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Survival of Kanagawa-positive strains of *Vibrio parahaemolyticus* in a brackish-water area

By N. H. KUMAZAWA and E. KATO

Department of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680, *Japan*

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SUMMARY

Vibrio parahaemolyticus was observed to overwinter in sediments and to be present in considerable numbers in sediments and *Clithon retropictus* (gastropod mollusc) during summer months at a brackish-water area along Hashizu Creek in Japan. The highest level of the organisms was $9{\cdot}3 \times 10^6$ and $2{\cdot}3 \times 10^7$/100 g in sediments and *C. retropictus* respectively.

Production of Kanagawa haemolysin was detected in approximately 12% and 20% of strains isolated from sediments and *C. retropictus* respectively at two stations in Hashizu Creek but were not detected at the other three stations. Two haemolysin-producing strains were isolated from water samples but none were isolated from *Corbicula japonica* (bivalve mollusc). These findings suggest that haemolysin producers are preserved principally in sediments and some shellfish in the brackish-water areas with restricted salinity conditions.

INTRODUCTION

Vibrio parahaemolyticus is an estuarine organism which can cause gastro-enteritis after consumption of contaminated sea-foods. Wide surveys on the ecology of the organism at Chesapeake Bay in the United States by Kaneko & Colwell (1973, 1978) revealed that the organism survived the winter and proliferated in estuarine sediments in parallel with rising water temperatures, and that some of the organisms in the estuary were associated with zooplanktons. The organism was also detected in various coastal animals, including molluscs (Ayres & Barrow, 1978; Baross & Liston, 1970; Baross, Liston & Morita, 1978; Earle & Crisley, 1975; El-Sahn, El-Banna & El-Tabey Shehata, 1982; Kuroda & Machida, 1976; Nakamura, Machida & Ishizaki, 1977) and crustaceans (Baross, Liston & Morita, 1978; Osakabe, Yamazaki & Kodama, 1973) in low levels. However, most of the strains isolated from these animals gave negative results in the Kanagawa test and were thus different from clinical isolates. Kanagawa-positive strains were detected among the environmental isolates in a frequency of only 10^{-2} or less (Ayres & Barrow, 1978; Sakazaki *et al.* 1968; Sutton, 1974; Wagatsuma, 1974). Attempts to show high accumulations of Kanagawa-positive organisms in living animals and the environment have been unsuccessful.

In the present study we detected high levels of *V. parahaemolyticus*, including

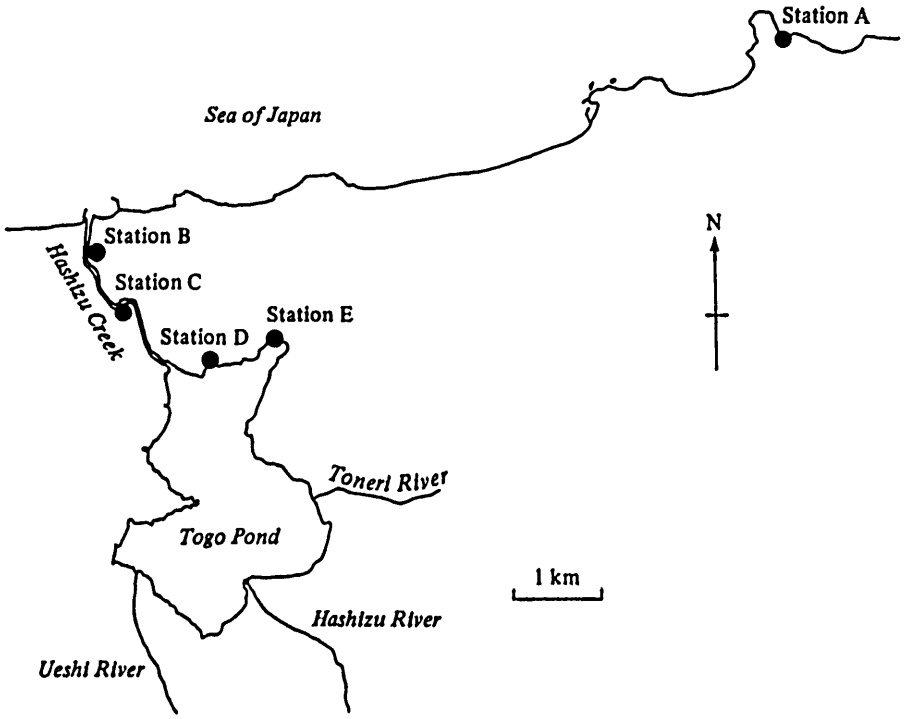

Fig. 1. Stations sampled at a coast of the Sea of Japan and a brackish-water area along Hashizu Creek and Togo Pond in Tottori Prefecture, Japan.

high rates of Kanagawa haemolysin producers from sediments and *Clithon retropictus* collected at Hashizu Creek, Japan, in the summer months.

MATERIALS AND METHODS

Sampling

Monthly sampling was carried out from September 1982 to September 1983 at five stations located on a coast facing the Sea of Japan and in a brackish-water area along Hashizu Creek and Togo Pond in Tottori Prefecture, Japan, as shown in Fig. 1. Togo Pond is a lagoon 4·2 km^2 in area connected with the Japan Sea via Hashizu Creek. Bottom sediments at stations B to E are composed of thick black muds, while station A is on a rocky shore without visible deposits of muddy sediments.

Monodonta labio, a marine gastropod mollusc, was collected at Station A. *Clithon retropictus*, a gastropod mollusc inhabiting the brackish-water area, and *Corbicula japonica*, a bivalve mollusc in the same habitat, were collected at stations B–E. Twenty to 100 specimens of each mollusc were collected at each station. Water samples were collected in glass bottles from a depth of 10 cm below the surface of the water, and samples of sediment were collected from the surfaces of the sediments, at each station. All samples were collected with aseptic precautions and were brought to the laboratory in a cool box at 15 °C or below for examination within 5 h of collection.

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Table 1. *Environmental parameters of water samples*

Station	Range of temperature (°C)	Salinity ‰ Mean	Salinity ‰ Range	Range of pH
A	10·0–28·0	34·0	26·4–41·0	8·0–8·4
B	6·5–28·4	8·9	1·6–18·4	7·2–8·7
C	5·0–29·5	6·5	2·0–14·5	7·1–8·9
D	5·6–31·6	4·2	1·3–8·7	7·1–9·1
E	5·0–31·7	4·5	1·3–8·3	7·3–9·2

Measurement of environmental parameters

The water temperature was measured at a depth of 10 cm. Salinity and pH were measured with an Iwaki Glass pH/ion meter, model 225.

Viable counts of V. parahaemolyticus

V. parahaemolyticus was isolated on Bromothymol-blue-lactose (BTB) agar plates (Nissui) supplemented with 5% NaCl and 1% sucrose at pH 7·4 (modified BTB agar), because this medium had been shown to be more effective than other selective media for recovering *V. parahaemolyticus* strains exposed to low temperatures.

Viable counts of *V. parahaemolyticus* in 100 g of mollusc or sediment were determined by a three-tube most-probable-number (MPN) method. One ml aliquots from appropriate decimal dilutions of homogenized mollusc soft tissue or sediment in Salt Polymixin Broth (SPB Nissui) were inoculated into MPN tubes with 10 ml of SPB and incubated at 37 °C for 18 h. SPB MPN tubes showing visible growth were subcultured to modified BTB agar plates at 37 °C for 18 h. Two large green colonies from each plate were purified by streaking for single colonies and identified as described below.

Viable counts of *V. parahaemolyticus* in 100 ml water samples were determined by filtration through membrane filters (Sartorius SM114) of 0·45 μm pore size and 47 mm diameter which were then mounted on the modified BTB agar plates and incubated at 37 °C for 18 h. Any large green colonies were purified and formally identified.

Isolates were identified as *V. parahaemolyticus* by the following characters: motile Gram-negative rods giving good growth on peptone agar containing 3% and 8% NaCl but no growth on peptone agar without added NaCl or with 10% NaCl; cytochrome oxidase and catalase positive; β-galactosidase negative, giving acid from glucose and mannitol but not from sucrose, lactose, rhamnose, dulcitol, inositol or salicin; neither H_2S nor visible gas produced in Triple Sugar Iron Medium (Nissui); Voges–Proskauer reaction negative; lysine and ornithine decarboxylase positive; arginine dihydrolase negative; indole produced in Lysine Indole Motility Medium (Nissui); bioluminescence negative.

Tests for production of Kanagawa haemolysin

The Kanagawa phenomenon was demonstrated by a clear zone of haemolysis around a lawn of test organism on Modified Wagatsuma Agar (Eiken) with 5% washed human erythrocytes, incubated for 24 h at 37 °C.

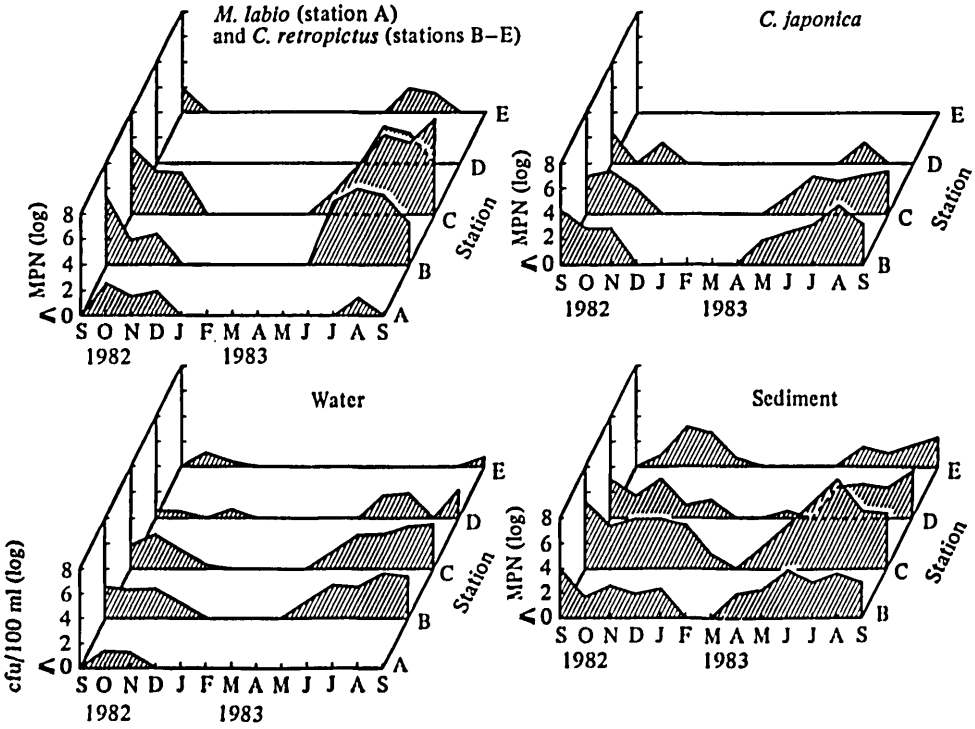

Fig. 2. Seasonal and regional variations of *V. parahaemolyticus* at five stations.

An immunological test was followed by a modified immunodiffusion method of Honda *et al.* (1980). Test organisms were inoculated in a single line on Biken agar containing 4 % NaCl, 3 % Na_2HPO_4, 3 % peptone, 0·5 % glucose and 1·5 % Noble agar (Difco) at pH 7·3 and incubated overnight at 37 °C. Anti-Kanagawa haemolysin rabbit serum absorbed on filter-paper strips (Toyo, no. 424, 4·0 mm in width) was then placed on the agar plate in parallel with the test colonies at a distance of about 5 mm from them. The plate was kept at room temperature overnight and examined for precipitin line formation.

RESULTS

Environmental parameters

Environmental parameters of water samples are shown in Table 1. Water temperatures at the four brackish-water stations fluctuated in parallel with each other, but the variations were more than at coastal station A. The area is shallow, so a significant difference in temperature between surface and bottom water was not observed.

Mean salinities of water samples in the four brackish-water stations were 4·2–8·9 g/litre, corresponding to 12–26 % of the values at station A. Values of pH of water samples were stable at station A, while those in the four brackish-water stations fluctuated irregularly but in parallel with each other.

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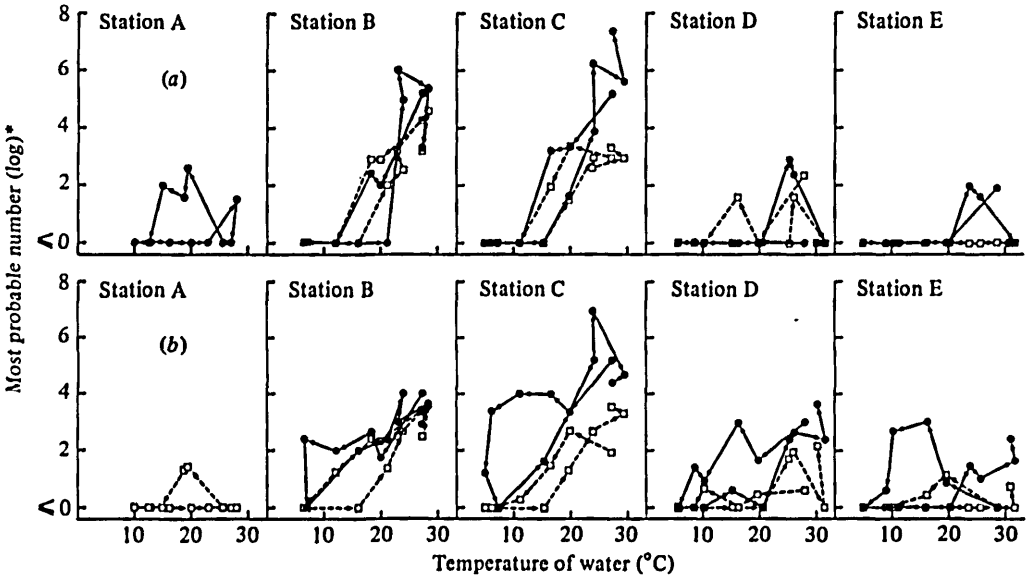

Fig. 3. Annual cycles of *V. parahaemolyticus* related to water temperature at five stations. (*a*) *M. labio* (station A) and *C. retropictus* (stations B–E) (●——●); *C. japonica* (□-----□); (*b*) sediment (●——●) and water (□·····□). * Bacterial number in water represented as colony forming units per 100 ml.

Seasonal and regional variations of V. parahaemolyticus

Seasonal and regional variations of viable counts per 100 g or ml of *V. parahaemolyticus* in molluscs, water and sediments collected at five stations are shown in Fig. 2. The organism was detected in *M. labio* at station A at levels of only 10^2 or less. However, levels of more than 10^4 were detected in *Cl. retropictus* at stations B and C in summer, with the highest levels of $2{\cdot}3 \times 10^7$ at station C and $9{\cdot}3 \times 10^5$ at station B. Levels found in water and in *Co. japonica* varied in a manner similar to those in *Cl. retropictus* with highest counts of $4{\cdot}3 \times 10^4$ in *Co. japonica* and $3{\cdot}8 \times 10^3$ in water recorded at station B.

The organism was detected in sediments over longer periods than in molluscs or water at the four brackish-water stations. At station C sediments gave more than 10^3 per 100 grams except in February and March 1983, with the highest count $9{\cdot}3 \times 10^6$ being recorded in July 1983. Annual variation of counts in sediments at stations B, D and E resembled those at station C but with levels of 10^3 or less.

In every month the highest counts were obtained at stations B and C; the counts decreased, and increases in counts occurred later, with increasing distance from these stations.

Annual cycles of V. parahaemolyticus *related to water temperature*

Annual variation of *V. parahaemolyticus* with water temperature is shown in Fig. 3. At station C levels of the organism in molluscs and water decreased in parallel with a fall in water temperature in late autumn and winter, while counts in sediments held relatively constant at approximately 10^4/100 g until the water temperature fell to 6 °C, when the organisms became undetectable. The critical temperature for the reappearance of the organism in spring was about 16 °C in

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Table 2. *Production of Kanagawa haemolysin from* Vibrio parahaemolyticus *strains isolated from various samples collected at a coast of the Sea of Japan and a brackish-water area along Hashizu Creek and Togo Pond as determined by Kanagawa phenomenon and immunological test*

Source of bacterial strain	Station	Kanagawa phenomenon		Immunological test	
		+	−	+	−
Monodonta labio	A	0*	9	0	9
Clithon retropictus	B	0	20	4	16
	C	0	24	5	19
	D	0	8	0	8
	E	0	9	0	9
Corbicula japonica	B	0	24	0	24
	C	0	24	0	24
	D	0	9	0	9
Sediment	B	0	32	4	28
	C	0	34	4	30
	D	0	27	0	27
	E	0	22	0	22
Water	A	0	4	0	4
	B	0	53	0	53
	C	0	31	1	30
	D	0	19	1	18
	E	0	9	0	9

* Number of bacterial strains.

sediments and 20 °C in molluscs and water. At any temperature there were more organisms present in autumn, when the counts were decreasing, than in spring, when they were increasing. Counts at stations B and C were always at their highest in summer, but the counts at the other three stations varied, even in summer.

Production of Kanagawa haemolysin from V. parahaemolyticus *strains*

A total of 358 strains of *V. parahaemolyticus* isolated in our study was examined for production of the Kanagawa haemolysin by demonstration of the Kanagawa phenomenon and by an immunological test. The results are summarized in Table 2. All strains were negative in the Kanagawa phenomenon in which all strains showed weak or no haemolysis, but 19 strains gave positive reactions in the immunological test. All but one of the haemolysin producers were isolated at stations B and C. At these sites, 9 of 44 strains isolated from *Cl. retropictus* and 8 of 66 strains from sediments produced haemolysin, as did one strain isolated from water. One haemolysin producer was isolated from water at station D. No haemolysin producers were isolated from *Co. japonica* or *M. labio.*

Seasonal variation in isolation of haemolysin producers is shown in Table 3. Haemolysin producers were isolated from *Cl. retropictus* more or less at any time when non-producers could be found.

In addition, 589 strains of *V. parahaemolyticus* were isolated from *Cl. retropictus*

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Table 3. *Seasonal and regional variations of Kanagawa haemolysin-producing* V. parahaemolyticus *as determined by immunological test*

Source of bacterial strain	Station*	Date of sampling: 1982 Sept.	Oct.	Nov.	Dec.	1983 Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.
Monodonta labio	A	—†	0/3‡	0/2	0/3	—	—	—	—	—	—	—	0/1	—
Clithon retropictus	B, C	2/6	2/6	2/6	—	—	—	—	—	0/3	1/6	0/6	1/6	1/6
	D, E	0/3	—	—	—	—	—	—	—	—	0/6	0/5	0/3	—
Corbicula japonica	B, C	0/6	0/6	0/6	—	—	—	—	—	0/6	0/6	0/6	0/6	0/6
	D, E	0/3	—	0/2	—	—	—	—	—	—	—	0/2	0/2	—
Sediment	B, C	0/6	0/6	0/6	0/6	0/4	0/2	—	2/5	2/6	0/6	1/6	2/6	1/3
	D, E	0/3	0/6	0/6	0/5	0/4	—	—	0/2	—	0/6	0/5	0/6	0/6
Water	A	—	0/3	0/1	—	—	—	—	—	—	—	—	—	—
	B, C	0/9	1/9	0/9	0/9	0/2	—	—	0/5	0/7	0/9	0/7	0/9	0/9
	D, E	0/3	0/6	0/3	0/4	—	—	—	—	—	0/1	1/2	0/3	0/6

* Data from stations B and C and those from stations D and E were combined because environmental parameters and incidence of the haemolysin producers were similar.

† No strain was isolated.

‡ Number positive/number tested.

collected at station C during October 1983. Of these, 134 (22·8%) were shown to produce the haemolysin by the immunological test while all strains again proved negative by the Kanagawa phenomenon.

DISCUSSION

Annual cycles of *V. parahaemolyticus* in molluscs, water and sediments, and production of Kanagawa haemolysin by the strains isolated were investigated along part of a coast of the Sea of Japan and of a brackish-water area along Hashizu Creek and Togo Pond in Japan. The results suggest that the organism proliferates in Hashizu Creek with rising water temperature and is carried up and down stream on the flood and ebb tides. Upstream, dilution with fresh water, and downstream, the increasing salinity of the sea, both lead to reduced proliferation of the vibrios.

In autumn, when bacterial counts were decreasing, the counts were nevertheless higher than those found at similar water temperatures in spring, when the counts were increasing. Direct temperature-dependence (Baross & Liston, 1970; Baross, Liston & Morita, 1978; Sutton, 1974) therefore does not completely describe the behaviour found.

Approximately 20% of strains isolated from *Cl. retropictus* and 12% of strains from sediments at stations B and C were shown by immunological methods to produce Kanagawa haemolysin, while no strain isolated from *Co. japonica* produced it. One strain from a water sample at station D also produced the haemolysin. No other strains isolated at stations A, D or E produced haemolysin, and it is possible that the one strain which did so had been washed down from Hashizu Creek. Haemolysin production seems to be associated with the restricted salinity of the brackish-water areas, and perhaps with specific molluscs. Vibrios which do not produce haemolysins seem to have a wider distribution.

Variations of the organism in sediments were characteristic. Sediments at stations B and C held levels of 10^2 or more vibrios/100 g until the water temperature fell to the lowest level. As haemolysin producers, however, were not detected from these sediments in autumn or winter, patterns of haemolysin production resembled those found in isolates from *Cl. retropictus*.

Most organisms isolated from oysters and clams have been reported to be negative for the Kanagawa phenomenon (Sutton, 1974; Wagatsuma, 1974). No strain isolated from *Co. japonica* or *M. labio* in our study produced haemolysin. Thus, most molluscs inhabiting coastal and estuarine areas do not normally carry high levels of haemolysin producers, although, exceptionally, environmental disturbance might temporarily allow them to sustain growth of these strains.

Kanagawa haemolysin producers isolated from *Cl. retropictus* and from sediments were different from clinical isolates in that isolates from clinical cases normally give clear zones of haemolysis on Wagatsuma agar (Honda *et al.* 1980) while the isolates described here were weak producers detected by immunological methods only. The strong producers showing clear haemolysis may be preserved in the environment in ways as yet undetermined, or may be selected variants of the weak producers more commonly isolated from environmental sources.

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