The growth of *Vibrio vulnificus* and the habitat of infected patients in Kumamoto

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SUMMARY **In Japan, *Vibrio vulnificus* (*V. vulnificus*) infection is very rare, and most infections have occurred in Kumamoto Prefecture (*1*), and especially around the Ariake and Yatsushiro seas. To investigate the relationship between the occurrence of *V. vulnificus* infection and environmental factors, including the salinity of seawater and the amount of rain in the Ariake and Yatsushiro seas, we measured the most probable number (MPN) of *V. vulnificus* in seawater and sea mud. In the Ariake Sea, we also observed the temperature and salinity of seawater at one site located on an estuary where the salinity is easily affected by river water and another site located offshore where seawater is little affected by river water. Furthermore, we investigated the MPN of *V. vulnificus* and observed the temperature and the salinity of seawater in 25 sites in the Ariake and Yatsushiro seas from July to August 2003 and 2004. In addition, we collected data on patients with *V. vulnificus* infections in Kumamoto from 1990 to 2006. The MPN of *V. vulnificus* differed by sampling site. More *V. vulnificus* were detected around the inland sea than the open sea, and the increase in *V. vulnificus* levels was affected by rainfall around inland sea areas with many rivers. *V. vulnificus* increases significantly in brackish water areas, and the salinity of seawater was as important as the seawater temperature. In other words, an area's topography and amount of rain are believed to be important factors for the occurrence of *V. vulnificus* infection. *V. vulnificus* infection has been regarded as an infection of hot districts. However, the salinity of seawater may be more important than temperature for the growth of *V. vulnificus*. Therefore, investigating these geographical and meteorological factors can help predict areas with a higher number of *V. vulnificus* infection outbreaks.**

Key Words: *Vibrio vulnificus*, tidelands, salinity of seawater, amount of rain, geographical factors, meteorological factors

Introduction

In Japan, there have been over 200 cases of *V. vulnificus* infection reported since 1978 (*2*). There are still many questions about the occurrence of *V. vulnificus* infections.

The current authors treated 43 *V. vulnificus* patients between 1990 and 2006 in Kumamoto Prefecture (Figure 1). In addition, there are several reports on *V. vulnificus* infections in Nagasaki (*3*) and Saga (*4*) prefectures but there are none on Kagoshima or Miyazaki prefectures, even though these prefectures neighbor Kumamoto and have higher average temperatures. This may be attributed to the fact that in these prefectures there are few areas with brackish water where *V. vulnificus* can grow.

Materials and Methods

Samples were prepared by using subsurface seawater

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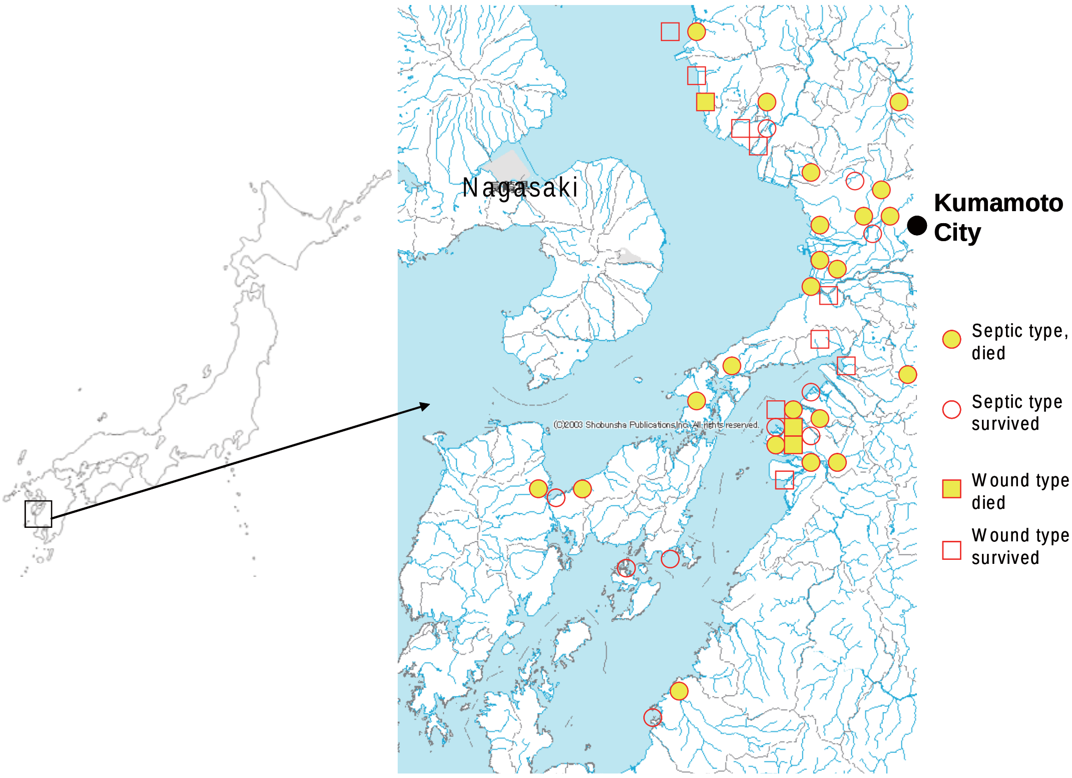

Figure 1. Patients with *V. vulnificus* infection in Kumamoto 1990-2006 (*n* = 43).

collected from Ariake Sea at a depth of between 1 m and 1.5 m. All samples were transported to the laboratory in insulated coolers and examined within 24-48 h of collection.

Seawater and mud samples were collected in sterilized glass bottles from sites A and B. Site A was located on the estuary of the Kikuchi River, where salinity is easily affected by river water, and site B, which was located offshore of the Ariake Sea 1.5 kilometers from Ooyano Island and where the salinity is little affected by river water. Samples were collected once a month from May to December, 2004.

Water samples were also collected in sterilized glass bottles from 25 different sites along the coast in Kumamoto (Figure 2) from July to August of 2003 and 2004.

The numbers of *V. vulnificus* in the seawater samples were estimated by the three-tube most-probable-number (MPN) method. Volumes of 10 mL and 1 mL of seawater were added to 10 mL and 1 mL of double-strength alkaline peptone water (APW), respectively. One mL of dilutions (10^{-1}-10^{-4}) in PBS was added to 10 mL APW. In addition, 500 mL of each seawater sample were filtered with a filter (pore size: 0.45 μm) and 40 mL APW were added to a tube containing the filter. After incubation at 35ºC for 18 h, 10 μL of the culture were streaked onto CHROM agar Vibrio and incubated at 35ºC for 18 h. Colonies

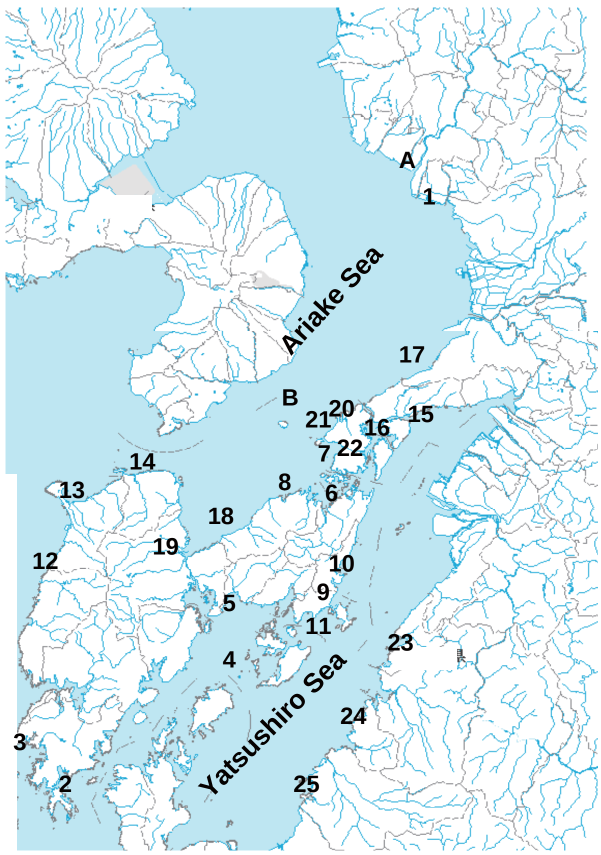

Figure 2. Water sampling sites along the coast in Kumamoto.

suspected of being *V. vulnificus* were confirmed to be *V. vulnificus* by the oxidase test, culture in triple sugar iron agar medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 2% NaCl, culture in lysine indol motility medium (Nissui Pharmaceutical Co. Ltd) containing 2% NaCl, culture in VP semi-solid medium (Nissui Pharmaceutical Co. Ltd) containing 2% NaCl, and growth in nutrient broth (Oxoid, Hampshire, UK) containing 0, 3, 8, and 10% NaCl. Furthermore, the suspected colonies were tested for the presence of the cytotoxin-haemolysin gene of *V. vulnificus* by PCR with the primer set used by Hill *et al*. (*5*).

To serve as a collection stomacher, 500 g of sea mud were placed in a pouch that as then sealed and immediately transported to the lab. Sea mud in the sample poucher was mixed by hand and every bag was handled uniformly. Twenty-five-gram samples were thinly scattered in dishes, dried for 2 h at 110ºC, and weighed after the sample was left in a desiccator for 40 min; then, the quantity of interstitial water was calculated. An additional 25 g of sea mud were diluted 10 × with PBS. This solution was added to 10 mL APW and diluted 10 × with PBS. *V. vulnificus* was identified and the MPN of the interstitial water per 100 mL was calculated by the method described above.

The seawater samples were collected in sterilized plastic bottles. Salinity was measured with a salt analyser (SAT-210, Toa Electronics, Tokyo, Japan). The temperature of seawater was measured with a thermometer. Seawater salinity (19-31‰) was not constant and may have been affected by weather or other factors rather than seasonal factors, although the temperature seemed to be affected by seasonal factors.

Results

The MPN of *V. vulnificus* in seawater and sea mud from May to December in 2004 is shown in Table 1. *V. vulnificus* numbers started to increase in seawater in May when the seawater temperature exceeded 20ºC

Table 1. *V. vulnificus* levels at Site A and Site B

Month	Site A				Site B			
	MPN (/100mL)		Sea water Temperature (ºC)	Salinity of seawater (‰)	MPN (/100mL)		Sea water Temperature (ºC)	Salinity of seawater (‰)
	Seawater	Sea mud*			Seawater	Sea mud*		
May	7	230,000	19	18.4	< 3	< 3	19.5	33.5
June	< 3	700	24.3	34.2	< 3	< 3	29	34.1
July	2,300	43,000	30.5	21.1	< 3	< 3	28	35.2
August	2,300	1,500	31	34.1	3	30	29.5	35.9
September	1,500	230,000	29.5	30.8	< 3	< 3	27	33.3
October	75	21,000	24	32.9	< 3	40	22.4	32.3
November	3	290	21	30	< 3	< 3	19	32.7
December	< 3	150	17.5	30.7	< 3	< 3	17	29

*MPN of interstitial water (/100mL)

Table 2. *Vibrio vulnificus* at 25 sites around Kumamoto (from July to August in 2003 and 2004)

Site	2003			2004		
	MPN (/100mL)	Amount of rain (mm)*	Salinity of seawater (‰)	MPN (/100mL)	Amount of rain (mm)*	Salinity of seawater (‰)
1	23,000	180	19.6	2300	0	34.1
2	< 3	30	33.7	< 3	0	37.3
3	< 3	30	33.5	< 3	0	37.8
4	< 3	35	31.8	< 3	0	36.5
5	7	174	30	< 3	0	37.2
6	< 3	208	26.1	< 3	24	34.8
7	4	208	26.1	3	24	34.7
8	4	208	25.3	3	24	34.4
9	4	205	21.4	< 3	4	34.1
10	3,800	205	22.1	< 3	4	34.4
11	< 3	205	24	< 3	4	35
12	< 3	129	33	< 3	6	36
13	< 3	30	32.7	< 3	6	36.2
14	< 3	174	30.8	< 3	6	35.5
15	93	128	25.6	< 3	6	35.3
16	430	128	24.1	< 3	6	36.5
17	230	128	26.8	3	6	35.9
18	< 3	278	32.7	3	34	35.9
19	< 3	174	31.3	< 3	4	37
20	36	44	31.9	< 3	70	35.8
21	6	44	31.3	< 3	70	36.4
22	< 3	44	31.7	< 3	70	36.4
23	21	251	30.3	< 3	30	35.6
24	9	251	31.6	< 3	0	35.4
25	23	204	31.6	< 3	2	35

* Amount of rain during the seven days before each seawater sampling

at site A, and the peak number of *V. vulnificus* was from July to October when the seawater temperature exceeded 30ºC. *V. vulnificus* inhabits sea mud more so than seawater, *V. vulnificus* was only isolated from seawater once in August at site B. In addition, the MPN was low, as shown in Table 1. *V. vulnificus* was isolated from sea mud in August and October, and the numbers were higher than from seawater. In addition, the salinity was stable. The increase in *V. vulnificus* was probably affected by the salinity, and *V. vulnificus* may have increased in number in sea mud more so than in seawater.

The MPN of *V. vulnificus* at twenty-five sites in Kumamoto in July and August in 2003 and 2004 is shown in Figure 1 and Table 2. The amount of rain in Table 2 shows the total rain during the seven days before each seawater sampling. There was more rainfall in 2003 than in 2004. In 2003, *V. vulnificus* was isolated from 14 of 25 sites but from only five sites in 2004. The salinity of seawater was higher in 2004 than in 2003 at all of the sites investigated. The difference in seawater salinity could have been caused by a difference in rainfall.

Kumamoto is one area where numerous *V. vulnificus* infections occur. Since 1990, 30 patients with *V. vulnificus* infection were seen at Kumamoto University Hospital and 13 patients were seen at 7 other hospitals in Kumamoto Prefecture (Table 3). However, there were no patients from islands facing the open sea.

Discussion

V. vulnificus infection is very rare, but its prognosis is poor once it occurs. *V. vulnificus* infection is divided into several clinical types: septic, wound, digestive, and other types. In Asian countries, including Japan, many

Table 3. *V. vulnificus* infection patients in Kumamoto from 1990 to 2006

No.	Year	Female/Male	Years	Type	Day of onset	Died/Suvived
1	1990	F	58	Septic type	1990.10.30	D
2	1991	M	56	Septic type	1991.7.8	S
3	1992	M	48	Septic type	1992.8.6	D
4	1993	F	64	Septic type	1993.8.15	D
5	1995	M	44	Wound type	1995.7.12	D
6		M	57	Septic type	1995.7.13	D
7	1996	M	55	Septic type	1996.7.22	D
8		M	56	Ingestive type	1996.7.31	S
9		M	67	Septic type	1996.9.18	D
10	1997	M	35	Wound type	1997.6.13	D
11		M	53	Septic type	1997.7.21	D
12	1999	F	66	Septic type	1999.6.6	D
13		F	74	Wound type	1999.7.8	S
14		M	38	Septic type	1999.8	D
15		M	67	Septic type	1999.9.21	D
16	2001	M	61	Septic type	2001.6.29	D
17		M	72	Septic type	2001.7.10	S
18		M	61	Septic type	2001.7.10	S
19		M	77	Septic type	2001.7.4	S
20		M	62	Wound type	2001.7.7	S
21		M	56	Septic type	2001.7.12	D
22		M	43	Septic type	2001.7.17	D
23		M	74	Wound type	2001.7.18	S
24		M	77	Wound type	2001.8.	S
25		M	68	Septic type	2001.8.14	D
26	2002	M	67	Septic type	2002.8.23	D
27		M	70	Septic type	2002.9.19	S
28		M	63	Wound type	2002.10.6	S
29	2003	M	77	Wound type	2003.7.24	D
30		M	77	Wound type	2003.8.15	S
31		F	70	Septic type	2003.9.5	S
32		M	32	Wound type	2003.9.7	S
33		M	70	Septic type	2003.10.8	D
34	2004	M	58	Septic type	2004.6.17	D
35		M	40	Septic type	2004.6.21	S
36		M	66	Septic type	2004.9.21	S
37	2005	M	59	Septic type	2005.7.15	D
38		M	61	Septic type	2005.7.12	D
39		M	59	Wound type	2005.7.28	S
40		M	55	Septic type	2005.7.30	D
41		M	70	Wound type	2005.9.25	S
42	2006	M	69	Septic type	2006.7.19	D
43		M	70	Septic type	2006.8.3	D

patients have the septic type, which is assumed to be a result of eating fresh marine products. *V. vulnificus* infections have been reported all over the world (*6-15*) and especially in warm areas in the summer. The water temperature suitable for growth of *V. vulnificus* is more than 20ºC (*16*), and adequate salinity is 15-25‰ (*17*), but its growth is suppressed when salinity exceeds 25‰. However, there are few studies of *V. vulnificus* in relation to environmental factors and therefore, many questions remain regarding the association of *V. vulnificus* and environmental factors.

There are few reports on *V. vulnificus* in sea mud. The current investigation found a higher concentration of *V. vulnificus* in sea mud than in seawater. For this reason, wound-type *V. vulnificus* infection may occur from sea mud without the patient even being in seawater. Furthermore, there was no clear difference between sites A and B in terms of the seawater temperature; however, the salinity changed greatly at site A in comparison to site B, and a much higher concentration of *V. vulnificus* was found at site A than site B. The salinity of seawater may be as important for the growth of *V. vulnificus* as seawater temperature.

Hoi *et al*. (*18*) isolated *V. vulnificus* from seawater in Denmark, and *V. vulnificus* grew rapidly with a higher seawater temperature. However, there were no changes in the salinity of seawater in Denmark. Hervio-Heath *et al*. (*19*) performed an environmental study in France and clearly confirmed the presence of *V. vulnificus* in Girond littoral in the Bordeaux district; salinities there were 10-20‰, which were lower than in other areas. However, the rate of detection of *V. vulnificus* is assumed to be low, and the salinity of Mediterranean seawater is more than 35‰, which is higher than in other seas. This could be the reason why there are few patients with *V. vulnificus* in areas around the Mediterranean Sea (*20*). *V. vulnificus* was detected in Chesapeake Bay (*21*) even at a time when the seawater temperature was less than 10ºC. However, in North Carolina (*22*), it was detected only when seawater temperature was more than 20ºC. The current study detected high levels of *V. vulnificus* in an inland sea even when seawater temperature was under 15ºC (data not shown). Motes *et al*. (*23*) reported that the MPN of *V. vulnificus* is very high in the Arabian Gulf Coast region. the above reports suggest that the salinity of seawater is important for the growth of *V. vulnificus*.

The current authors reported the first outbreak of *V. vulnificus* infection in Japan in 2001 (*24*) and focused on rainfall and related factors at the time of the outbreak. Since 1990, forty-three patients have been confirmed to have the infection (Table 3) in Kumamoto. Patients were concentrated in a coastal area where there is a large river.

At sites 15, 16, and 17 in the peninsula area, the amount of rainfall in July to August 2003 was 128 mm and that in 2004 was 6 mm. The salinity in 2003 was between 24.1‰ and 26.8‰ and that in 2004 was between 35.3‰ and 36.5‰. The MPN of *V. vulnificus* in 2003 and 2004 was from 93 to 430 and < 3, respectively. At sites 2, 3, 12, 13, and 14 in island areas, in contrast, the salinity of seawater was stable though there was much more rainfall in 2003 than in 2004, and *V. vulnificus* was not detected either in 2003 or 2004. Based on these results, *V. vulnificus* is unlikely to grow in island areas facing the open sea but instead proliferate in island seas following rain. In actuality, no patients with *V. vulnificus* were found around sites 2, 3, 12, 13, and 14 in island areas even though the inhabitants eat a great deal of raw fish.

Thus, the amount of rain and the salinity of seawater are believed to be associated with the occurrence of a *V. vulnificus* infection as much as seawater temperature. Geographical and meteorological factors are believed to greatly influence the occurrence of *V. vulnificus* infection.

The onset of this illness is abrupt, rapidly progressing to septic shock with a high mortality rate. Clinicians managing patients with chronic liver disease need to educate their patients about the risk associated with the consumption of raw shellfish.

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