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SHORT REPORT
Isolation of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from wild aquatic birds in Japan

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SUMMARY

Vibrio parahaemolyticus and *Vibrio vulnificus* were isolated from faecal samples of wild aquatic birds in winter. Although *V. parahaemolyticus* and *V. vulnificus* were present in low numbers in seawater in the area where the faecal samples of the birds were collected, the pathogens were isolated from the faeces of the birds. This study demonstrates that wild aquatic birds are a vehicle for *V. parahaemolyticus* and *V. vulnificus* to survive in winter.

Vibrio parahaemolyticus and *Vibrio vulnificus* are important pathogens of the *Vibrio* genus. It is known that *Vibrio* infections occur through the consumption of contaminated seafoods and contact of wounds with contaminated seawater. In Japan, *V. parahaemolyticus* is one of the most common pathogens of foodborne infections. More than 94 cases of *V. vulnificus* infection including 68 deaths were reported during 1998–2003, and an average occurrence of 425 cases per year has been estimated [1]. Contaminations of seafood and seawater with *V. vulnificus* have also been reported in Japan [2–4]. To avoid infection, the investigation of the distribution of pathogens in the environment is important.

Population size of *V. parahaemolyticus* and *V. vulnificus* in seawater is correlated with seawater temperature [5–7], and most infections by *V. parahaemolyticus* or *V. vulnificus* occur during summer. It was believed that these pathogens were dead during cold weather conditions. However, recent studies have indicated that cold temperatures induce a 'viable but non-culturable (VNC)' state in these pathogens [8, 9]. Moreover, the possibility that *V. vulnificus* overwinters in certain conditions has also been indicated [10]. DePaola *et al.* [11] isolated pathogens from fish at higher densities than in sediment or seawater during winter. Previously, a few reports stated that vibrios, including *V. cholerae* and *V. parahaemolyticus*, were isolated from aquatic birds such as the gull [12–14]. However, the isolation of *V. vulnificus* from the faeces of birds has never been reported. Furthermore, there are a few reports on the isolation of *V. vulnificus* from seawater or enrichment samples in the winter season. This study demonstrates that wild aquatic birds are a vehicle of *V. vulnificus* and *V. parahaemolyticus* in winter.

A total of 616 fresh faecal samples from birds such as the black-tailed gull (*Larus carassirostris*), herring gull (*Larus argentatus*), black-headed gull (*Larus ridibundus*), mallard (*Anas platyrhynchos*), European widgeon (*Anas penelope*), and common teal (*Anas crecca*), were collected along a section of

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Table 1. *Detection of* V. vulnificus *and* V. parahaemolyticus *in wild aquatic birds in Japan*

	Herring gull or Black-tailed gull		Black-headed gull		Duck		Total (%)	
Date	VP	VV	VP	VV	VP	VV	VP	VV
2002								
29 Jan.	2/2*	0/2	2/2	0/2			4/4 (100)	0/4 (0)
24 Feb.	22/32	0/32	11/19	0/19	1/3†	0/3†	34/54 (63·0)	0/54 (0)
4 Mar.	10/18	0/18	7/15	0/15			17/33 (51·5)	0/33 (0)
4 Apr.	8/28	0/28	11/25	0/25			19/53 (35·8)	0/53 (0)
28 Oct.	1/1	0/1	2/2	1/2			3/3 (100)	1/3 (33·3)
12 Nov.	8/12	0/12	13/17	0/17	1‡/19§	0/19§	22/38 (57·9)	0/38 (0)
24 Dec.			12/20	0/20			12/20 (60)	0/20 (0)
2003								
31 Aug.	15/15	15/15					15/15 (100)	15/15 (100)
16 Sep.	41/47	42/47					41/47 (87·2)	42/47 (89·4)
15 Oct.	33/50	26/50					33/50 (66)	26/50 (52)
18 Nov.	35/40	6/40					35/40 (87·5)	6/40 (15)
2004								
21 Dec.	19/30	3/30			13/18†	0/18†	32/48 (66·7)	3/48 (6·3)
2005								
31 Jan.	16/30	0/30			14/32†	0/32†	30/62 (48·4)	0/62 (0)
22 Feb.	0/15	0/15	9/15	0/15	5‖/20†	0/20†	14/50 (28)	0/50 (0)
9 Mar.					9/39	0/39	9/39 (23)	0/30 (0)
1 Apr.			1/10	0/10	14/40	0/40	15/50 (30)	0/50 (0)
Total	216/320	86/320	68/125	1/125	57/171	0/171	341/616	87/616
(%)	(67·5)	(26·9)	(54·4)	(0·8)	(33·3)	(0)	(55·4)	(14·1)

VP, *Vibrio parahaemolyticus*; VV, *Vibrio vulnificus*.
* Number of detected sample/total number of tested sample.
† Mallard.
‡ Common teal.
§ Ten European widgeon and nine common teal.
‖ A TRH-positive strain (O10KUT) was isolated.

coastline in Kumamoto prefecture, southern Japan, from January to April 2002, October to December 2002, August to November 2003, in December 2004, and January to April 2005 (Table 1, Fig.). Because herring gulls/black-tailed gulls and black-headed gulls did not visit the area between May and July, we could not collect their faecal samples during this period. Immediately after each group of herring gulls/black-tailed gulls or black-headed gulls left from the breakwater, their faecal samples were quickly collected, and ~1 g of each was put into 10 ml alkaline peptone water (APW). The samples were transported immediately to our laboratory without cooling within 1 h. To detect *V. parahaemolyticus* and *V. vulnificus*, each sample in 10 ml of APW was incubated at 35 °C for 18 h. One loop (10 μl) of the culture was inoculated onto CHROMagar Vibrio (CHROMagar, Paris, France) and incubated at 35 °C for 18 h. Colonies suspected as *V. parahaemolyticus* and *V. vulnificus* were confirmed 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, and 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*, and the *tdh* and *trh* genes of *V. parahaemolyticus* by the PCR method with the primer set used by Hill *et al.* [15] and Tada *et al.* [16] respectively. As a result, *V. parahaemolyticus* was detected in bird faecal samples at high levels on most sampling dates even in winter [Fig. (*a*)]. *V. vulnificus* was detected in October 2002,

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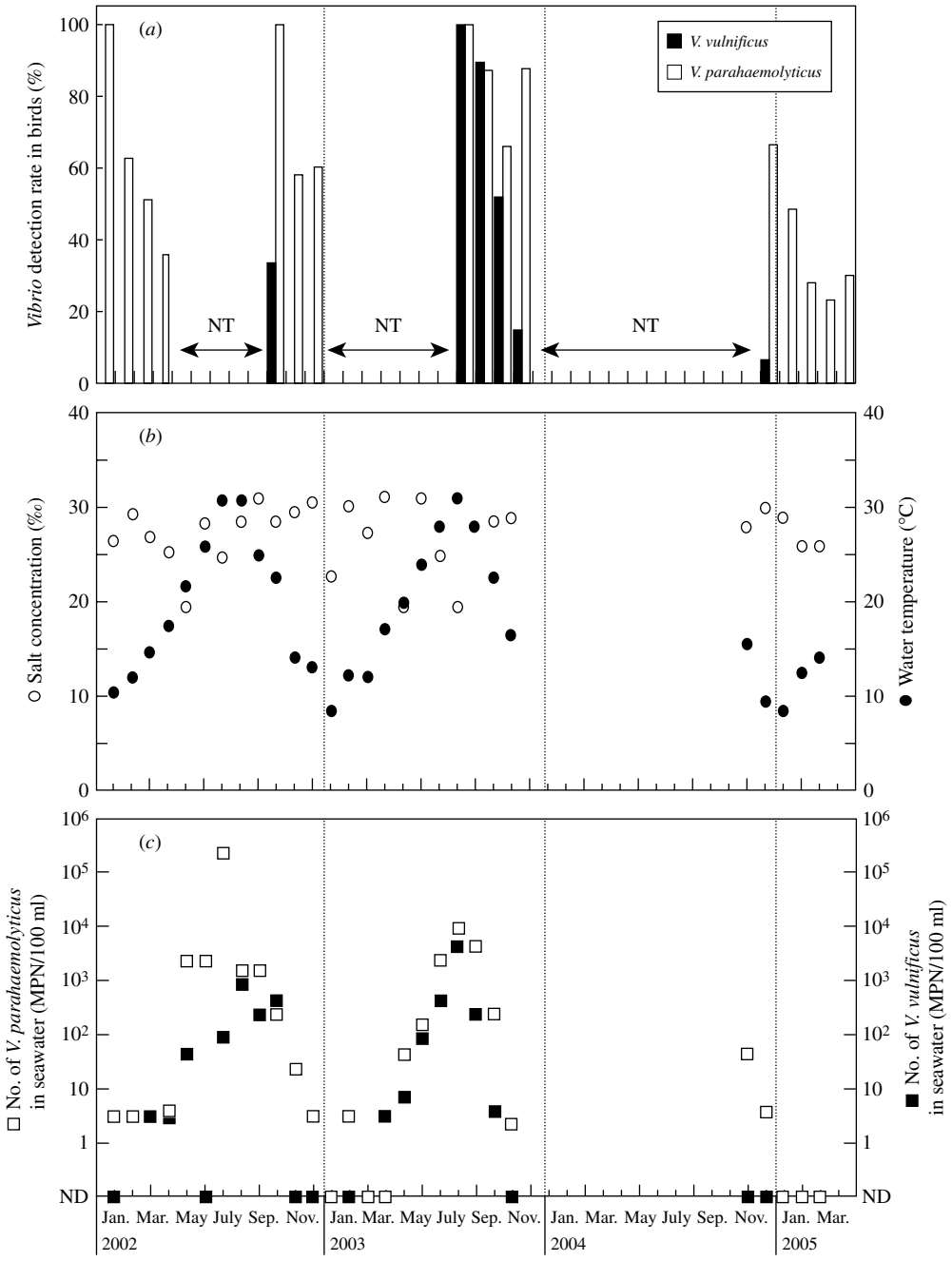

Fig. Detection of *V. parahaemolyticus* and *V. vulnificus* in bird faecal samples and the environment. (*a*) The detection ratio of *Vibrio* in the bird faecal samples; NT, not tested. (*b*) The salt concentration and water temperature of seawater near the place the bird faecal samples were collected. (*c*) The numbers of *V. parahaemolyticus* and *V. vulnificus* in seawater; ND, not detected.

between August and November 2003, and in December 2004.

The seawater samples were collected in sterilized plastic bottles in areas where the faecal samples of the birds were also collected. 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 throughout the four seasons [Fig. (*b*)]. Seawater salinity might be affected by weather or other factors rather than seasonal factors, while the temperature seemed to be affected by seasonal factors. The temperature was $<15\,^{\circ}C$ between December and March and $>24\,^{\circ}C$ between June and September

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Table 2. *Serotypes of* Vibrio parahaemolyticus *isolates in January–April, 2002*

	K antigen																								
	6	8	12	17	20	29	30	31	32	33	34	42	43	44	45	46	51	58	60	61	66	68	71	UT	Total
O antigen																									
1								1	5	4														6	16
2																									0
3	1	2			1	1										1	1	1	1			1	1	13	24
4		7	1			1		1			3	2		2	1						1			13	32
5				4			2				1		1							1				8	17
6							1																	1	2
7																									0
8																								4	4
9																									0
10																					3			12	15
11																									0
UT																					1			5	7
Total	1	9	1	5	1	2	3	2	5	4	4	2	1	2	1	1	1	1	1	1	5	1	1	62	117

UT, Untypable.

[Fig. (*b*)]. Most bird faecal samples were collected in periods when the seawater was <15 °C [Fig. (*a*, *b*)].

The numbers of *V. parahaemolyticus* and *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 of double-strength APW, and 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 was filtered with a filter (pore size: 0·45 μm) and 40 ml APW was added to a tube contained with the filter. After incubation at 35 °C for 18 h, 10 μl of the culture was streaked onto CHROMagar Vibrio and incubated at 35 °C for 18 h. The suspected colonies were confirmed by the method described above. The results showed that the population number of *V. parahaemolyticus* in seawater samples was associated with temperature [Fig. (*c*)]. The population was <100 MPN/100 ml between November and April and >1000 MPN/100 ml between July and September. The population number of *V. vulnificus* was also associated with seasonal factors and tended to be lower than that of *V. parahaemolyticus*. The population number of *V. vulnificus* was <10 MPN/100 ml and >100 MPN/100 ml between November–April and July–September respectively [Fig. (*c*)]. The number of vibrios in water seems to be related to temperature. Vibrios were isolated from bird faecal samples even if they were in low numbers in seawater. *V. parahaemolyticus* was detected with a higher rate than *V. vulnificus* [Fig. (*a*, *b*)] both in bird faecal samples and seawater.

The details of detection of vibrios in birds are shown in Table 1. *V. parahaemolyticus* was detected at the highest rate in herring gull/black-tailed gull samples (more than 67·5% of samples) (Table 1). The rates of *V. parahaemolyticus* detection in black-headed gulls and ducks were 54·4 and 33·3% respectively. *V. vulnificus* was detected at the highest rate in herring gulls/black-tailed gulls (>26·9%). However, the rates of *V. vulnificus* detection in black-headed gulls and ducks were 0·8 and 0% respectively. In all bird faecal samples, the detection rate of *V. parahaemolyticus* (55·4%) was higher than that of *V. vulnificus* (14·1%).

Serotyping of *V. parahaemolyticus* was performed for 117 strains of *V. parahaemolyticus* isolated between January and April 2002 by the agglutination test with antisera against O and K antigens (Denka Seiken Co. Ltd, Tokyo, Japan). As a result, various combinations of O and K serotypes were observed (Table 2). More than half of the strains had untypable K antigenicity. It is suggested that many unknown types of antigenicity exist in the environment. Although *tdh*-positive strain was not isolated through this study, a *trh*-positive strain (serotype O10:KUT) was isolated from ducks in February 2005. Demonstrating that aquatic birds can carry pathogenic strains of *V. parahaemolyticus*.

Aquatic birds catch and eat shellfish at low tide. At high tide, herring gulls/black-tailed gulls and black-headed gulls stay around the breakwater in separate groups. Aquatic birds spit out their undigested food on the breakwater. This undigested food on the breakwater makes it easy to identify what they feed on. Their food includes small crab, small squilla and other shellfish. These shellfish may carry *V. vulnificus* and *V. parahaemolyticus*, and infect birds through ingestion. *V. parahaemolyticus* was also detected in the faecal samples of birds that consumed seaweed (i.e. mallard, European widgeon and common teal). This suggests that the number of *V. parahaemolyticus* in seaweed was similar to that in shellfish.

The number of cases of *V. vulnificus* infection in Kumamoto prefecture were one, three and two during the years 2002, 2003 and 2004 respectively. Furthermore >100 MPN/100 ml of *V. vulnificus* was detected in seawater in the summer, between July and September, of every year. These facts indicate the existence of vehicles for the pathogen to survive in water. In this study, we analysed aquatic bird faecal samples for the presence of *V. parahaemolyticus* and *V. vulnificus* in order to understand their role in the presence of these pathogens in seawater or shellfish in winter. The detection rate in the faecal samples showed a decline with the decline in the detection rate in seawater. However, *V. parahaemolyticus* and *V. vulnificus* were sometimes isolated only from the faecal samples of birds and not from nearby seawater. Birds might act as a potential vehicle in the transmission of these pathogens. It is not known whether vibrios colonize the birds' intestines. However, *V. parahaemolyticus* and *V. vulnificus* were easily recovered from part of the samples in this study by direct plating of bird faeces (data not shown). This indicates the possibility of the growth of vibrios in the intestine. Further study should concentrate on the growth of vibrios in bird intestine. In addition, during winter vibrio cells existing in a VNC state might change to a culturable state once they enter the intestine of aquatic birds. It seems that environments such as seawater or shellfish might be exposed with the culturable cells.

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DECLARATION OF INTEREST

None.

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