producing strains would accumulate in estuaries where *C. corona* resides, through repeated passages of the strains in the gut of *C. corona*. In our previous studies, a TDH-producing strain, D3, was found to survive in the gut of a juvenile estuarine neritid gastropod, *C. retropictus*, at a level 100 times higher than TDH-non-producing strain R13 [7]. Based on this evi-

dence, TDH-producing strains were suspected to accumulate in estuaries containing juvenile *C. retropictus* [7]. In fact, TDH-producing strains were approximately 20% of *V. parahaemolyticus* organisms detected in juvenile *C. retropictus* in our field survey at Hashizu Estuary, though the rates in muddy sediment and water there were approximately 12 and 1%, respectively [5]. In estuaries, *C. corona* seems to accumulate TDH-producing strains in a manner similar to that by juvenile *C. retropictus*. TDH-producing strains grown in estuaries could flow out to the sea and contaminate fishing ports or fishing farms near the estuaries.

TDH-producing and TDH-non-producing organisms were eliminated slowly from another estuarine neritid gastropod, *C. sowerbianus* (Fig. 2), while the marine neritid gastropods, *Nerita albicilla* and *Heminerita japonica*, have been shown to eliminate *V. parahaemolyticus* strains within 7 days after ingestion [4, 6]. Thus, *C. sowerbianus* seems to retain *V. parahaemolyticus* strains for a longer period than marine neritid gastropods do even if they have no ability to accumulate TDH-producing vibrios. In our present study, gastropods were inhibited from re-ingesting excreted *V. parahaemolyticus* by UV-irradiation of the recirculating water. At estuaries where *C. sowerbianus* could repeatedly ingest *V. parahaemolyticus* strains, it appears that *C. sowerbianus* would retain *V. parahaemolyticus* organisms. The reason estuarine neritid gastropods are able to retain TDH-producing strains could not be explained in the present study. It might be related to the fact that *V. parahaemolyticus* is an estuarine organism. Estuarine invertebrates are likely to evolve to co-exist with other estuarine organisms, including *V. parahaemolyticus*. Such interactions may, over the long-term, have resulted in adaptation of TDH-producing organisms to survive in the gut of estuarine invertebrates.

Prior to the present study, estuarine neritid gastropods

were found to retain TDH-producing vibrios at similar levels to or at higher levels than TDH-non-producing ones. Neritid gastropods able to preserve TDH-producing strains at lower levels than TDH-non-producing strains have not yet been found, suggesting that TDH or the related molecule from *V. parahaemolyticus* seems to contribute to colonization in the gut of the gastropods. Mechanisms of the colonization will be investigated in the near future.

C. corona and *C. sowerbianus* are the dominant neritid gastropods in many estuaries of tropical and subtropical areas in East Asia including the Southwest Islands of Japan. Our data indicate that *C. corona* would be an important reservoir of TDH-producing vibrios in estuaries it inhabits.

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