



Original Article

Distribution of Pathogenic *Vibrio* Species in the Coastal Seawater of South Korea (2017-2018)



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ABSTRACT

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Objectives: Pathogenic *Vibrio* species are widely distributed in warm estuarine and coastal environments, and can infect humans through the consumption of raw or mishandled contaminated seafood and seawater. For this reason, the distribution of these bacteria in South Korea was investigated.

Methods: Seawater samples were collected from 145 coastal area points in the aquatic environment in which *Vibrio* species live. Environmental data (i.e., water temperature, salinity, turbidity, and atmospheric temperature) was collected which may help predict the distribution of the species (data not shown). Seawater samples were filtered, and incubated overnight in alkaline peptone water, at 37°C. Using species-specific polymerase chain reaction methods, screening tests were performed for the *hlyA*, *ctxA*, *vvhA*, and *tlh* genes. Clones of pathogenic *Vibrio* species were isolated using 3 selective plating media.

Results: In 2017, total seawater isolation rates for *Vibrio vulnificus*, *Vibrio cholerae* (non-pathogenic, non-O1, non-O139 serogroups), and *Vibrio parahaemolyticus* were 15.82%, 13.18%, 65.80%, respectively. However, in 2018 isolation rates for each were 21.81%, 19.40%, and 70.05%, respectively.

Conclusion: The isolation rates of pathogenic *Vibrio* species positively correlated with the temperature of seawater and atmosphere, but negatively correlated with salinity and turbidity. From 2017 to 2018, the most frequent seawater-isolated *Vibrio* species were *V. parahaemolyticus* (68.10%), *V. vulnificus* (16.54%), and non-toxicogenic *V. cholerae* (19.58%). Comprehensive monitoring, prevention, and control efforts are needed to protect the public from pathogenic *Vibrio* species.

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Introduction

More than 100 *Vibrio* bacterial species which are ubiquitous in seawater, marine, and estuarine environments, have been identified worldwide. Among all *Vibrio* species, the most clinically significant include *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* [1,2]. Cholera is an infectious disease caused by *V. cholerae*; a symptom of which is severe diarrhea and this may lead to dehydration, and death if untreated. Moreover, cholera outbreaks are seasonal, and in specific biogeographical

zones with warmer temperatures, and ecology [1]. Cholera is an ancient disease and *V. cholerae* reportedly has killed millions of people from warm tropical to cold temperate regions of the world. Evidently, *Vibrio* infections occur during summer and early fall, corresponding to the period of warmer seawater temperatures [3]. Almost all cases of food-borne *Vibrio* infections are associated with a recent history of handling or consuming seafood (primarily raw oysters) [1-5].

By the end of the 20th century, it was reported that *V. cholerae* was completely eliminated from most parts of the

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developed world. However, it remained endemic to many tropical regions [4]. In recent years, this species has been detected in temperate regions and in many developed countries worldwide [6-11]. Its extreme diversity has facilitated its prevalence in North America, and among the prominent halophilic *Vibrio* species, *V. parahaemolyticus* is the leading cause of seafood-associated disease globally [7, 9]. In the 1950s, seafood-borne *Vibrio* species were reported, wherein a strain which caused the pandemic outbreak and its variants, were identified in far East Japan [3,12]. In South Korea, the main cause of seafood-borne disease was determined to be infection by *V. parahaemolyticus*, leading to 18 outbreaks (223 patients) in 2010, 9 outbreaks (133 patients) in 2011, 11 outbreaks (195 patients) in 2012, 5 outbreaks (40 patients) in 2013, 7 outbreaks (78 patients) in 2014, 5 outbreaks (25 patients) in 2015, 22 outbreaks (251 patients) in 2016, and 9 outbreaks (354 patients) in 2017 (Korea Ministry of Food and Drug Safety, 2019). Similarly, *V. vulnificus* was another leading cause of seafood-borne illness and mortality in Korea, resulting in deaths from infection, and specifically 371 patients and 183 deaths in a 7-year period from 2011 to 2017 [Korea Centers for Disease Control and Prevention (KCDC), 2019]. According to the US Centers for Disease Control and Prevention, since 2011, the incidence of *Vibrio* infections has increased dramatically in the United States [13].

Many surveillance and research studies regarding human pathogenic *Vibrio* species, ranging from bacterial characterization, to antimicrobial resistance, distribution, and human infection, have been reported in South Korea [14-19]. In 2001 and 2016, 2 toxigenic *V. cholerae* outbreaks were reported to be due to the ingestion of raw fish and raw seafood in South Korea [17,20]. Since South Korea has specific peninsular plateau geographical characteristics, and its people traditionally favor seafood consumption, KCDC has been investigating the seawater distribution of *Vibrio* species, and analyzing the genetic characteristics of isolated strains and their relationship with environmental factors, to use these data for the prevention of human infectious disease [21,22]. Based on these results, the KCDC aims to prevent the emergence of human infection due to the abnormal growth of pathogenic *Vibrio* species resulting from climate change. These surveillance results may be helpful to track the reasons for epidemic outbreaks of *Vibrio* species.

Materials and Methods

1. Sample collection and amplification

Seawater samples were collected bimonthly during 2017-2018 from 145 South Korean coastal area collection points. Sample collection was performed by 11 Coastal

Quarantine stations and 2 Metropolitan Institutes of Health and Environment (Jeollanam-do and Incheon; Figure 1). More than 500 mL of seawater was collected within 1 meter of the seawater surface. Collected seawater samples were filtered using a 0.45- μ m nitrocellulose membrane filter paper (Pall laboratories, NY, USA). After filtration, membranes were incubated in 5 mL of alkaline peptone water (Oxoid, Basingstoke, UK) for 8-16 hours, at 37°C.

2. DNA extraction

For DNA extraction, 1 mL of seawater-enriched broth sample was used. Seawater samples were pelleted by centrifugation at 12,000 x g for 2 minutes, at room temperature. After discarding the supernatant, 200 μ L of distilled water was added to each sample. Samples were then heated to 100°C for 10 minutes, and subsequently cooled to -20°C for 10 minutes. DNA was collected by centrifugation at 12,000 x g for 2 minutes, at room temperature. The extracted DNA samples were stored at -20°C until required.

3. Screening of *Vibrio* species

Specifically-designed primers were used to amplify a fragment from each of the species-specific genes: *V. cholerae* (*hlyA* and *ctxA*), *V. vulnificus* (*vvhA*), and *V. parahaemolyticus* (*tlh*). The *Vibrio* 4-plex real-time polymerase chain reaction



Figure 1. Study area comprising the 145 sampling points along the whole coastal region of South Korea.

(PCR) Kit (Kogenebiothech, Seoul, South Korea) was used for genetic characterization, according to the manufacturer's real-time PCR protocol (Applied Biosystems, CA, USA).

4. *Vibrio* species culture and colony isolation

Seawater-enriched broth samples were spread on selective medium plates, namely CHROMagar *Vibrio* (CHROMagar, Paris, France), thiosulfate citrate bile salt agar (Difco, MI, USA), and Tryptic Soy Agar (Difco, Michigan, USA), and incubated for > 16 hours at 37°C. After incubation, colonies that were suspected as *Vibrio* species were picked from each plate, and were confirmed using the VITEK II (BioMérieux, Paris, France) system and real-time PCR.

5. Genetic determination of *V. vulnificus*

Colonies that were suspected to be of *V. vulnificus* were extracted from each plate and confirmed by PCR methods for environmental and clinical types. Tri-primer PCR methods [23] based on targeted 16S rRNA genes, were performed using a Bio-Rad Thermal Cycler (Bio-Rad, CA, USA) using Maxime PCR PreMix I-star taq (Intronbio, Seongnam, South Korea) containing 2.5 U i-Star Taq DNA polymerase, 2.5 mM of each dNTP, 1×reaction buffer, and 1×gel loading dye as stabilizer.

Conventional tri-primer PCR was performed for amplification of the 16S rRNA gene [23] and specifically, the 237-bp (Vib 2F and 3R) and 825-bp (Vib 1F and 3R) fragments of 16S rRNA to distinguish between environmental and clinical types. The 16S rRNA tri-primer set which was used included Vib 1F (5'- GTGGTAGTGTTAATAGCACT-3'), Vib 2F (5'- TCTAGCGGAGACGCTGGA-3'), and Vib 3R (5'- GCTCACTTTCGCAAGTTGCC-3'). The PCR conditions were 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C

for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a single extension cycle at 72°C for 5 minutes.

Results

From 2017 to 2018, and for the 145 surveillance points, the annual percentages of seawater-isolated, laboratory-confirmed, pathogenic *Vibrio* cases reported in South Korea in 2017 were: *hlyA*, 28.52% (678/2,377 cases), *ctxA*, 0% (0/2,377 cases), *vvhA*, 33.61% (799/2,377 cases), and *tlh*, 77.32% (1,838/2,377 cases). In 2018 pathogenic *Vibrio* cases were: *hlyA*, 35.06% (987/2,815 cases) *ctxA*, 0% (0/2,815 cases), *vvhA*, 43.09% (1,213/2,815 cases), and *tlh*, 74.85% (2,107/2,815 cases), which were an increase on 2017 figures (Table 1). Similarly, isolation of bacteria increased in 2018 relative to those in 2017 (Table 1). However, there was no significant difference in mortality rates of *V. vulnificus*-infected patients between 2017 and 2018. In 2017 and 2018, the occurrence of 46 *V. vulnificus* septicemia cases related to eating contaminated seafood, raw fish, and oysters were reported (Table 2). Mortality rates for patients suffering septicemia caused by *V. vulnificus* were 52.2% (24/46) in 2017, and 42.6% (20/47) in 2018 (Table 2). Of note, septicemia cases where the infection was caused by *V. vulnificus* resulted in the highest death rates among all foodborne infectious diseases.

Analysis of seawater-isolated *V. vulnificus* by 16S rRNA-based classification revealed 4 environmental and 81 clinical type cases in 2017. In 2018, there was an increase in both clinical ($n = 38$) and environmental types ($n = 489$) compared to those in the previous year (Table 3; Figure 2). From 2017 to 2018, the monthly distribution of seawater-isolated, laboratory-confirmed pathogenic strains, had the highest numbers of

Table 1. PCR amplification, and positive isolation of 3 pathogenic *Vibrio* species in the coastal seawater of South Korea from 2017 to 2018.

Year	Target genes	No. PCR positive samples	PCR positive (%)	No. bacterial isolates	Positive isolation (%)	No. total samples
2017	<i>hlyA</i>	678	28.52	376	15.82	2,377
	<i>ctxA</i>	0	0	0	0	
	<i>vvhA</i>	799	33.61	313	13.17	
	<i>tlh</i>	1,838	77.32	1,564	65.80	
2018	<i>hly</i>	987	35.06	641	21.81	2,815
	<i>ctxA</i>	0	0	0	0	
	<i>vvh</i>	1,213	43.09	546	19.40	
	<i>tlh</i>	2,107	74.85	1,972	70.05	

PCR amplification of *Vibrio* species-specific genes including *hlyA*, *ctxA* (*V. cholerae*), *vvhA* (*V. vulnificus*), and *tlh* (*V. parahaemolyticus*). PCR = polymerase chain reaction.

Table 2. Occurrence of septicemia cases and deaths caused by *Vibrio vulnificus*, during 2017 and 2018.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2017	0	0	0	1	0* (1)†	1 (1)	2	13 (8)	19 (11)	9 (3)	1	0	46 (24)
2018	0	0	0	1	0	2	7 (3)	16 (8)	10 (6)	11 (3)	0	0	47 (20)

* Number of patients with septicemia caused by *V. vulnificus*.

† Number of patient deaths from septicemia caused by *V. vulnificus*.

Table 3. Classification of *Vibrio vulnificus* seawater isolates based on tri-primer PCR amplification of 16S rRNA.

Year	Environmental-type	Clinical-type	No. total isolates
2017	4	81	313
2018	38	489	546

PCR amplification for tri primer methods.

PCR = polymerase chain reaction.

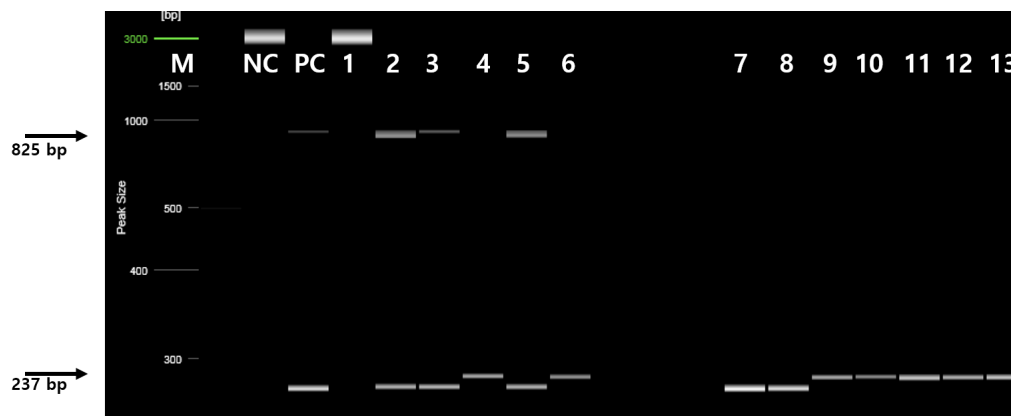


Figure 2. Conventional PCR results of the amplification of the 16S rRNA gene.

Lane 9-13: clinical type and lanes 2, 3, and 5: environmental type of *Vibrio vulnificus*.

M = maker; NC = negative control; PC= positive control (237 and 825 bp), clinical type (237 bp) and environmental type (237 and 825 bp); PCR = polymerase chain reaction.

V. cholerae non-O1/non-O139 in August 2017 and July 2018, *V. vulnificus* in August 2017 and September 2018, and *V. parahaemolyticus* in August 2017 and September 2018 (Tables 4 and 5).

Discussion

Researchers have historically paid attention to disease caused by *V. cholerae* and placed relatively minor medical focus on other *Vibrio* species, some of which are now emerging as pathogens that can cause human disease with mild to severe

symptoms [24]. The surveillance work in this study was representative of the *Vibrio* species and specifically, the coastal seawater distribution of pathogenic *Vibrio* species in South Korea. However, it should be noted that the relationship to human infections may not be adequately represented.

Before the endemic outbreak of toxigenic *V. cholerae* in 2016, the KCDC had been investigating seawater distribution of *Vibrio* species based on only 3 surveillance points for each of the 11 coastal quarantine stations [17,20,21,25]. Following the endemic outbreak, the surveillance points were extended to 13, for each of the 11 coastal quarantine stations. Thus, the surveillance areas for pathogenic *Vibrio* species had been

Table 4. Monthly occurrence and distribution of pathogenic *Vibrio* species (detected by real-time PCR and colony isolation), from seawater samples collected at 145 points, from 35 coastal sites in South Korea during 2017.

Month	No. of samples	No. of sites	No. of positives					
			<i>V. cholerae</i> non-O1/non-O139		<i>V. vulnificus</i>		<i>V. parahaemolyticus</i>	
			PCR-positive	Colony isolation	PCR-positive	Colony isolation	PCR-positive	Colony isolation
Jan	66	3	12 (18.18)	2 (3.03)	0 (0)	0 (0)	28 (42.42)	20 (30.30)
Feb	70	3	13 (18.57)	4 (5.71)	0 (0)	0 (0)	27 (38.57)	25 (35.71)
Mar	70	3	13 (18.57)	10 (14.29)	5 (7.14)	1 (1.43)	23 (32.86)	17 (24.29)
Apr	290	13	38 (13.10)	21 (7.24)	21 (7.24)	12 (4.14)	130 (44.83)	96 (33.10)
May	290	13	63 (21.65)	35 (12.03)	39 (13.40)	16 (5.50)	233 (80.07)	196 (60.48)
Jun	290	13	81 (27.93)	55 (18.97)	79 (27.24)	37 (12.76)	263 (90.69)	204 (70.34)
Jul	290	13	107 (36.90)	66 (22.76)	129 (44.48)	56 (19.31)	267 (92.07)	223 (76.90)
Aug	290	13	133 (45.86)	73 (25.17)	193 (66.55)	71 (24.48)	272 (93.79)	248 (85.52)
Sep	290	13	94 (32.87)	50 (17.48)	181 (63.29)	66 (22.76)	251 (87.76)	238 (83.22)
Oct	290	13	90 (31.47)	39 (13.64)	136 (47.55)	48 (16.55)	244 (85.31)	222 (77.62)
Nov	70	3	17 (25.76)	12 (18.18)	11 (16.67)	6 (8.57)	54 (81.82)	50 (75.76)
Dec	70	3	17 (25.76)	9 (13.64)	5 (7.58)	0 (0)	46 (68.18)	45 (69.70)

Data are presented as *n* (%).

PCR = polymerase chain reaction.

Table 5. Monthly occurrence and distribution of pathogenic *Vibrio* species as detected by real-time PCR and colony isolation from seawater samples collected at 145 points from 35 coastal sites in South Korea, 2018.

Month	No. of samples	No. of sites	No. of positives					
			<i>V. cholerae</i> non-O1/non-O139		<i>V. vulnificus</i>		<i>V. parahaemolyticus</i>	
			PCR-positive	Colony isolation	PCR-positive	Colony isolation	PCR-positive	Colony isolation
Jan	70	3	19 (27.14)	13 (18.57)	2 (2.86)	0 (0)	31 (44.29)	29 (41.43)
Feb	70	3	12 (17.14)	9 (12.86)	2 (2.86)	0 (0)	19 (27.14)	16 (22.86)
Mar	70	3	20 (28.57)	16 (22.86)	5 (7.14)	1 (1.43)	20 (28.57)	20 (28.57)
Apr	290	13	52 (17.93)	36 (12.41)	26 (8.97)	12 (4.14)	80 (27.59)	74 (25.52)
May	290	13	66 (22.76)	41 (14.14)	40 (13.79)	14 (4.83)	169 (58.28)	147 (50.69)
Jun	290	13	95 (32.76)	64 (22.07)	101 (34.83)	28 (9.66)	244 (84.14)	218 (75.17)
Jul	290	13	150 (51.72)	87 (30.0)	182 (62.76)	71 (24.48)	252 (86.90)	231 (79.66)
Aug	290	13	343 (47.31)	229 (21.38)	465 (64.14)	194 (26.76)	658 (90.76)	619 (85.38)
Sep	290	13	125 (43.10)	62 (21.38)	217 (74.83)	133 (45.86)	280 (96.55)	273 (94.14)
Oct	290	13	80 (27.59)	44 (15.17)	156 (53.79)	85 (29.31)	261 (90.00)	254 (87.59)
Nov	70	3	13 (8.57)	8 (11.43)	12 (17.14)	5 (7.14)	53 (75.71)	52 (74.29)
Dec	70	3	12 (17.14)	5 (7.14)	5 (7.14)	3 (4.29)	40 (57.14)	39 (55.71)

Data are presented as *n* (%).

PCR = polymerase chain reaction.

expanded to monitor coastal areas in Korea.

As previously described, *V. vulnificus* infections in South Korea are the leading cause of septicemia and high mortality, due to seafood-borne and seawater-borne illnesses. To control and prevent human infectious diseases caused by pathogenic *Vibrio* species, plans for future surveillance projects and survey points should consider analyzing the results of marine environmental monitoring projects. Parameters should include temperature changes due to sub-tropical climate, seawater flow, coastal geographical conditions, seawater exchange cycle, and population movement according to urban planning. The surveillance should also consider changes in the distribution patterns of pathogenic *Vibrio* species in the marine environment, the direct relevance to patient disease outbreaks, and death. This should be considered in terms of algae seawater migration, resident time spent in seawater (reflective of the topographical factors of the Korean coast), and the possibility of contact with pathogenic *Vibrio* species.

In conclusion, this report describes the need to prevent *Vibrio* species infections in the general public, and for the surveillance of pathogens in Korean coastal waters. Continuous coastal water monitoring of *Vibrio* pathogens, associated with the early detection of infectious agents, could reduce infection risks, and help to understand the natural ecology and distribution of *Vibrio* pathogens. However, due to the changing nature of the tropical climate, it is necessary to prepare for outbreaks of human infections caused by non-seasonal growth of *Vibrio* species. Therefore, comprehensive monitoring, prevention, and control efforts are required as part of the strategy to protect the public from foodborne and waterborne diseases.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

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