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Season-Specific Occurrence of Potentially Pathogenic *Vibrio* spp. on the Southern Coast of South Korea

Doris Y. W. Di,[a] Anna Lee,[b] Jeonghwan Jang,[c] Dukki Han,[a] Hor-Gil Hur[a]

School of Earth Sciences and Environmental Engineering, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea[a]; Centers for Disease Control and Prevention, Osong-eup, Cheongju-si, Chungcheongbuk-do, Republic of Korea[b]; BioTechnology Institute, University of Minnesota, Saint Paul, Minnesota, USA[c]

ABSTRACT *Vibrio* species are widely distributed in warm estuarine and coastal environments, and they can infect humans through the consumption of raw and mishandled contaminated seafood. In this study, we aimed to isolate and observe the distribution of enteropathogenic *Vibrio* spp. from environments of the southern coast of South Korea over a season cycle. A total of 10,983 isolates of *Vibrio* spp. were obtained from tidal water and mud samples over a 1-year period from five sampling sites along the southwest coast of South Korea. We found that *Vibrio alginolyticus* ($n = 6,262$) and *Vibrio parahaemolyticus* ($n = 1,757$) were ubiquitous in both tidal water and mud year round, whereas *Vibrio cholerae* ($n = 24$) and *Vibrio vulnificus* ($n = 130$) were seasonally specific to summer. While all *V. cholerae* isolates were nontoxigenic (non-O1 and non-O139), more than 88% of *V. vulnificus* isolates possessed the virulence factor elastolytic protease (encoded by *vvp*). Interestingly, *V. parahaemolyticus*, which was omnipresent in all seasons, contained the virulence factors thermostable direct hemolysin (encoded by *tdh*) and thermostable direct hemolysin-related hemolysin (encoded by *trh*) in larger amounts in June (29 *trh*-positive strains) and September (14 *tdh*-, 36 *trh*-, and 12 *tdh*- and *trh*-positive strains) than in December (4 *trh*-positive strains) and February (3 *tdh*-positive strains), and virulence factors were absent from isolates detected in April. To understand why virulence factors were detected only in the warm season and were absent in the cold season although the locations are static, long-term monitoring and particularly seasonal study are necessary.

IMPORTANCE The presence of enteropathogenic *Vibrio* species (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*), which cause acute diarrheal infection, septicemia, and wound infections upon ingestion through food and water, is usually associated with temperature. The World Health Organization (WHO) has estimated that there are 1.4 to 4.3 million cases and 28,000 to 142,000 deaths per year worldwide caused by cholera disease. In South Korea alone, consumption is as much as 52.4 kg of fish and shellfish per year per capita. Our findings suggested that seasonally specific acceleration of these possible pathogenic *Vibrio* spp. may threaten seafood safety and increase the risk of illness in South Korea, where local people consume raw fish during warmer months.

KEYWORDS *Vibrio parahaemolyticus*, *Vibrio vulnificus*, pathogens

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Address correspondence to Hor-Gil Hur, hghur@gist.ac.kr.

Vibrio species are omnipresent and widely distributed in aquatic environments all over the world (1). The occurrence of *Vibrio* spp. is commonly associated with temperature, especially a temperate climate. Generally, *Vibrio* species are periodically detectable in summer but less common in winter, whereas the *Vibrio* population variation is low in tropical and subtropical waters (1). Reports have shown a significant association between rising seawater temperature and an increase in the number of

Vibrio infections, suggesting that global warming could be a factor in the emergence of *Vibrio* diseases in temperate areas due to its influence on resident bacterial communities (2). Over the past 25 years, marine-related illnesses along the east coast of the United States have risen steadily in correlation with El Niño occurrences (3). It has also been suggested that increases in seawater temperature, as a prospective consequence of climate change, may be responsible for *Vibrio* infection outbreaks in Israel, Denmark, Spain, Chile, and the United States (4). For example, the emergence of *Vibrio* infections in the Baltic Sea area was associated with increased sea surface temperature (4, 5). In Bangladesh, the risk of cholera was found to be 2 times higher 6 weeks after a 5°C increase in river, pond, and lake water temperature, and water temperature was found to have a direct correlation with cases of cholera (6).

The geographical distribution of *Vibrio* spp. in the environment is confined to warm and temperate regions at water temperatures ranging from 20°C to 30°C (7), which is the optimum growth temperature for these bacteria. Increases in surface seawater temperatures will undoubtedly increase human contact with *Vibrio* spp. and disease transmission (4). A study conducted in Helgoland Roads, North Sea, Germany, showed that the abundance of *Vibrio* spp. in seawater is higher in summer (3.4×10^4 cells/ml) and significantly lower in winter (5×10^2 cells/ml) (8). However, the seasonal distribution of *Vibrio* populations could be species specific. Böer et al. conducted a study at 10 recreational beaches along the German North Sea for 2 years and found that *V. alginolyticus* and *V. parahaemolyticus* were ubiquitous year round, whereas *V. vulnificus* was limited to the summer months (9).

In the past decade, unusual, incremental increases in seawater temperature along the shoreline have been linked to epidemic outbreaks of *Vibrio*-associated illness caused by *V. parahaemolyticus* in areas such as Chile, Peru, the United States, Europe, and Asia. Studies conducted all over the world have highlighted the environmental factors that affect the abundance and distribution of *V. parahaemolyticus*, such as water salinity, temperature, turbidity, and the levels of chlorophyll and organic matter in suspension (10). Although the mechanism underlying human infection by *V. parahaemolyticus* is not completely understood, there are two hemolysins that are commonly recognized as pathogenicity indicators: thermostable direct hemolysin (TDH), which is a pore-forming protein involved in bacterium invasion, and TDH-related hemolysin (TRH), which plays a significant role in virulence (10–12). Most of the clinical isolates of *V. parahaemolyticus* possess *tdh* and/or *trh*; however, a relatively low number of environmental isolates were found to carry these genes (10, 13). *V. vulnificus* is an opportunistic human pathogen. Although human infections are rare, *V. vulnificus* disease causes the highest hospitalization (91.3%) and mortality (34.4%) rates of all foodborne diseases in the United States (14–16). One of the possible virulence determinants in *V. vulnificus*, *vvp*, which encodes elastolytic protease, may be a causative agent for skin lesions (16–20). It is well known that epidemic cholera is caused mainly by toxigenic *V. cholerae* O1 and O139. The serotype O1 has been further divided into the classical (CL) and El Tor (ET) biotypes (21, 22). The pathogenicity of *V. cholerae* depends on a combination of properties, including the presence of cholera toxin (CT), encoded by *ctxA*, and the colonization and adhesion factor encoded by *tcpA* (22, 23). Studies have shown seasonal patterns for this species, with increased occurrence and incidence of wound infections at warmer temperatures (24).

In recent years, with the changing climate, specifically, rising air and sea temperatures and intensifying monsoons, the ecology of pathogenic bacteria that affect public health has been a great concern. More study on the ecology of enteropathogenic *Vibrio* species in the environment where human activities occur is especially important to those who rely on catching marine products to make a living and/or as a source of food (9). The current study focused on investigating the seasonal dynamics of enteropathogenic *Vibrio* species in the coastal tidal waters and mud along the southwestern coast of South Korea over a season cycle. The environmental factors that may affect the seasonal occurrence of *Vibrio* species and populations are also discussed.

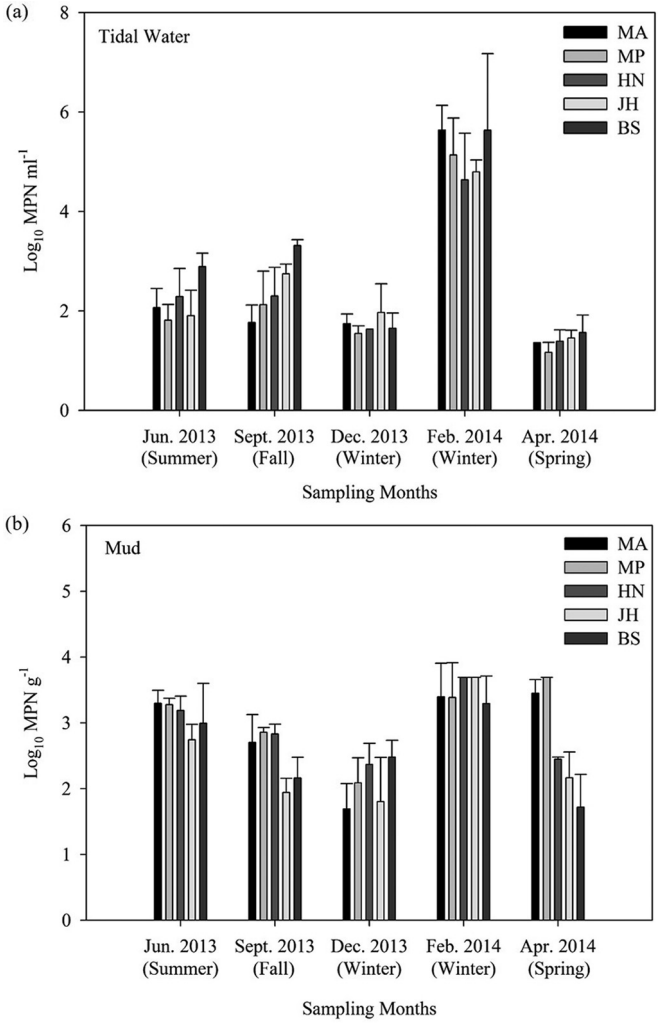

FIG 1 Distribution of presumptive *Vibrio* spp. ($\log_{10}$ MPN) in tidal water (a) and mud (b). Each bar in each month represents five sampling sites (MA, Muan; MP, Mokpo; HN, Haenam; JH, Jangheung; and BS, Boseong).

RESULTS

Seasonal variation in *Vibrio* spp. with environmental factors. The most probable number (MPN) of presumptive *Vibrio* spp. was found to differ significantly by month ($P < 0.05$) but not by sampling site ($P > 0.05$) (Fig. 1) as determined by analysis of variance (ANOVA). This shows that the MPN of presumptive *Vibrio* spp. in both habitats is affected by season and not location, although the total human population and major land uses differed at the sampling sites (see Table S1 in the supplemental material). Figure 1a shows that the average MPN of presumptive *Vibrio* spp. detected in tidal water in February was significantly different (*post hoc*, $P < 0.05$) from that in other months. The mean $\log_{10}$ MPN per milliliter of tidal water in February is 5.17, which is 3 logs higher than those in June (2.19) and September (2.45) and 4 logs higher than those in December (1.71) and April (1.39). The average MPN of presumptive *Vibrio* spp. detected in mud in February was significantly different ($P < 0.05$) from those in September, December, and April but not significantly different ($P > 0.05$) from that in June (Fig. 1b). The mean $\log_{10}$ MPNs per gram of mud in each season are 3.10 (June), 2.50 (September), 2.09 (December), 3.49 (February) and 2.70 (April). The overall average $\log_{10}$ MPN in mud is higher than that in tidal water. In addition, sampling month was found to have significant effect ($P < 0.05$) on temperature and pH in both tidal water and mud, as well as on salinity and biological oxygen demand (BOD) in tidal water (see

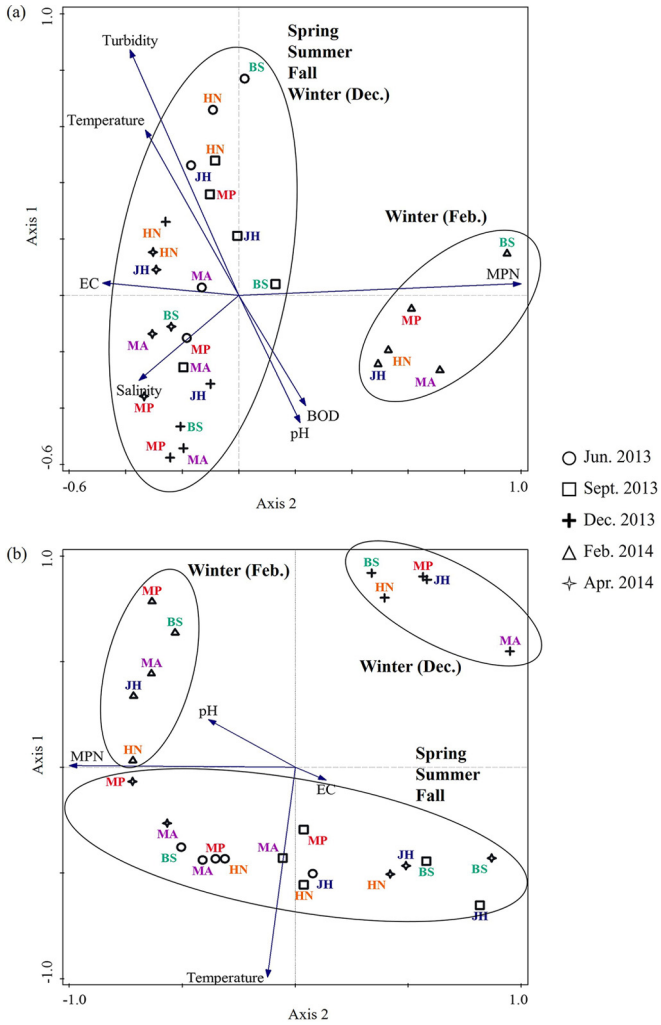

FIG 2 Principal-component analysis (PCA) projections of scores for correlation of the most probable number (MPN) and environmental parameters (BOD, EC, pH, salinity, temperature, and turbidity) from tidal water (a) and mud (b) in different seasons. A projection score near 1.0 indicates that the variables are perfectly correlated, whereas a score near −1.0 indicates that the variables are perfectly inversely correlated. Variables with vectors projected in the same plane could be considered positively correlated. Closely correlated variables are grouped in black ovals, indicated by months and sampling sites (MA, Muan; MP, Mokpo; HN, Haenam; JH, Jangheung; and BS, Boseong).

Fig. S1 and S2 in the supplemental material). However, electric conductivity (EC) was found to be temporally specific in tidal water but spatially specific in mud ($P < 0.05$). Location had no significant effect ($P > 0.05$) on the tested parameters, except for EC in mud. Tidal water turbidity did not differ significantly according to season or location ($P > 0.05$) (see Table S4 in the supplemental material).

Principal-component analysis (PCA) was performed to investigate the correlation between the MPN of presumptive *Vibrio* spp. in the samples and environmental variables such as pH, temperature, salinity, EC, BOD, and turbidity according to sampling site and season (Fig. 2). The MPN in tidal water samples was found to be seasonally independent, whereas the MPN in mud samples was seasonally dependent, and the clusters were distinctly separated by the direction of the temperature projections. In tidal water (Fig. 2a), two major clusters of the variables were observed, a large cluster comprised of four sampling months (June, September, December, and April) and a small cluster comprised of a single month, February. The environmental variables distributed unevenly, with MPN and turbidity of tidal water having the longest projection score of near 1.0, which indicates that the variable is perfectly correlated. The

TABLE 1 *Vibrio* species identification for the isolates collected from tidal water

Sampling season	*Vibrio* species	No. (%) of isolates from tidal water at[a]:					Total no. (%) of isolates
		MA	MP	HN	JH	BS	
June 2013	*V. alginolyticus*	184 (76.0)	177 (77.0)	157 (71.4)	159 (65.2)	95 (40.6)	772 (66.0)
	V. cholerae	0	0	0	0	1 (0.4)	1 (0.1)
	V. parahaemolyticus	10 (4.1)	6 (2.6)	19 (8.6)	39 (16.0)	18 (7.7)	92 (7.9)
	V. vulnificus	0	0	2 (0.9)	0	32 (13.7)	34 (2.9)
	Other *Vibrio* spp.	48 (19.8)	47 (20.4)	42 (19.1)	46 (18.9)	88 (37.6)	271 (23.2)
September 2013	*V. alginolyticus*	68 (29.6)	119 (51.5)	112 (59.6)	93 (41.5)	120 (51.7)	512 (46.3)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	34 (14.8)	50 (21.6)	15 (8.0)	54 (24.1)	61 (26.3)	214 (19.4)
	V. vulnificus	0	0	0	0	5 (2.2)	5 (0.5)
	Other *Vibrio* spp.	128 (55.7)	62 (26.8)	61 (32.4)	77 (34.4)	46 (19.8)	374 (33.9)
December 2013	*V. alginolyticus*	119 (53.6)	36 (41.4)	212 (92.6)	159 (70.7)	51 (22.1)	577 (58.0)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	12 (5.4)	18 (20.7)	0	20 (8.9)	90 (39.0)	140 (14.1)
	V. vulnificus	0	0	0	0	0	0
	Other *Vibrio* spp.	91 (41.0)	33 (37.9)	17 (7.4)	46 (20.4)	90 (39.0)	277 (27.9)
February 2014	*V. alginolyticus*	51 (54.3)	17 (7.7)	115 (69.3)	42 (20.2)	41 (31.1)	266 (32.4)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	7 (7.4)	3 (1.4)	5 (3.0)	10 (4.8)	10 (7.6)	35 (4.3)
	V. vulnificus	0	0	0	0	0	0
	Other *Vibrio* spp.	36 (38.3)	201 (91.0)	46 (27.7)	156 (75.0)	81 (61.4)	520 (63.3)
April 2014	*V. alginolyticus*	89 (46.1)	236 (94.8)	189 (88.7)	204 (85.0)	164 (67.8)	882 (77.6)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	0	0	0	1 (0.4)	4 (1.7)	5 (0.4)
	V. vulnificus	0	0	0	0	0	0
	Other *Vibrio* spp.	104 (53.9)	13 (5.2)	24 (11.3)	35 (14.6)	74 (30.6)	250 (22.0)

[a] Total number of bacterial isolates grown on TCBS agar. All colonies grown on TCBS agar were selected, not exceeding 252 isolates for each sampling site. Locations: MA, Muan; MP, Mokpo; HN, Haenam; JH, Jangheung; BS, Boseong.

differences in the variables in February compared to those in the other months were mostly affected by MPN, as observed by the direction of the projection arrow. MPN in tidal water was positively correlated with pH ($r = 0.257$, $P = 0.215$) and BOD ($r = 0.120$, $P = 0.569$) but negatively correlated with temperature ($r = -0.318$, $P = 0.122$), salinity ($r = -0.224$, $P = 0.282$), EC ($r = -0.301$, $P = 0.144$), and turbidity ($r = -0.198$, $P = 0.343$). In contrast, mud samples from April (spring), June (summer), and September (fall) were closely correlated but were separated from those collected in December (winter) and February (winter) by temperature (Fig. 2b). Although three clusters were observed in the mud samples, two small clusters comprised of variables from December and February, considered as one group, differed from those from April, June, and September by the projection arrow and the projection score of temperature. This indicates that the variables observed in winter were different from those in the other seasons, explained by temperature. In addition, the MPN in mud samples was positively correlated with pH ($r = 0.290$, $P = 0.159$) and temperature ($r = 0.002$, $P = 0.991$) but negatively correlated with EC ($r = -0.106$, $P = 0.613$).

Relative abundance and genotypic diversity of enteropathogenic *Vibrio* spp. Among the 11,772 phenotypically identified isolates of *Vibrio* spp., 10,983 isolates were genetically identified as *Vibrio* spp. by genus-specific 16S rRNA gene amplification, accounting for 93.7% and 92.9% of the presumptive *Vibrio* isolates from tidal water and mud, respectively (see Table S2 in the supplemental material). Among the enteropathogenic *Vibrio* sp. isolates identified, *V. cholerae* ($n = 24$) and *V. vulnificus* ($n = 130$) were found to be season specific, whereas *V. alginolyticus* ($n = 6,262$) and *V. parahaemolyticus* ($n = 1,757$) were season independent (Tables 1 and 2). *V. cholerae* was isolated in June only, with one strain from tidal water and 23 strains from mud samples. Thirty-four strains (2 and 32 from Haenam [HN] and Boseong [BS], respectively) of *V. vulnificus* were isolated in June and five strains (all from BS) were isolated in September from tidal

TABLE 2 *Vibrio* species identification for the total isolates collected from mud samples

Sampling season	*Vibrio* species	No. (%) of *Vibrio* isolates from mud at[a]:					Total no. (%) of isolates
		MA	MP	HN	JH	BS	
June 2013	*V. alginolyticus*	173 (68.7)	170 (67.5)	34 (13.5)	79 (31.6)	119 (47.8)	575 (45.8)
	V. cholerae	2 (0.8)	0	20 (7.9)	1 (0.4)	0	23 (1.8)
	V. parahaemolyticus	28 (11.1)	37 (14.7)	67 (26.6)	73 (29.2)	77 (30.9)	282 (22.5)
	V. vulnificus	2 (0.8)	0	59 (23.4)	29 (11.6)	1 (0.4)	91 (7.3)
	Other *Vibrio* spp.	47 (18.7)	45 (17.9)	72 (28.6)	68 (27.2)	52 (20.9)	284 (22.6)
September 2013	*V. alginolyticus*	106 (46.9)	55 (25.7)	82 (39.6)	107 (52.2)	114 (51.4)	464 (43.2)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	68 (30.1)	98 (45.8)	88 (42.5)	54 (26.3)	60 (27.0)	368 (34.3)
	V. vulnificus	0	0	0	0	0	0
	Other *Vibrio* spp.	52 (23.0)	61 (28.5)	37 (17.9)	44 (21.5)	48 (21.6)	242 (22.5)
December 2013	*V. alginolyticus*	200 (87.0)	146 (70.9)	104 (46.0)	177 (82.7)	94 (47.7)	721 (67.2)
	V. cholerae	0	0	0	0	0	0 (0.0)
	V. parahaemolyticus	6 (2.6)	34 (16.5)	79 (35.0)	4 (1.9)	69 (35.0)	192 (17.9)
	V. vulnificus	0	0	0	0	0	0 (0.0)
	Other *Vibrio* spp.	24 (10.4)	26 (12.6)	43 (19.0)	33 (15.4)	34 (17.3)	160 (14.9)
February 2014	*V. alginolyticus*	137 (60.1)	114 (60.3)	172 (71.1)	211 (84.1)	138 (56.1)	772 (66.8)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	61 (26.8)	28 (14.8)	29 (12.0)	12 (4.8)	49 (19.9)	179 (15.5)
	V. vulnificus	0	0	0	0	0	0
	Other *Vibrio* spp.	30 (13.2)	47 (24.9)	41 (16.9)	28 (11.2)	59 (24.0)	205 (17.7)
April 2014	*V. alginolyticus*	107 (45.0)	178 (77.7)	153 (63.0)	187 (76.3)	96 (39.5)	721 (60.2)
	V. cholerae	0	0	0	0	0	0
	V. parahaemolyticus	97 (40.8)	16 (7.0)	53 (21.8)	13 (5.3)	71 (29.2)	250 (20.9)
	V. vulnificus	0	0	0	0	0	0
	Other *Vibrio* spp.	34 (14.3)	35 (15.3)	37 (15.2)	45 (18.4)	76 (31.3)	227 (18.9)

[a]Total number of bacterial isolates grown on TCBS agar. All colonies grown on TCBS agar were selected, not exceeding 252 isolates for each sampling site. Locations: MA, Muan; MP, Mokpo; HN, Haenam; JH, Jangheung; BS, Boseong.

water, whereas 91 strains (2, 59, 29, and 1 from Muan [MA], HN, Jangheung [JH], and BS, respectively) were isolated in June from mud samples. In contrast, *V. alginolyticus* and *V. parahaemolyticus* were isolated from both tidal water and mud samples collected in all sampling months and at all sites, except for tidal water samples collected in December and April from MA, MP, and HN, which did not contain *V. parahaemolyticus*. In general, in all seasons, the occurrence of *V. parahaemolyticus* was higher and more stable in mud samples than in tidal water. The occurrence of *V. alginolyticus* was higher than that of *V. parahaemolyticus* in both tidal water and mud. The environmental parameters were tested for correlation with the abundance of *V. alginolyticus* and *V. parahaemolyticus*. The $\log_{10}$ MPN of *Vibrio* spp. in tidal water ($r^2 = 0.172$, $P = 0.039$) and temperature in mud ($r^2 = 0.178$, $P = 0.036$) were found to have a significant linear relationship with *V. alginolyticus* abundance (Table 3). The $\log_{10}$ MPN of *Vibrio* spp. per milliliter of tidal water ranged from 1.17 to 5.64 (Fig. 1a), and the temperature of mud ranged from 3.0°C to 28.5°C (Fig. S2). However, the strength of the relationship is weak, as the coefficient of determination (r) values are less than 1. Despite this, none of the environmental variables were found to have a significant correlation with the abundance of *V. parahaemolyticus* in either tidal water or mud. Thus, the assumption can be made that changes in temperature may affect the number of *V. alginolyticus* bacteria in mud.

Distribution of virulence genes among potential human-pathogenic strains of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The distribution of virulence traits among the three potential human pathogens *V. cholerae* ($n = 24$), *V. parahaemolyticus* ($n = 1{,}757$), and *V. vulnificus* ($n = 130$) is summarized in Table 4. These virulence (*ctxA*), biotype (*tcpA*), and serogroup (*rfb*) genes were not detected in the 24 *V. cholerae* strains isolated from tidal water and mud. A possible virulence determinant, an elastolytic protease (*vvp*) gene of *V. vulnificus* strains that can cause infection in humans, was detected in most of the strains isolated. Among the 34 *V. vulnificus* isolates from tidal

TABLE 3 Statistical analysis of the relationship between the occurrences of ubiquitous *V. alginolyticus* and *V. parahaemolyticus* with different environmental parameters for tidal water and mud

Environmental parameter	Linear regression[a]			
	V. alginolyticus		*V. parahaemolyticus*	
	r^2 value	*P* value	r^2 value	*P* value
Tidal water				
Log_{10} MPN	**0.172**	**0.039**	0.016	0.550
Temp (°C)	0.074	0.189	0.001	0.882
pH	0.003	0.789	0.055	0.259
Salinity (ppt)	0.034	0.379	0.004	0.767
BOD (mg $liter^{-1}$)	0.001	0.898	0.000	0.998
Turbidity (NTU[b])	0.081	0.168	0.008	0.679
Mud				
Log_{10} MPN	0.003	0.807	0.000	0.974
Temp (°C)	**0.178**	**0.036**	0.099	0.126
pH	0.032	0.391	0.103	0.119
EC (S m^{-1})	0.010	0.635	0.013	0.593

[a]Significance level, 0.05. Boldface indicates significant linear relationships.
[b]NTU, nephelometric turbidity units.

waters in June, 30 strains (88.2%) contained *vvp*. Five isolates from tidal water in September also contained *vvp* genes. Of the 91 *V. vulnificus* isolates from mud samples in June, 88 strains (96.7%) contained *vvp*. Although *V. parahaemolyticus* was isolated year round, pathogenic strains were detected mostly in June (29 strains with *trh*) and September (14 strains with *tdh*, 36 strains with *trh*, and 12 strains with *tdh* and *trh*). Of the 1,757 *V. parahaemolyticus* strains, 69 contained *trh*, including 41 and 28 strains from tidal water and mud, respectively. Among the 41 strains with *trh* from tidal water, 6, 34, and 1 were found in June, September, and December, respectively. Among the 28 strains with *trh* from mud, 23, 2, and 3 were found in June, September, and December, respectively. Most strains with *trh* were found in September ($n = 34$) in tidal water and in June ($n = 23$) in mud. Seventeen strains of *V. parahaemolyticus* possessed *tdh*, with 13 from tidal water and 4 from mud samples. Among the four strains with *tdh* from mud samples, one strain was found in September and three strains were found in February. Twelve strains of *V. parahaemolyticus* from tidal water in September contained both *tdh* and *trh*.

DISCUSSION

In this study, *V. alginolyticus* and *V. parahaemolyticus* were present year round, whereas *V. vulnificus* and *V. cholerae* were specific to the summer season. More

TABLE 4 Distribution of virulence genes among isolates of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*

Origin	Sampling season	No. (%) of positive strains[a]								
		V. cholerae ($n = 24$)					*V. parahaemolyticus* ($n = 1,757$)			*V. vulnificus* ($n = 130$)
		ctxA	*rfb* O1	*rfb* O139	*tcpA* El Tor	*tcpA* classical	*tdh*	*trh*	*tdh* + *trh*	*vvp*
Tidal water	June 2013	0	0	0	0	0	0	6 (6.5)	0	30 (88.2)
	September 2013	0	0	0	0	0	13 (6.1)	34 (15.9)	12 (5.6)	5 (100.0)
	December 2013	0	0	0	0	0	0	1 (0.7)	0	0
	February 2014	0	0	0	0	0	0	0	0	0
	April 2014	0	0	0	0	0	0	0	0	0
Mud	June 2013	0	0	0	0	0	0	23 (8.2)	0	88 (96.7)
	September 2013	0	0	0	0	0	1 (0.3)	2 (0.5)	0	0
	December 2013	0	0	0	0	0	0	3 (1.6)	0	0
	February 2014	0	0	0	0	0	3 (1.7)	0	0	0
	April 2014	0	0	0	0	0	0	0	0	0

[a] Presence of genes as determined by PCR. Genes: *ctxA*, cholera toxin (CT); *rfb* O1, serotype O1-specific *rfb* region; *rfb* O139, serotype O139-specific *rfb* region; *tcpA* El Tor, El Tor toxin-coregulated pilus (TCP); *tcpA* classical, classical TCP; *tdh*, thermostable direct hemolysin (TDH); *trh*, TDH-related hemolysin; *vvp*, elastolytic protease.

interestingly, the virulence genes (*tdh* and *trh*) in *V. parahaemolyticus* were absent in April in both tidal water and mud samples and were present more frequently in June and September than in other months. The presence and seasonality of *Vibrio* spp. are similar to the results obtained in German coastal waters, where the presence of *Vibrio* spp. was significantly associated with temperature and salinity (2). It has been known that *V. parahaemolyticus* and *V. alginolyticus* are ubiquitous year round in the German North Sea, where salinity is high. Both species are known to respond strongly to increased water temperatures and seasonal cycles (2). In the central Wadden sea of northern Europe, *V. alginolyticus* and *V. parahaemolyticus* were omnipresent, whereas *V. vulnificus* was limited to summer months, suggesting that water temperature is the most important factor in the area (9).

In contrast, *V. vulnificus* was detected only in the summer months in the North Sea in Germany, whereas non-O1 and non-O139 *V. cholerae* strains occurred irregularly and did not follow any seasonal patterns (2). In French Mediterranean coastal lagoons, *V. vulnificus* was limited to summer in water, sediment, and shellfish samples (18). In a Danish marine environment, low numbers of *V. vulnificus* were present in water and sediment, with 0.8 to 19 CFU/liter from June to mid-September in water and 0.04 to 11 CFU/g from July to mid-November in sediment (25). In fact, *V. vulnificus* proliferates when the water temperature exceeds 18°C, usually in the range of 9 to 31°C and at low to moderate salinity (1 to 34 ppt) (18), and often the number dropped to nearly undetectable levels if the temperature dropped below 10°C (18, 19, 26). In this study, salinity and pH in tidal water were found to be significantly lower in summer (June). Possible reasons for this phenomenon might be freshwater discharge or submarine discharge due to the monsoon season in South Korea (27). Other than environmental factors, biological factors such as marine plankton, specifically zooplankton, show a stronger association with *Vibrio* species than phytoplankton (1). As many as 10^9 cells/g (wet weight) of *Vibrio* species were counted from plankton, which is higher than the *Vibrio* colony count from the surrounding water column (1). In the Chesapeake Bay, almost all the total viable count of plankton is composed of *Vibrio* species. When winter is over, vibrios released from the sediment attach to zooplankton and reproduce rapidly as the temperature increases (28). Other factors, such as *V. vulnificus*-positive fish, were found to be inversely correlated to salinity (Pearson's correlation coefficient $r = -0.91077$, $P < 0.0001$) and positively correlated to temperature (Pearson's correlation coefficient $r = 0.62481$, $P < 0.0298$) in the northern Gulf of Mexico (24). *V. parahaemolyticus* was frequently reported from tropical and subtropical oysters (99% positive in Brazil, 94% in India, 100% in the United States, and 71% in Taiwan) (10).

Most environmental *V. parahaemolyticus* strains are considered nonpathogenic because of the low frequencies of detection of *tdh* and *trh*. It has been shown that in general, only 1 to 2% of environmental *V. parahaemolyticus* strains contain *tdh*; however, this amount is sufficient to have a large impact on human health, particularly in tropical developing countries (29). In the highly populated regions along the coasts of South Carolina and Georgia in the United States, environmental isolates of *V. parahaemolyticus* containing both *tdh* and *trh* were detected at relatively low levels of 4.3% and 0.3%, respectively (12). In Galicia, Spain, the pattern of virulence genes observed in *V. parahaemolyticus* was that *trh*$^+$ strains were most common in fall, when the seawater is warmer and less saline, and *tdh*$^+$ strains were most common in the winter and spring (30). However, in contrast to what was observed in Spain, various studies of the distinct geographical distributions of the temperature-mediated affinity of *tdh*$^+$ and *trh*$^+$ strains have suggested that *trh*$^+$ strains dominate in cold waters, whereas *tdh*$^+$ strains dominate in warmer waters (31–34). Similar to our study, the year-round presence of *V. alginolyticus* and *V. parahaemolyticus* has been reported by others (2, 9). Non-O1/non-O139 *V. cholerae* and *V. vulnificus*, which were found in this study, showed season-specific prevalence and were found only in summer in tidal water and mud samples; however, in Germany, *V. cholerae* followed no seasonal pattern (2). *V. parahaemolyticus* strains containing *trh* were present across the summer and fall, with the highest occurrence in summer (6.5%) for mud samples and in fall (15.9%) for tidal water. Our

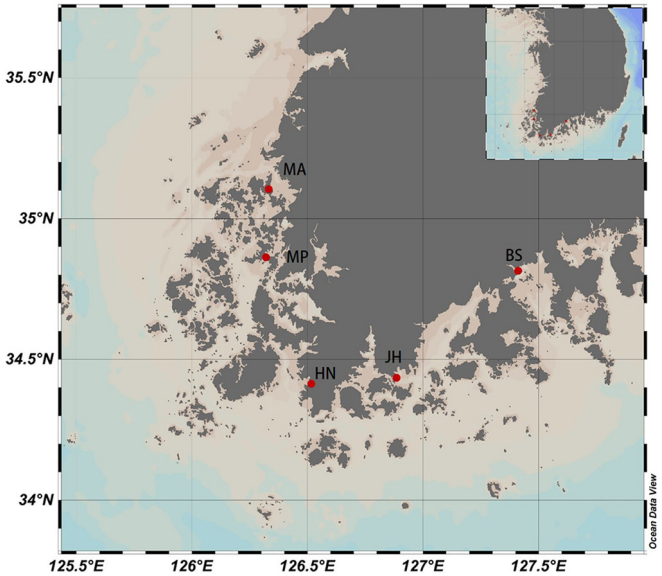

FIG 3 Study area for five sampling points along the southern coastal region of South Korea: Muan (MA), Mokpo (MP), Haenam (HN), Jangheung (JH), and Boseong (BS). The map was generated using the Ocean Data View software (R. Schlitzer, 2015 [odv.awi.de]).

results are similar to the findings in Galicia, Spain, where the prevalence of the *trh* gene peaked in fall, when the seawater temperature is higher (30). In contrast, the *tdh* gene was found in fall (6.1%) in tidal water and was rarely detected in mud samples. This result does not agree with the findings from other studies, where the *tdh* gene was found across the winter and spring in seawater in Galicia, Spain (30), and in the warmer waters in other places (31–34). *V. parahaemolyticus* strains containing *trh* and *tdh* genes also decreased to near zero in December and February and were absent in April, even though the temperature gradually increased. The *vvp* genes of *V. vulnificus* were detected in most strains isolated in June from both tidal water and mud samples and in September from tidal water, suggesting a higher possibility of causing diseases in humans during these times. In the United States, cases of oyster-associated human infections in warm-water months were equivalent to the amount of *V. vulnificus* cells present in seawater and shellfish (35).

In conclusion, *Vibrio* spp. isolated from southern South Korean coastal tidal water and mud samples showed species-specific seasonal patterns, with *V. cholerae* and *V. vulnificus* limited to summer. The other enteropathogenic species *V. alginolyticus* and *V. parahaemolyticus* were the dominant species in these areas year round. Interestingly, the MPN of *Vibrio* spp. in tidal water and temperature in mud showed a significant linear relationship with *V. alginolyticus*. *Vibrio* spp. were consistently more abundant in mud than in tidal water, suggesting that mud could serve as a reservoir for *Vibrio* spp., especially in winter. Moreover, the virulence traits of these enteropathogenic *Vibrio* spp. were restricted to warmer months, even though some species were present in all seasons. This year-round study suggests that the warmer season-specific virulence factors of *Vibrio* species and the risk to the human population of contagion by these pathogens might accelerate as the temperature increases due to global warming. Thus, it is necessary for long-term monitoring of these enteropathogenic *Vibrio* spp. to fully understand their distribution patterns in the region.

MATERIALS AND METHODS

Sampling and isolation of *Vibrio* strains from tidal water and mud samples. Tidal water and mud samples were collected from five sites, Muan (MA), Mokpo (MP), Haenam (HN), Jangheung (JH), and Boseong (BS), located along the coast of southern South Korea (Fig. 3) in June (summer), September (fall), and December (winter) 2013 and in February (winter) and April (spring) 2014. The sampling locations, major land use and activities, and human populations are described in Table S1 in the supplemental

material. Each sample was collected in triplicate from three points in the site that were approximately 10 m apart. Tidal water and mud samples were transported to the laboratory in sealed sterile plastic bottles and plastic bags on ice on the day of sampling. Approximately 2 liters of coastal sea surface tidal waters were collected, during either flood tide or ebb tide. Temperature, pH, salinity, electric conductivity (EC), and turbidity were measured *in situ* using a YSI 63 instrument (YSI Inc., Yellow Springs, OH) and a turbidimeter (TN-100; Thermo Scientific Eutech Products, Vernon Hills, IL). Biological oxygen demand (BOD) was measured with a YSI Pro 20-BOD probe (YSI Inc., Yellow Springs, OH) as previously described (36). A most-probable-number (MPN) estimate of *Vibrio* sp. numbers was done using standard MPN tables for a three-tube series in triplicate (15, 37). One milliliter of each tidal water sample was diluted using 9 ml of alkaline peptone water (APW), and 10^{-1} to 10^{-6} dilutions were made in 9 ml of APW in triplicates. After inoculation, all tubes were incubated overnight at 37°C. *Vibrio* strain isolation was conducted by filtering 1-ml, 10-ml, and 100-ml aliquots of tidal waters onto the surfaces of 0.45-μm-pore size membranes (Advantec, Tokyo, Japan). The membrane filters were incubated on thiosulfate-citrate-bile-sucrose (TCBS) (Difco, Detroit, MI) agar plates at 37°C for 18 to 24 h. Presumptive *Vibrio* isolates, which appeared as green and yellow colonies, were randomly selected, streaked, and incubated under the same conditions. All single colonies were enriched using APW and preserved at −70°C in TSG buffer (tryptic soy broth, 1% sodium chloride, and 24% glycerol) (38) in 96-well tissue culture test plates (SPL Life Sciences, Pucheon, South Korea) until use. For mud samples, about 10 cm of the upper layer of mud was removed, and mud samples underneath at about 5-cm depth (0.5 kg) were collected in duplicate. The temperature and EC of the mud sediment were measured *in situ* with ProCheck (Decagon Devices, Pullman, WA). The pH was measured using a pH Spear (Thermo Scientific Eutech Products) by suspending 10 g of air-dry mud soil into 10 ml of deionized water according to procedures described previously (39). For bacterial enrichment, 10 g of the mud samples was added to 40 ml of APW in a 50-ml plastic centrifuge tube (Corning Inc., Corning, NY) with shaking at 200 rpm on an orbital shaker (HB-203S; Hanbaek Scientific, Bucheon, South Korea) as previously described (40). After shaking for 2 h, the tubes were then centrifuged at 90 × *g* (lightly) for 10 min (Z 36 HK; Hermle, Wehingen, Germany). Subsequently, 1-ml, 10-ml, and 30-ml aliquots of the supernatant were filtered, and *Vibrio* strains were isolated as described above for the tidal water samples. For MPN enumeration, 10 g of each mud sample was added to 90 ml of APW, and 10^{-1} to 10^{-6} dilutions were made in 9 ml of APW in triplicates. The tubes were incubated overnight at 37°C (15, 37).

DNA extraction. The isolated strains were cultivated by stamping on TCBS agar plates using a microplate replicator (Boekel Scientific, Feasterville, PA) and were grown overnight at 37°C. DNA was extracted as described previously (41). Briefly, single colonies were picked and suspended in 150 μl of 0.05 M NaOH in a 96-well PCR plate (Axygen Scientific, Union City, CA). The cells were heated at 95°C for 15 min in a thermal cycler (Mastercycler Gradient; Eppendorf, Hamburg, Germany) and then centrifuged for 10 min at 82 × *g* (Z400; Hermle, Wehingen, Germany).

Confirmation of genus (*Vibrio*) and identification of species. All extracted DNA was tested by PCR to confirm the genus *Vibrio* and by multiplex PCR to identify the species using the primers listed in Table 5 and an A200 Gradient thermal cycler (LongGene, Hangzhou, China). Four *Vibrio* strains, *V. alginolyticus* KCTC 12696^T, *V. cholerae* NCCP 11179, *V. parahaemolyticus* KCTC 2471, and *V. vulnificus* KCTC 2959^T, were used as positive controls, and *Escherichia coli* NCCP 10004 and a nontemplate control were also used as negative controls. For genus identification, the amplification reaction mixtures contained 1× PCR buffer, 0.2 mM each deoxyribonucleoside triphosphate (dNTP), 1 U of nTaq-HOT polymerase (Enzynomics, Daejeon, South Korea), 10 pmol/μl forward and reverse primers (Macrogen, Daejeon, South Korea), and 1 μl of template in a final reaction volume of 10 μl. The program consisted of a 3-min initial denaturation step at 94°C, followed by 25 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min and a final elongation step at 72°C for 10 min. Amplified products were examined using a rapid automated gel electrophoresis system (QIAxcel Advanced System; Qiagen, Valencia, CA). Isolates identified as *Vibrio* were further tested by using multiplex PCR to determine the species. Amplification reaction mixtures contained 1× PCR buffer, 0.2 mM each dNTP, 0.1 U of nTaq-multi-HOT polymerase, 0.4 pmol/μl each primer (Macrogen, Daejeon, South Korea), and 1 μl of template in a final reaction volume of 10 μl. The PCR amplification program was as follows: a 3-min initial denaturation step, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final elongation step at 72°C for 7 min. Amplified products were examined using the QIAxcel Advanced System. *Vibrio* genus and species were determined by verifying the sizes of the amplified fragments (Table 5). Strains with species identified using the multiplex PCRs were further confirmed by 16S rRNA sequencing using universal primers (Macrogen, Daejeon, South Korea). Ten strains of each identified species for *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were randomly selected from the stock cultures for sequencing (see Table S3 in the supplemental material).

Detection of virulence genes in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Strains identified as *V. cholerae* ($n = 24$), *V. parahaemolyticus* ($n = 1,757$), and *V. vulnificus* ($n = 130$) were tested for the presence of virulence factors. Multiplex PCR was conducted using the primers listed in Table 5. *V. cholerae* NCCP 11179, *V. parahaemolyticus* DSM 25722, and *V. vulnificus* KCTC 2959^T were used as positive controls. *E. coli* NCCP 10004 and a nontemplate control were included as negative controls. Amplification reaction mixtures contained 1× PCR buffer, 0.2 mM each dNTP, 1 U of nTaq-multi-HOT polymerase (Enzynomics, Daejeon, South Korea), 0.5 to 1 pmol/μl each primer (Macrogen, Daejeon, South Korea), and 1 μl of template in a final reaction volume of 20 μl. PCR amplifications were carried out as follows: a 10-min initial denaturation step, followed by 30 cycles of 94°C for 30 s, 51 to 60°C for a 30-s annealing step, and 72°C for 1 min and a final elongation step

TABLE 5 Primers used in this study

Species	Target gene[a]	Primer	Sequence (5′ to 3′)	Size (bp)	Reference
Vibrio spp.	16S rRNA	567F	GGCGTAAAGCGCATGCAGGT	120	44
		680R	GAAATTCTACCCCCCTCTACAG		
V. alginolyticus	*dnaJ*	VM-F[b]	CAGGTTTGYTGCACGGCGAAGA	144	45
		V.al2-MmR	GATCGAAGTRCCRACACTMGGA		
V. cholerae	*ctxA*	CTX-F	GCAGTCAGGTGGTCTTATGC	308	46
		CTX-R	CGTGCCTAACAAATCCCGTC		
	dnaJ	VM-F	CAGGTTTGYTGCACGGCGAAGA	375	45
		VC-Rmm	AGCAGCTTATGACCAATACGCC		
	rfb	O1-F	GTTTCACTGAACAGATGGG	192	46
		O1-R	GGTCATCTGTAAGTACAAC		
	rfb	O139-F	AGCCTCTTTATTACGGGTGG	449	46
		O139-R	GTCAAACCCGATCGTAAAGG		
	tcp	tcpA_72F	CACGATAAGAAAACCGGTCAAGAG		22
		tcpA_477R	CGAAAGCACCTTCTTTCACGTTG	451 (El Tor), 620 (classical)	
		tcpA_647R	TTACCAAATGCAACGCCGAATG		
V. parahaemolyticus	*dnaJ*	VM-F	CAGGTTTGYTGCACGGCGAAGA	96	45
		VP-MmR	TGCGAAGAAAGGCTCATCAGAG		
	tdh	tdh-F	TCCATCTGTCCCTTTTCCTGC	278	This study
		tdh-R	CGAACACAGCAGAATGACCG		
	trh	trh_F	TACCTTTTCCTTCTCCAGGTTCGG	122	47
		trh_R	TCGTTTTATGTTTCGGTTTGTCCAGT		
V. vulnificus	*dnaJ*	VM-F	CAGGTTTGYTGCACGGCGAAGA	412	45
		VV-Rmm	GTACGAAATTCTGACCGATCAA		
	vvp	vvp_F	GACGTTCAAGCTGACGATGC	598	This study
		vvp_R	CACGCCCACTTGGTTAAACG		

[a]Genes: *dnaJ*, housekeeping gene that encodes heat shock protein 40, for identification of *Vibrio* species; *ctxA*, cholera toxin; *rfb*, serotype O1/O139-specific *rfb* region; *tcp*, El Tor/classical toxin-coregulated pilus (TCP); *tdh*, thermostable direct hemolysin (TDH); *trh*, TDH-related hemolysin; *vvp*, elastolytic protease.
[b]Universal forward primer for five *Vibrio* species.

at 72°C for 7 min. Amplified products were examined by agarose gel electrophoresis (2%) and ethidium bromide staining.

Statistical analysis. Principal-component analysis (PCA) was performed to examine the component distribution and correlation of the MPN of the presumptive *Vibrio* spp. with environmental parameters using CANOCO v5.0 (Microcomputer Power, Ithaca, NY). One-way ANOVA, Pearson's correlation, Spearman correlation, and linear regression were performed using SPSS Statistics v17.0 (SPSS Institute, Cary, NC) with a significance level of 0.05. One-way ANOVA was used to examine the mean difference between seasons and sites for all environmental parameters. A *post hoc* test was performed based on the least significant difference (LSD) at a significance level of 0.05. Pearson's correlation, Spearman correlation, and linear regression were used to investigate the correlations between the occurrence of omnipresent *Vibrio* spp. (*V. alginolyticus* and *V. parahaemolyticus*) and environmental parameters. By assuming that all variables are normally distributed, including those for the low-concentration samples, values below the limit of detection (LOD) were neglected (42, 43).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02680-16.

TEXT S1, PDF file, 1.2 MB.

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All of the authors of this study declare that there are no conflicts of interest.

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