Distribution of *Vibrio* Species Isolated from Aquatic Environments with TCBS Agar

Hidekazu UCHIYAMA

Department of Public Health, School of Allied Health Sciences, Kitasato University, Kanagawa

Abstract

Environmental bacteria grown on TCBS agar plates (TCBS strains) were investigated for the presence of *Vibrio cholerae* in aquatic environments. TCBS strain counts were 0.01 ~ 0.001 times the total viable counts in pairs of the same samples. The TCBS strains were of two types which required NaCl (salt strain) and did not require NaCl (non-salt strain) to grow in peptone water. Non-salt strains made up 85.3 ~ 92.1% of TCBS strains isolated from river water. TCBS strains isolated from an estuary contained 40.9% of non-salt strains and 57.4% of salt strains. Salt strains made up 69.2 ~ 86.8% of TCBS strains isolated from seawater. The percentages of *Vibrio* species in TCBS strains were 11.9 ~ 47.9%. *V. alginolyticus* and *V. parahaemolyticus* were isolated from seawater. *V. vulnificus* was only isolated from estuary water. *V. cholerae* non-O1 was isolated from both river water and estuary water which had low salinity. *V. fluvialis* was isolated from all three aquatic environments. This investigation suggests that *Vibrio* species were present in each sample station and that *V. cholerae* existed in river water.

Key words: *Vibrio cholerae*, ecology, river, sea, TCBS

Introduction

Vibrio species exist in aquatic environments such as rivers, ponds, wells and the sea. *Vibrio* species include pathogenic vibrios¹⁾. *V. cholerae*, *V. parahaemolyticus* and other vibrios were isolated from estuary and seawater in a bay, and there was a suggestion that pathogenic vibrios were autochthonous bacteria²⁾. Infectious diseases due to pathogenic vibrios have occurred from polluted seafood and polluted seawater in a bay and along a coast^{3, 4)}. Therefore, it is necessary to clarify the ecology of pathogenic bacteria in nature to prevent infectious diseases from the natural environment.

V. cholerae grow in peptone water without NaCl, therefore the organism does not require NaCl for growth in peptone water. *V. parahaemolyticus* is a halophilic bacteria and can not grow in peptone water without NaCl; the organism grows in peptone water with 3% NaCl. *Vibrio* species members have different NaCl requirements and require, or tolerate, various concentrations of NaCl in peptone water to grow. Therefore, *Vibrio* species can be isolated from river water, estuary water and seawater with thiosulfate citrate bile salts agar (TCBS agar). The author investigated the relationship between the NaCl

requirement and the distribution of *Vibrio* species in various aquatic environments.

Materials and Methods

1. Sampling sites

Water samples were collected from the stations shown in Fig. 1. The river surface water samples were collected from the midstream of Sagami River (Showa-hash, station 1), the estuary of the river (Banyu-hash, station 2) and from the midstream of Sakai River (Negishi-hash, station 3). The Sagami River flows through the center of Kanagawa Prefecture and its water is supplied as drinking water. The Sakai River is polluted by sewage drains. Stations 1 and 3 differed in their water quality. Water samples were collected with a 500 ml sterile Heyroht water sampler (Shibata scientific technology Ltd.) and the same sampler was used several times for one collection. The period of river water sampling was from June to October, 1991 and samples were collected about once a month. Seawater samples were taken from the surface (0 m depth) from five stations of Tokyo Bay and two stations of Sagami Bay in sterile glass bottles. These sampling stations were located in various parts of Tokyo Bay (longitude and latitude ; station 4 : 35° 34'N 139° 55'E, station 5 : 35° 30'N 139° 51'E, station 6 : 35° 26'N 139° 45'E, station 7 : 35° 21'N 139° 42'E, station 8 : 35° 03.8'N 139° 31.7'E) and Sagami Bay (longitude and latitude ; station 9 : 35° 00.5'N 139° 23.0'E, station 10 : 34° 55.8'N 139° 24.9'E). The seawater samples were

Received Dec. 17 1998/Accepted Sep. 20 1999

Reprint requests to: Hidekazu UCHIYAMA,

Department of Public Health, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Sagami-hara, Kanagawa 228-8555, Japan

TEL:+81(42)778-8073 (Laboratory) FAX:+81(42)778-9628 (Faculty office)

Fig. 1 Stations sampled in River, Estuary and Bay areas.

collected between August 30 and September 2, 1993.

2. Collection of samples

As soon as river water was collected, various volumes (5, 10, 20, 200 ml) were filtrated with a membrane filter (Millipore filter HA; pore size $0.45 \mu\text{m}$, 47 mm diameter). The membrane filters were placed onto alkaline peptone water (APW; peptone: Daigo 10g, NaCl 5g, double distilled water 1000 ml, pH 8.6) plates (APW plus agar 15g per 1000 ml) and kept at air temperature for about 2 h while being transported to the laboratory. This was done because stressed cells, due to unfavorable conditions, if any, can be resuscitated by this procedure⁹⁾. The membrane filters were transferred from APW plates to TCBS agar plates in the laboratory⁹⁾. The river water samples in full 2 liter bottles to test water quality and count bacteria in the aquatic environment were transported to the laboratory and cooled with ice after collection.

The seawater samples were filtrated with a membrane filter (nuclepore filter, GE Co., pore size $0.40 \mu\text{m}$) on a vessel immediately after collection. A polycarbonate filter was used because it is routinely used and polycarbonate filters are better than cellulose filters for counting bacteria⁶⁾. The filters were placed onto APW plates for 1h, and then transferred to TCBS agar (Eiken) plates. These membrane filters were held at 20 °C for 1 ~ 3 days on the vessel and transported to the laboratory at air temperature. It was assumed that the number of *Vibrio* species isolated from river water and seawater was not influenced by either filters, because the *Vibrio* species are large enough to be held by both filters.

3. Organisms grown on TCBS agar plate (TCBS strains)

The membrane filters on APW plates were transferred to TCBS agar plates and incubated at 30 °C for 24 ~ 48 h in the laboratory in the case of river water. However, the membrane filters on the TCBS agar plates were kept at 20 °C for 48 h in the laboratory in the case of seawater until colonies on the TCBS agar plates were observable. TCBS strain counts were made using a plate colony count method on the TCBS agar plates and

calculated per 100 ml sample volume. Twenty to 30 colonies on the TCBS agar plates were randomly picked and these were pure cultured on a TCBS agar and *Vibrio* agar medium (VAM: peptone 10g, 'Lab-Lemco' powder:oxid 3g, yeast extract: Difco 3g, NaCl 5g, agar 15g, double distilled water 1000 ml, pH 7.5). These strains were stored in VAM, and later, identified.

4. Identification of TCBS strains

TCBS strains isolated from seawater were pure cultured, stored and identified by a medium to which was added artificial sea salt (NaCl 30g, KCl 3g, $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$ 3g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1g, per 1000ml), except for the TCBS agar. Stored TCBS strains were precultured with VAM at 36 °C overnight. These strains were identified at the genus level by the following tests: gram reaction, morphology, spore, catalase, oxidase, oxidation-fermentation test, gas from glucose, nitrate reduction, motility, indole, H_2S production and growth in peptone water with 0, 3, 7, 10% NaCl⁷⁾. Additional tests used to identify *Vibrionaceae* were as follows; Voges-Proskauer test, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase in Moeller's medium, gelatinase, O/129 sensitivity, acid from sucrose, mannitol, inositol, arabinose, and *d*-tartaric acid utilization⁸⁾.

5. Organism counts in aquatic environments

Total viable counts (TVC) in aquatic environments were made by a plate colony count method with standard method agar (SMA; Nissui). The river water samples were diluted with 0.85% NaCl solution, and spread on SMA plates. These media were incubated at 36 °C for 24 h. The estuary samples were spread on SMA and on SMA with artificial sea salt (SMA-s). The TVC in the estuary samples was made with either colonies on SMA or SMA-s. The TVC in the seawater samples was made on SMA-s. The seawater samples were spread on the medium in the vessel. The medium was then stored at 20 °C for 1 ~ 3 days, transferred to air temperature, and then incubated at 36 °C for 12 h in the laboratory.

6. Water quality examination methods

Air temperature was measured with a mercury thermometer. Water temperature was measured with a Pettenkoffer's water-thermometer. pH was measured by colorimetry. The amount of ammonia nitrogen (NH_4^+) was measured using the Nessler method and phosphate ions (PO_4^{3-}) were measured using an ammonium molybdate method. The chlorine anion (Cl^-) was measured using the selective electrode method and electrical conductivity (EC) was measured with an electrical conductivity meter. The salinity concentrations were measured using a refractometer (ATAGO ; S/Mill) with a scale of 1‰. The water quality of bay water was measured for only salinity with a refractometer.

Results

1. Water quality

Table 1 shows the water quality of the 3 river stations. Fluctuations in various environmental parameters at station 1 were compared to other stations. The water quality at station 3 underwent a change due to a dirty drain flow. At station 2, salinity underwent a change with the change of volume of seawater and fresh water. Salinity was converted from a concentration of Cl^- and the salinity at station 1 was 0.005 ~

0.02‰, station 3 0.04 ~ 0.11‰, and station 2 0.04 ~ 31.3‰. The salinities of the bay waters were 26 ~ 33‰.

2. TVC and TCBS strain counts

The number of bacteria per 100 ml water are shown in Table 2. This ratio reflects a selection of bacteria from the TCBS agar. TCBS strain counts were 0.01 ~ 0.001 times that of TVC in the aquatic environments. TCBS strain counts fluctuated widely in the estuary samples, but there were few changes in Tokyo Bay and Sagami Bay.

3. NaCl requirement of the TCBS strain

There were two types of TCBS strains, those which required NaCl (salt strain) and those which did not require NaCl (non-salt strain) to grow in peptone water. Table 3 shows the rate of salt strain and non-salt strain TCBS strains isolated from each station. At each station, 1.8 ~ 24.6% of the TCBS strains did not grow in peptone water with various concentrations of NaCl added, and these strains needed not only peptone, but also other nutrition. The rate of the non-salt strain was 85.3 ~ 92.1% for

the TCBS strains isolated from river water, 40.9% for TCBS strains isolated from estuary water and 6.2 ~ 7.0% for TCBS strains isolated from seawater in the bay. The TCBS strain isolated from river water contained many non-salt strains. On the other hand, the rates of salt strain in the TCBS strains were 2.0 ~ 10.1% from river water, 57.4% from estuary water and 69.2 ~ 86.8% from seawater in the bay.

4. Vibrionaceae in aquatic environments

Table 4 shows the results of the identification of TCBS strains. The TCBS strains were *Vibrionaceae*, *Acinetobacter*, *Enterobacteriaceae*, *Alcaligenes* and others. The rates of *Vibrionaceae* in the TCBS strains were 26.6 ~ 38.6% from river water, 71.0% from estuary water, and 36.9 ~ 47.4% from seawater in the bay.

5. Vibrio species in aquatic environments

Table 5 shows the number of *Vibrio* species and others in the genus *Vibrionaceae*. The rates of *Vibrio* species in the TCBS

Table 1 Ranges of various environmental parameters at the three river stations¹⁾

Parameters ²⁾	Sagami River	Sakai River	Estuary
Air temperature (°C)	21.0 ~ 36.0 ³⁾	21.2 ~ 35.0	22.0 ~ 36.6
Water temperature (°C)	15.2 ~ 22.4	18.4 ~ 27.1	17.8 ~ 29.9
pH	7.4 ~ 7.6	7.3 ~ 7.6	7.4 ~ 7.6
E.C. (μ S/cm)	112.4 ~ 154.0	235.0 ~ 467.0	142.5 ~ >2000
Cl ⁻ (mg/l)	3.3 ~ 12.0	23.0 ~ 64.0	25 ~ 19000
NH ₄ ⁺ (mg/l)	0.1	1.0 ~ 10.0	0.5 ~ 3.0
PO ₄ ³⁻ (mg/l)	0.05 ~ 0.1	0.1 ~ 1.0	0.1 ~ 0.5
TVC (log CFU/100ml)	5.5 ~ 6.2	5.6 ~ 7.6	4.1 ~ 6.2
TCBS strains (log CFU/100ml)	1.4 ~ 3.0	3.4 ~ 3.7	2.3 ~ 4.7

1) The period of river water sampling was from June to October, 1991.

2) Abbreviations: E.C., electric conductivity; Cl⁻, chloride ion; NH₄⁺, ammonia nitrogen; PO₄³⁻, phosphate ion; TVC, total viable count; TCBS strains, total count of colonies on TCBS agar plates.

3) Minimum ~ maximum values.

Table 2 Microbes in river and sea water

Assay	Number of microbes in each sampling area				
	Sagami River (n = 6) ²⁾	Sakai River (n = 6)	Estuary ¹⁾ (n = 7)	Tokyo Bay (n = 4)	Sagami Bay (n = 2)
TVC ³⁾ (log CFU/100ml)	5.8 ± 0.22 ⁴⁾	6.9 ± 0.66	5.5 ± 0.64	4.7 ± 0.22	5.5 ± 0.20
TCBS strains ⁵⁾ (log CFU/100ml)	2.6 ± 0.54	3.5 ± 0.14	3.4 ± 0.86	2.9 ± 0.11	2.4 ± 0.08
Rate ⁶⁾	-3.3 ± 0.61	-3.5 ± 0.66	-2.2 ± 1.60	-1.8 ± 0.30	-3.1 ± 0.10

1) Sagami River.

2) Number of samples paired with TVC and TCBS strains.

3) Total viable count.

4) Mean ± standard deviation.

5) Total count of colonies on TCBS agar plates.

6) log TCBS strains/TVC.

Table 3 Growth in peptone water containing 0% and 3% NaCl

Growth at NaCl ¹⁾		Rate of TCBS strains grown from the following areas					
		Sagami River (n = 6) ³⁾	Sakai River (n = 6)	Estuary ²⁾ (n = 8)	Tokyo Bay (n = 5)	Sagami Bay (n = 2)	
0%	3%						
+	+	88.1 % (89) ⁴⁾	74.3 % (81)	37.9 % (64)	7.0 % (8)	6.2 % (4)	
+	-	4.0 (4)	11.0 (12)	3.0 (5)	0 (0)	0 (0)	
-	+	2.0 (2)	10.1 (11)	57.4 (97)	86.8 (99)	69.2 (45)	
-	-	5.9 (6)	4.6 (5)	1.8 (3)	6.1 (7)	24.6 (16)	
Total		100 (101) ⁵⁾	100 (109)	100 (169)	100 (114)	100 (65)	

1) Concentration of NaCl added to peptone water. Symbols: +, growth; -, no growth.

3) Number of water samples.

2) Sagami River.

4) Number of strains.

5) Number of strains isolated with TCBS agar.

Table 4 Genus member rates in the TCBS strains

Genus	Rate of genus members from the following areas					
	Sagami River	Sakai River	Estuary ¹⁾	Tokyo Bay	Sagami Bay	
<i>Vibrionaceae</i>	38.6 % (40) ²⁾	26.6 % (29)	71.0 % (120)	47.4 % (54)	36.9 % (24)	
<i>Acinetobacter</i>	25.7 (26)	21.1 (23)	5.9 (10)	8.8 (10)	6.2 (4)	
<i>Enterobacteriaceae</i>	17.8 (18)	15.6 (17)	18.9 (32)	8.8 (10)	33.8 (22)	
<i>Alcaligenes</i>	1.0 (1)	10.1 (11)	3.6 (6)	2.6 (3)	7.7 (5)	
<i>Moraxella</i>	5.9 (6)	0 (0)	0 (0)	14.0 (16)	7.7 (5)	
<i>Pseudomonadaceae</i>	6.9 (7)	12.8 (14)	0 (0)	5.3 (6)	3.1 (2)	
<i>Flavobacterium</i>	0 (0)	0 (0)	0 (0)	2.6 (3)	0 (0)	
Others ³⁾	2.9 (3)	13.8 (15)	0.6 (1)	10.5 (12)	4.6 (3)	
Total	100 (101) ⁴⁾	100 (109)	100 (169)	100 (114)	100 (65)	

1) Sagami River.

2) Number of genus members identified.

3) Other genus members and unknown bacteria.

4) Number of strains isolated with TCBS agar as shown in table 3.

Table 5 Number of species in the genus *Vibrionaceae* in river and sea water

Species	Number of organisms isolated from the following areas				
	Sagami River	Sakai River	Estuary ¹⁾	Tokyo Bay	Sagami Bay
<i>V. alginolyticus</i>	—	—	7	3	12
<i>V. campbellii</i>	—	—	—	1	—
<i>V. cholerae</i>	24	13	7	—	—
<i>V. costicola</i>	—	—	—	2	—
<i>V. fluvialis</i>	2	—	2	1	1
<i>V. gazogenes</i>	—	—	—	2	—
<i>V. harveyi</i>	—	—	5	1	—
<i>V. logei</i>	—	—	4	13	2
<i>V. marinus</i>	—	—	3	3	2
<i>V. metschnikovii</i>	—	—	1	—	—
<i>V. mimicus</i>	—	—	7	—	—
<i>V. natriegens</i>	—	—	—	1	—
<i>V. parahaemolyticus</i>	—	—	11	11	2
<i>V. proteolyticus</i>	—	—	1	—	—
<i>V. splendidus</i>	—	—	3	—	1
<i>V. vulnificus</i>	—	—	30	—	—
<i>Listonella pelagia</i> (<i>V. pelagius</i>)	—	—	19	2	—
<i>Aeromonas</i>	5	12	8	2	—
<i>Plesiomonas</i>	—	—	—	7	1
Unknown ²⁾	9	4	12	5	3
Total	40 ³⁾ (101) ⁴⁾	29 (109)	120 (169)	54 (114)	24 (65)

1) Sagami River.

2) Minidefinition for *Vibrionaceae*.3) Number of genus *Vibrionaceae*.

4) Number of strains isolated with TCBS agar as shown in table 4.

seawater in the bay.

Discussion

Bacteria in aquatic environments were surveyed for bacterial numbers and direct viable counts ⁹⁾. However, bacteria that could grow on an artificial nutrient agar medium made up only a part of the environmental bacteria. The distribution of heterotrophic bacteria in oceans has been carefully investigated and reported by Shimidu *et al.*, and it was reported that *Vibrionaceae* were scarce but widely distributed ⁹⁾. In seawater from Tokyo Bay, it was reported that the main species of bacterial flora was *Acinetobacter* ¹⁰⁾. Maeda reported on the composition of flora in Sagami River water showing that *Flavobacterium* made up 31.3 ~ 43.8%. Along with *Acinetobacter* and *Moraxella*, these 3 genus occupied 50 ~ 75% of the total heterotrophic bacteria ¹¹⁾.

In this investigation, *Vibrionaceae* was isolated from each point of the aquatic environment with TCBS agar. TCBS agar and SMA contained similar concentrations of nutrients which support the growth of environmental bacteria. However, TCBS agar includes selective agents and a number of TCBS strains and TVC had no correlation ¹²⁾. TVC reflected the concentration of nutrition in water environments for bacteria growth, because TVC correlated with NH_4^+ , PO_4^{3-} , E.C., Cl^- ¹²⁾. Therefore, TCBS strain counts were compared with TVC isolated from the same sample and the TCBS strain counts were 0.01 ~ 0.001 times those of TVC. *Vibrionaceae* in the TCBS strains was between 26.6 ~ 71.0%. It is estimated that the rate of *Vibrionaceae* in environmental bacteria is low, although *Vibrionaceae* members were isolated from all the aquatic environments tested in this study. The rates of *Vibrionaceae* in the TCBS strains increased in the order of river water, seawater and estuary water. The TVC in river water was higher than that of seawater, because the concentration of nutrients in river water was more than that of seawater (Table 2). Therefore, it is thought that *Vibrionaceae* members were isolated more than anywhere else in estuary water, because the estuary received river water which contained many nutrients and seawater provided suitable salinity for these bacteria to grow.

TCBS agar was developed to isolate *V. cholerae* and *V. parahaemolyticus* from clinical materials ¹³⁾ because bacterial growth, except for *Vibrio* species, is inhibited by selective agents in TCBS agar, though resistant or tolerable bacteria to selective agents grow on it, and the medium is applied in many cases to isolate *Vibrio* species from various aquatic environments ^{14,15)}. The

strains isolated from each area were 25.7% (26/101) from Sagami River water, 11.9% (13/109) from Sakai River water, 47.8% (81/169) from the estuary, 33.3% (38/114) from seawater in Tokyo Bay, and 30.8% (20/65) from seawater in Sagami Bay. The highest rate of *Vibrio* species was found in the estuary, and the lowest rate was from Sakai River. *Vibrio* species made up 11.9 ~ 47.8% of the TCBS strains isolated from the three aquatic environments; river water, estuary water and seawater in the bay.

V. cholerae was isolated from estuary and river water but the organism was not isolated from seawater in the bay. No strains of *V. cholerae* isolated from the aquatic environments agglutinated with O1 antiserum. *V. alginolyticus*, *V. fluvialis* and *V. parahaemolyticus* were isolated from seawater in the bay. *V. cholerae*, *V. metschnikovii*, *V. mimicus* of the non-salt strain and *V. vulnificus* of the salt strain were isolated from the estuary water. *V. fluvialis* was isolated from river water, estuary water and

ratio of the genus in TCBS strains does not always show the ratio of that genus, except in *Vibrionaceae*, in aquatic environments. Pathogenic vibrios were detected only 3.0×10^{-2} cells/ml in the TCBS medium but *V. cholerae* were detected in about 2.0 cells/ml in aquatic environments using a 16S-rRNA hybridization method after incubation in APW with a most probable number method¹⁶⁾. Kapper *et al.* reported that *V. cholerae* can be occasionally detected by incubation for 1 week at 10°C¹⁴⁾. After samples are filtrated the filters were incubated on APW plates for 1 ~ 2 h at air temperature for the stress cells to resuscitate, then the filters were transferred onto TCBS agar plates. Though viable, but nonculturable, bacteria¹⁷⁾ and injured bacteria¹⁸⁾ in aquatic environments did not grow on TCBS agar because of selective agents, however, a few bacteria could be grown on it with an APW plate treatment method. The existence of stressed cells in aquatic environments requires further study.

Vibrio species were isolated from aquatic environments with a wide range salinity with TCBS agar. Within the TCBS strains, the non-salt strains were isolated chiefly from river water, and the salt strains were isolated mostly from bay seawater. It was assumed that aquatic environments such as seawater or fresh water determined whether the strain could grow in peptone water with or without NaCl. Among the TCBS strains isolated from seawater a few non-salt strains that grow in both 0‰ and 3‰ NaCl were included. On the other hand, TCBS strains isolated from river water contained not only non-salt strains but also salt strains. The Sakai River water was taken from a polluted stream and the concentration of Cl⁻ of the Sakai River sample was higher than that of the Sagami River. It is assumed that the reason why the ratio of the salt strain in the Sakai River water was higher than that of the Sagami River water was due to the above reasons, or that salinity-resistant or tolerable organisms existed in the river water from the beginning. No clear evidence to prove this theory was obtained.

The distribution of pathogenic vibrios was surmised from the station data where vibrios were isolated. The seasonal quality change in river water was slight except for water temperature during the period of this investigation. The quality of the Sakai River water was influenced by inflow drains and the quality of the estuary water was influenced by inflow seawater. *V. cholerae* was isolated from 5 out of 6 samples from the Sagami river water and 2 out of 6 samples from the Sakai river water. *V. cholerae* was isolated from estuary water of low salinity between 0.04 ~ 0.56 ‰ NaCl and the organisms were not isolated from high salinity samples of 8.6 ~ 31.3 ‰ NaCl. *V. cholerae* was isolated from river water during the period of this investigation. *V. cholerae* existed in river water, because this organism was isolated from river and estuary water and it was a non-salt strain and could grow due to nutrients in the river water¹⁹⁾. *V. metschnikovii* and *V. mimicus*

were isolated from estuary water of low salinity. Estuary water seems to be the most suitable habitat for *V. vulnificus*, because the organism was isolated only from estuary water and in 7 out of 8 samples isolation of the organism was not related to salinity. This organism existed in an aquatic environment of salinity between 7 ~ 16‰²⁰⁾. *V. fluvialis* and *V. splendidas* were isolated from estuary water of low and high salinity. It appears that *V. fluvialis* existed in various aquatic environments, because the organism was isolated from not only seawater, but also from other low salinity aquatic environments in which salinity was under 6‰²¹⁾. *V. alginolyticus* and *V. parahaemolyticus* were organisms present in seawater, and they were isolated from seawater and estuary water. *V. parahaemolyticus* was isolated in the summer season from June to September²²⁾. During this investigation, *V. parahaemolyticus* was isolated from estuary water at a high salinity of 8.6 ~ 31.3‰. Halophilic bacteria were also isolated from sea fish and sea products²³⁾. The other members of the *Vibrio* species seems to be present in seawater because they were isolated from estuary water at a high salinity of 8.6 ~ 31.3 ‰ as well as from seawater.

The pathogenicity of pathogenic vibrios isolated from the aquatic environments was not examined in this study. It was reported that pathogenic vibrios were isolated from fish and other sea products in seawater from estuaries and the coast^{15, 24)}. This investigation showed that many members of the *Vibrio* species existed in these places where fresh water and seawater are mixed. Therefore, there is a possibility that these organisms are taken up by fish and other sea creatures and it was reported that marine products had pathogenic vibrios in their self-systems²⁵⁾. Thus, pathogenic vibrios in marine products can go on to infect to humans.

The above shows that *Vibrio* species occupied a low percentage among the organisms isolated with TCBS agar in various aquatic environments. However, pathogenic vibrios were widely distributed in the aquatic environments. TCBS agar was used to isolate bacteria in aquatic environments with various salinities. The results show that non-salt strains existed in fresh water and that salt strains existed in seawater. Therefore, these results suggest that *V. cholerae* is present in river water.

Acknowledgments

I thank Professor Kouichi Ohwada and Dr Masahiko Nishimura, Ocean Research Institute, University of Tokyo, for kindly providing me with the collected seawater during the KT - 93 - 3 cruise and for their helpful advice. Thanks are also due to the captain and crews of R/V Tansei - Maru, for helping me with sampling. This work was supported in part by a grant AKPS (All Kitasato Project Study) No. 5 - 3 - 3 from Kitasato University.

References

- 1) Shinoda S. Pathogenic vibrios and food sanitation of marine products. *Jpn J Toxicol Environ Health* 1992; 38: 99-109.
- 2) Colwell R R, Kaper J, Joseph S W. *Vibrio cholerae*, *Vibrio parahaemolyticus* and other vibrios. *Science* 1977; 198: 394-6.
- 3) Tison D L, Nishibuchi M, Seidler R J, Siebeling R J. Isolation of non-O1 *Vibrio cholerae* serovers from Oregon coastal environments. *Appl Environ Microbiol* 1986; 51: 444-5.
- 4) Kaysner C A, Abeyta C Jr, Wekell M M, DePaol A Jr, Stott R F, Leitch J. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. *Appl Environ Microbiol* 1987; 53: 1349-51.
- 5) Venkateswaran K, Nakano H, Okabe T, Takayama K, Matsuda O, Hashimoto H. Occurrence and distribution of *Vibrio* spp., *Listonella* spp., and *Clostridium botulinum* in the Seto Inland Sea of Japan. *Appl Environ Microbiol* 1989; 55: 559-67.

- 6) Hobie J E, Daley R J, Jasper S. Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* 1977; 33: 1225-8.
- 7) Cowan S T, Steel K J. Manual for the identification of medical bacteria. London: Cambridge at University Press, 1965.
- 8) West P A, Colwell R R. Identification and classification of *Vibrionaceae*...an overview. In: Colwell R R ed. *Vibrios in the environment*. USA: John Wiley & Sons, 1984: 285-366.
- 9) Simidu U, Kogure K, Tsukamoto K. Distribution of *Vibrionaceae* in sea. *Nihon Biseibutsu Seitai Gakkaiho* 1987; 1: 65-74.
- 10) Simidu U, Kaneko E, Taga N. Microbiological studies of Tokyo Bay. *Microb Ecol* 1977; 3: 173-91.
- 11) Maeda S. The flora of aerobic heterotrophic bacteria in the river Sagami. *Jap J Limnol* 1980; 41: 163-71.
- 12) Uchiyama H. A number of *Vibrio cholerae* non-O1 isolated from aquatic environments. *J Jpn Assoc Infect Dis* 1998; 72: 720-6.
- 13) Kobayashi T, Enomoto S, Sakazaki R, Kuwahara S. A new selective isolation medium for the *Vibrio* group (Modified Nakanishi's medium - TCBS agar). *Jpn J Bacteriol* 1963;18: 387-92.
- 14) Kaper J, Lockman H, Colwell R R, Joseph S W. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Appl Environ Microbiol* 1979; 37: 91-103.
- 15) Schandevyl P, Dyck E V, Piot P. Halophilic *Vibrio* species from seafish in Senegal. *Appl Environ Microbiol* 1984; 48: 236-8.
- 16) Nishimura M, Kita-Tsukamoto K, Kogure K, Ohwada K. Enumerations of *Vibrio cholerae* in aquatic environments by MPN-16S rRNA hybridization Method. *Bull Jpn Soc Microb Ecol* 1992; 7: 43-6.
- 17) Oliver J D, Nilsson L, Kjelleberg S. Formation of non-culturable *Vibrio vulnificus* cells and its relationship to the starvation state. *Appl Environ Microbiol* 1991; 57: 2640-4.
- 18) Amy P S, Pauling C, Morita R Y. Starvation-survival of a marine *Vibrio*. *Appl Environ Microbiol* 1983; 45: 1041-8.
- 19) Uchiyama H. Survival of *Vibrio cholerae* non-O1 in freshwater river. *Jpn J Public Health* 1997; 44: 547-57.
- 20) Kelly M T. Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurrence in a Gulf Coast environment. *Appl Environ Microbiol* 1982; 44: 820-4.
- 21) Nishibuchi M, Roberts N C, Bradford Jr H B, Seidler R J. Broth medium for enrichment of *Vibrio fluvialis* from the environment. *Appl Environ Microbiol* 1983; 46: 425-9.
- 22) Nishio T, Kida M, Shimouchi H. Ecological studies on *Vibrio parahaemolyticus*. I. Distribution in sea water and sea mud. *Med J Hiroshima Univ* 1967; 15: 615-8.
- 23) Molitoris E, Joseph S W, Krichevsky M I, Sindhuhardja W, Colwell R R. Characterization and distribution of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated in Indonesia. *Appl Environ Microbiol* 1985; 50: 1388-94.
- 24) Kaysner C A, Abeyta C Jr, Wekell M M, DePaola A Jr, Stott R F, Leitch J M. Incidence of *Vibrio cholerae* from estuaries of the United States West Coast. *Appl Environ Microbiol* 1987; 53: 1344-8.
- 25) Kaysner C A, Tamplin M L, Wekell M M, Stott R F, Colburn K G. Survival of *Vibrio vulnificus* in shellstock and shucked oysters (*Cassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. *Appl Environ Microbiol* 1989; 55: 3072-9.