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Distribution of *Vibrio* species isolated from bivalves and bivalve culture environments along the Gyeongnam coast in Korea: Virulence and antimicrobial resistance of *Vibrio parahaemolyticus* isolates

Jong Soo Mok[a,*], Ara Ryu[a], Ji Young Kwon[b], Byeori Kim[a], Kunbawui Park[a]

[a] Food Safety and Processing Research Division, National Institute of Fisheries Science, Busan, 46083, Republic of Korea
[b] Southeast Sea Fisheries Research Institute, National Institute of Fisheries Science, Tongyeong, 53085, Republic of Korea

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ABSTRACT

Vibrio species, including *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, are common pathogens causing seafood-borne illnesses worldwide. In 2017, we monitored the distributions of pathogenic *Vibrio* strains in seawater and bivalves collected along the Gyeongnam coast in Korea, a major source of bivalve shellfish, particularly oysters, as well as products of the raw seafood industry. In addition, we determined the features of virulence and antibiotic resistance in *V. parahaemolyticus* isolates. Among these pathogenic *Vibrio* strains, *V. parahaemolyticus* was present at the highest level in both seawater samples (23.1%) and bivalves (39.4%). Importantly, *V. parahaemolyticus* were detected at high levels (> 75%) in oysters during the summer and frequently present during the oyster-harvesting season, ranging from 12.5% to 50.0%. All strains positive for the virulence genes were isolated from oysters, which are commonly consumed raw in many countries, and the oyster growing water. More than 90.0% of *V. parahaemolyticus* isolates were susceptible to 16 of the 23 antimicrobials tested, which are effective against *V. parahaemolyticus* illness. More than half of the isolates were resistant to at least three antimicrobials; in particular, three antibiotics (ampicillin, cefazolin, and streptomycin) should be excluded as treatment options for *V. parahaemolyticus* infections due to the higher resistance of the isolates. The consumption of raw seafood, including oysters, is common in Korea; therefore, to ensure seafood safety, continuous monitoring of *Vibrio* strains, as well as their virulence and antimicrobial resistance, is necessary in marine food sources.

1. Introduction

Vibrio species are autochthonous bacteria that are natural inhabitants in estuarine and marine environments worldwide (Joseph, Colwell, & Kaper, 1982; Thompson, Iida, & Swings, 2004; Oh et al., 2011; Letchumanan, Chan, & Lee, 2014; Centers for Disease Control and Prevention [; CDC], 2019a). Approximately 12 *Vibrio* spp. can cause human illness, known as vibriosis, and have emerged as a severe threat to human health worldwide (CDC, 2019a; Robert-Pillot, Copin, Himber, Gay, & Quilici, 2014). The major species causing human illness are *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae* (Baker-Austin, Stockley, Rangdale, & Martinez-Urtaza, 2010; Park, Mok, Kwon, Ryu, & Shim, 2019; Robert-Pillot et al., 2014; Silva et al., 2018). *V. parahaemolyticus* is the most common pathogen causing seafood-borne illnesses in many countries, due to the consumption of raw or undercooked seafood, especially bivalve shellfish such as oysters (CDC, 2019a; Elmahdi, DaSilva, & Parveen, 2016; Park et al., 2018a). In the USA, the most common *Vibrio* species causing human illness is also *V. parahaemolyticus*, which is estimated to cause 45,000 illnesses annually; moreover, most people become infected by eating raw or undercooked shellfish, particularly oysters (CDC, 2019a).

Although not all *V. parahaemolyticus* strains are pathogenic in humans (Xie, Wu, Zhang, Xu, & Cheng, 2017), this strain causes the highest incidence of seafood-associated bacterial infections in Korea (Korea Ministry of Food and Drug Safety [KMFDS], 2018). The virulence of *V. parahaemolyticus* is mainly attributed to the presence of two major genes: *tdh* (encoding thermo–stable direct hemolysin) and *trh* (encoding *tdh*-related hemolysin) (Gutierrez West, Klein, & Lovell, 2013; Kang et al., 2017; Xie et al., 2017). Thus, the presence of *tdh*- and/or *trh*-positive *V. parahaemolyticus* strains in marine food sources, particularly oysters, is considered a major public health risk (Park et al., 2018b; Xie et al., 2017).

* Corresponding author.
E-mail address: mjs0620@korea.kr (J.S. Mok).

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Another problem is the occurrence and prevalence of antimicrobial resistant *V. parahaemolyticus* in marine environments. Since the discovery of penicillin in the 1920s, antimicrobials have been used for the treatment of infectious diseases (Aarestrup & Wegener, 1999; Xie et al., 2017). Antimicrobial resistance has emerged in a wide range of infectious agents and has evolved in a variety of bacteria, including *V. parahaemolyticus* (Cabello, 2006; Mazel & Davies, 1999; Park et al., 2018a). This emergence may be related to the misuse of antimicrobials to prevent and treat bacterial infections in aquaculture systems as well as in humans and agriculture (Letchumanan et al., 2015; Yang et al., 2017). In particular, *V. parahaemolyticus* strains with multiple antimicrobial resistance may pose a severe threat to public human health and the commercial aquaculture industry (Al-Othrubi, Kqueen, Mirhosseini, Hadi, & Radu, 2014; Kang et al., 2017; Kim, Eum, Kim, & Park, 2016b; Lesmana et al., 2001; Ottaviani, Susini, Montagna, Monno, & D'Annibale, 2013; Shaw et al., 2014).

Many *Vibrio* species including *V. parahaemolyticus* have been isolated from marine food sources (e.g., shellfish, fish, and seawater). Gyeongnam province, along the southern coast in Korea, is a major source of bivalve shellfish, particularly oysters, as well as other products of the raw seafood industry. The Food and Agriculture Organization of the United Nations (FAO, 2019) reported that Korea was the world's 2nd largest producer of oysters, accounting for almost 5.6% of global production (5,858,341 tons) in 2017. According to Statistics Korea (2019), Korea produced 341,524 tons of oysters in 2018, the largest amount of shellfish produced in the country. In particular, Gyeongnam province, located in the south of Korea, produced the largest amount of oysters in Korea, accounting for ~75% of oyster products. To decrease outbreaks caused by the consumption of raw or undercooked contaminated bivalves, it is important to elucidate the distribution and virulence of potentially pathogenic *Vibrio* strains in aquatic sources. Moreover, for the proper control and prevention of illnesses associated with this bacterium, the antimicrobial resistance of *V. parahaemolyticus* must be monitored. Accordingly, in this study, we determined the incidence of *Vibrio* strains in seawater samples and bivalve shellfish from the Gyeongnam coast in 2017. In addition, we evaluated the features of virulence and antibiotic resistance of *V. parahaemolyticus* isolates.

2. Materials and methods

2.1. Sample collection

The study region is the major shellfish production area along the Gyeongnam coast in Korea. Samples of bivalve shellfish and seawater were collected monthly from 18 fixed sampling stations in commercial shellfish harvesting areas along the Gyeongnam coast from January to December 2017 (Fig. 1). Samples of bivalve shellfish, such as oysters (*Crassostrea gigas*; stations 1, 5, 6, 7, 8, 9, 10, 11, and 12), mussels (*Mytilus galloprovinciallis*; stations 3 and 4), and ark shells (*Scapharca broughtonii*; station 2), were collected from fixed sampling locations in commercial shellfish farms. Surface seawater samples were collected from each sampling location (stations 1, 2, 5–10, and 12–18) into presterilized polyethylene bottles (1000 mL). A total of 127 shellfish samples were collected from fixed sampling stations, including 95 oysters, 24 mussels, and 8 ark shells, and 173 seawater samples were collected from each station at the same time as the shellfish samples.

All samples were maintained at temperatures between 5° and 10 °C in cooler during transport to the laboratory for analysis of *Vibrio* species. Surface water temperature and salinity were measured at the same depths at which the seawater samples were collected, with a YSI 556 Multiprobe System (YSI, Yellow Springs, OH, USA).

2.2. Analysis of Vibrio species

All samples used for the analysis of *Vibrio* species, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholera*, were analyzed immediately after their arrival in the laboratory. The shellfish samples were immediately washed with tap water and shucked. According to the Bacteriological Analytical Manual of the United States Food and Drug Administration (US FDA, 2018), *Vibrio* species in the shellfish and water samples were enumerated through the most probable number (MPN) method. The MPN method is three-tube test using three 10-fold serial dilutions. Briefly, the shellfish meat (200 g) was homogenized with 200 mL of phosphate-buffered saline (PBS; 2.5 mM KH_2PO_4; pH 7.2) using a blender (Waring, Torrington, CT, USA). A 1:10 dilution was prepared by transfer of 20 g of shellfish homogenate (1:1 dilution) into 80 mL of PBS and subsequent serial dilution with PBS. For detection of *Vibrio* species, each PBS-diluted homogenate was placed in 10 mL of alkaline peptone water (APW; pH 8.5 ± 0.2) containing 2% NaCl and incubated for 18–24 h at 35 °C. For analysis of *Vibrio* species, the PBS-diluted seawater samples were placed directly into 10 mL APW containing 2% NaCl and incubated for 18–24 h at 35 °C. Approximately 10 μL aliquots of positive APW culture including shellfish and seawater samples were streaked onto thiosulfate citrate bile salt agar (TCBS; Difco, Detroit, MI, USA) and incubated for 24 h at 35 °C. Thereafter, the colonies suspected to be *Vibrio* spp. were picked from the TCBS agar plates. Presumptive colonies were screened for oxidase production and fermentation activity on triple sugar iron agar (Difco). The *Vibrio* strains were then confirmed with a VITEK system (BioMerieux Vitek, Marcy l'Etoile, France). The results are expressed as MPN/100 mL for seawater samples and MPN/100 g for shellfish meat samples.

In addition, the serotype of *V. cholerae* isolates was determined by slide agglutination with antiserum kits (polyvalent *V. cholerae* O1 antiserum, monovalent Inaba and Ogawa antisera, and monoclonal O139 antiserum; Joongkyeon, Goyang, Korea) for *V. cholerae* O1 and O139, according to the manufacturers' instructions. *V. cholerae* isolates that did not agglutinate with either O1 or O139 antisera were confirmed to belong to non-O1 or non-O139 serogroups.

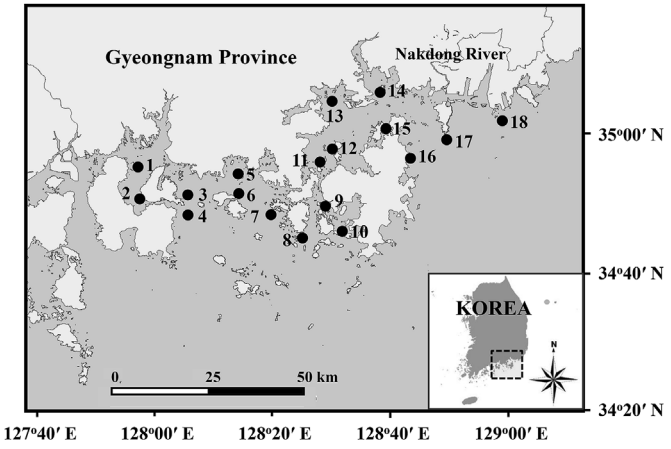

Fig. 1. Sampling stations of seawater and bivalve shellfish such as oysters (stations 1, 5, 6, 7, 9, 10, 11, and 12), mussels (stations 3 and 4), and ark shells (station 2) along the Gyeongnam coast.

2.3. Virulence genes in V. parahaemolyticus isolates

The presence of virulence genes in *V. parahaemolyticus* isolates was determined through polymerase chain reaction (PCR) with a thermal cycler (Takara Bio Inc., Otsu, Japan). The primer sets VPD-1/VPD-2 and VPR-1/VPR-2 (Takara Bio Inc.) were used for amplification of the *tdh* and *trh* genes, respectively. PCR amplification was performed with the following conditions: 35 cycles at 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s. All amplified products were confirmed with a G BOX (Syngene, Cambridge, UK) gel documentation system. The expected size of the amplified DNA was 251 bp for the *tdh* gene and 250 bp for the *trh* gene.

To validate the PCR performance, positive control templates (VP1 and VP2; Takara Bio Inc.) were used for the *tdh* and *trh* toxin genes, respectively.

2.4. Antimicrobial susceptibility tests of V. parahaemolyticus isolates

The susceptibility of *V. parahaemolyticus* isolates to antimicrobials was examined through the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016) and our previous study (Yang et al., 2017). Briefly, Muller-Hinton agar (Difco) and a total of 23 antibiotic disks (BBL, Sparks, MD, USA) were selected for antimicrobial susceptibility tests. The following 23 antimicrobial disks were used in this study (with concentrations per disk given in parentheses): ampicillin (AM; 10 μg), piperacillin (PIP; 100 μg), amoxicillin/clavulanic acid (AMC; 20 μg and 10 μg, respectively), piperacillin-tazobactam (TZP; 100 μg and 10 μg, respectively), cefotaxime (CTX; 30 μg), cefotetan (CTT; 30 μg), cefazolin (CZ; 30 μg), cefixime (CFM; 5 μg), imipenem (IPM; 10 μg), meropenem (MEM; 10 μg), aztreonam (ATM; 30 μg), amikacin (AN; 30 μg), gentamicin (GM; 10 μg), kanamycin (K; 30 μg), streptomycin (S; 10 μg), tetracycline (TE; 30 μg), ciprofloxacin (CIP; 5 μg), ofloxacin (OFX; 5 μg), norfloxacin (NOR; 10 μg), nalidixic acid (NA; 30 μg), trimethoprim/sulfamethoxazole (SXT; 1.25 μg and 23.75 μg, respectively), trimethoprim (TMP; 5 μg), and chloramphenicol (C; 30 μg). The results were classified as resistant (R), intermediately resistant (I), or susceptible (S) according to the CLSI guidelines (CLSI, 2016). *Escherichia coli* ATCC 25922 was used as a quality control organism. The multiple antimicrobial resistance (MAR) index of the isolates was defined as *x/y*, where *x* represents the number of antimicrobial agents to which the isolate was resistant, and *y* represents the total number of antimicrobial agents against which an individual isolate was tested (Titilawo, Sibanda, Obi, & Okoh, 2015).

2.5. Statistical analysis

All statistical analyses were performed in R software (ver. 2.4.3) for Windows (R Development Core Team, 2018). Duncan's multiple-range tests were used to compare differences between bacterial occurrence and environmental parameters at a 95% confidence level with “agricolae package” in the R program, which has a broad functionality in the design of experiments or in the experimental data analysis (de Mendiburu, 2019).

2.6. Key resource table

Resource	Source	Identifier
Antibodies		
VPR- 6 1/VPR-2		
Chemical		
AM		
AMC		
amikacin		
amoxicillin/clavulanic acid		
ampicillin		
AN		
ATM		
aztreonam		
cefazolin		
cefixime		
cefotaxime		
cefotetan		
CFM		
chloramphenicol		
CIP		
ciprofloxacin		
CTT		
CTX		
CZ		
gentamicin		
GM		
imipenem		
IPM		
iron		
kanamycin		
KH2PO4		
MEM		
meropenem		
NA		
NaCl		
nalidixic acid		
NOR		
norfloxacin		
ofloxacin		
OFX		
PBS		
phosphate-buffered saline		
PIP		
piperacillin		
streptomycin		
tazobactam		
TE		
tetracycline		
thiosulfate		
TMP		
trimethoprim		
trimethoprim/sulfamethoxazole		
VP2		
ProteinPeptide		
tdh		

3. Results

3.1. Water temperature and salinity

The monthly variations in surface water temperature and salinity at the sampling stations along the Gyeongnam coast in 2017 are shown in Fig. 2. The monthly mean water temperature ranged from 8.4 ± 1.9 °C to 27.2 ± 0.6 °C. The temperatures were higher during the summer, with the highest temperature recorded in August. Therefore, the mean water temperature exhibited large seasonal variations. The mean water salinity varied from 28.98 ± 1.55 practical salinity units (psu) to 33.43 ± 0.73 psμ, with the highest salinity recorded in January. The water temperature of the survey area was relatively high in summer and low in winter; the salinity was relatively high in winter and low in summer. The water temperature and salinity did not differ significantly among sampling stations, with the exception of a station 16 for salinity (data not shown).

3.2. Distribution of Vibrio species

Table 1 and Supplementary Table A summarize the distributions of *Vibrio* species isolated from the seawater samples and bivalve shellfish

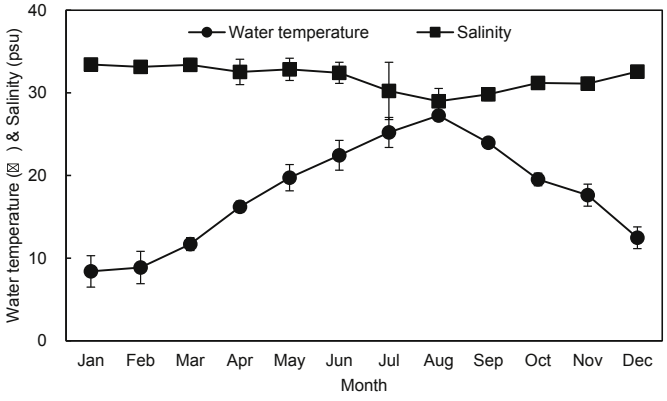

Fig. 2. Monthly variations in water temperature and salinity along the Gyeongnam coast in 2017. Scale bars represent standard deviations.

Table 1
Distribution of pathogenic *Vibrio* strains in seawater samples and bivalve shellfish collected along the Gyeongnam coast in 2017.

Type of samples	Total number of samples	Positive number of samples (%)		
		V. parahaemolyticus	*V. vulnificus*	*V. cholerae*
Seawater	173	40 (23.1)	3 (1.7)	2 (1.2)
Bivalves	127	50 (39.4)	0 (0)	1 (0.8)

obtained in 2017 along the Gyeongnam coast. *Vibrio* strains were detected in 40 (23.1%), 3 (1.7%), and 2 (1.2%) samples for *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, respectively, among 173 seawater samples from 15 monitoring stations. Among 127 bivalve shellfish samples from 11 stations, *Vibrio* spp. were detected in 50 (39.4%), 0 (0%), and 1 (0.8%) samples for *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, respectively. All *V. cholerae* isolates were confirmed as non-O1 or non-O139 strains through slide agglutination tests.

The seasonal and spatial variations of *V. parahaemolyticus* isolated from the seawater samples and bivalve shellfish obtained in 2017 along the Gyeongnam coast are shown in Figs. 3–5. The monthly mean levels of *V. parahaemolyticus* in seawater and shellfish samples ranged from < 3.0 to 22.9 MPN/100 mL and from < 30 to 800 MPN/100 g, respectively (Fig. 3 and Supplementary Table B). The numbers of *V. parahaemolyticus* in the seawater samples varied from < 3.0 to 240 MPN/100 mL, and the highest numbers were detected in September (Fig. 3A). Among the bivalve shellfish samples, *V. parahaemolyticus* ranged from < 30 to 4600 MPN/100 g, and the highest numbers were detected in August (Fig. 3B). During the survey period, the detection rate of *V. parahaemolyticus* in seawater samples was high from summer to early autumn, especially in September (73.3%), August (66.7%), and

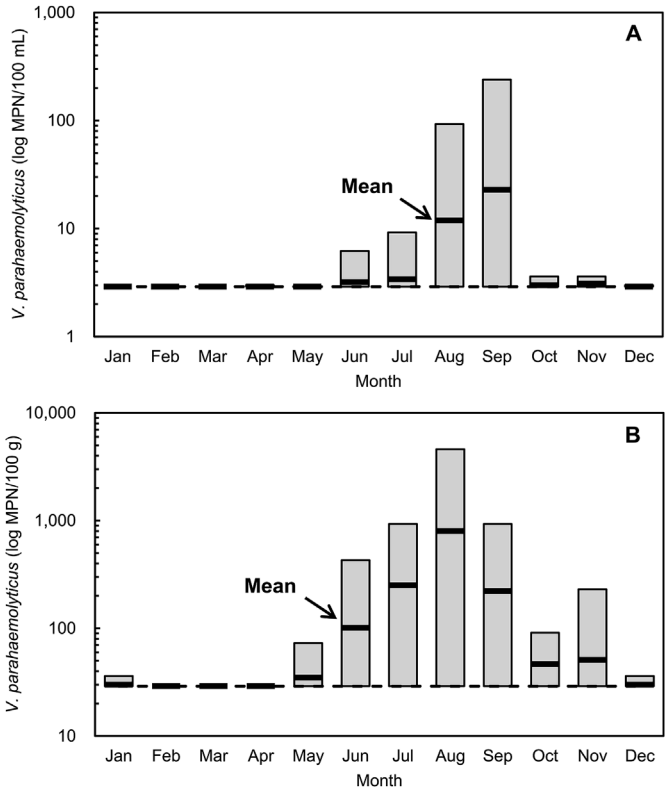

Fig. 3. Range (□) and mean value (−) of *Vibrio parahaemolyticus* numbers in seawater (A) and bivalve (B) samples collected along the Gyeongnam coast in 2017. Detection limits (———) were 3.0 MPN/100 mL for seawater and 30 MPN/100 g for bivalves.

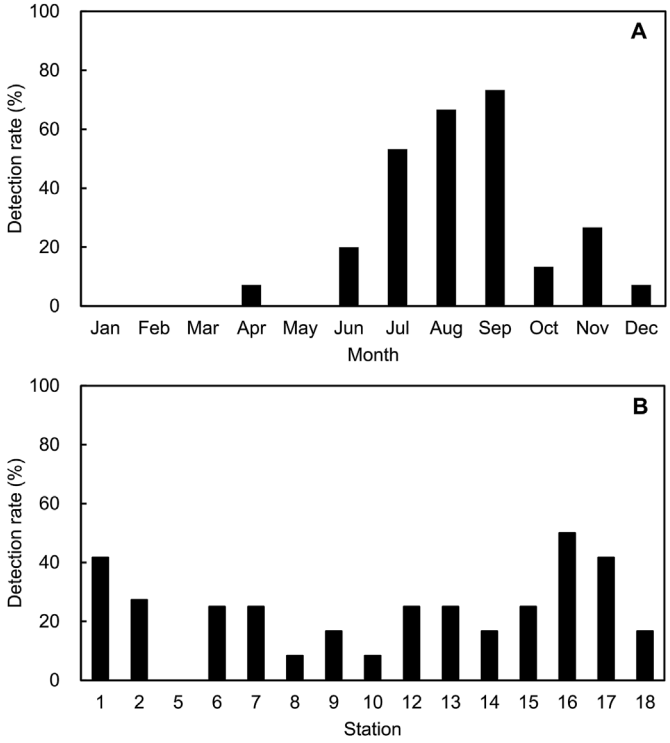

Fig. 4. Seasonal (A) and stationary (B) variations in *Vibrio parahaemolyticus* in seawater samples collected along the Gyeongnam coast in 2017.

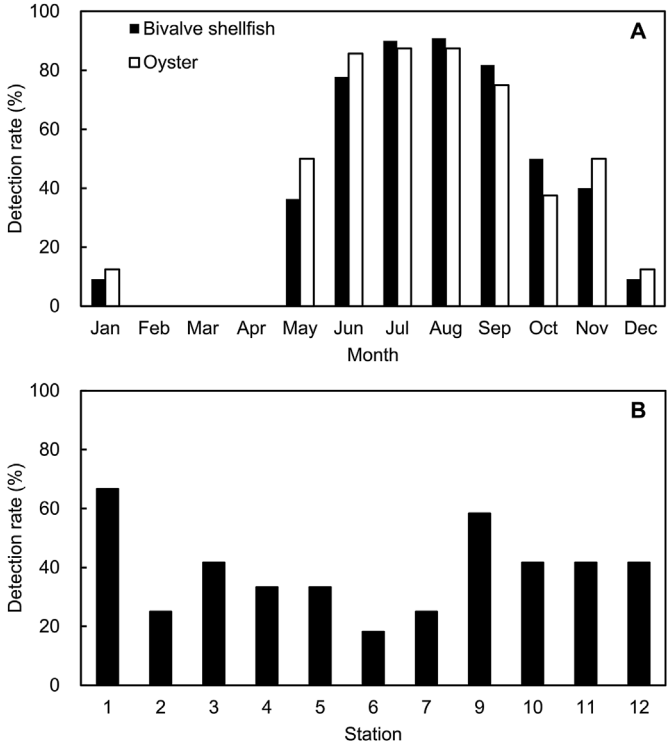

Fig. 5. Seasonal (A) and stationary (B) variations in *Vibrio parahaemolyticus* in bivalve shellfish samples, such as oysters (stations 1, 5, 6, 7, 9, 10, 11, and 12), mussels (stations 3 and 4), and ark shells (station 2), collected along the Gyeongnam coast in 2017. The monthly detection rates of *V. parahaemolyticus* in oysters were calculated by using the oyster samples collected from only oyster sampling stations.

July (53.3%) (Fig. 4A). In contrast, the strain detection rate was low from December to May of the following year, ranging from 0.0% to 7.1%. In particular, no strains were detected in January, February, March, and May, during which the seawater temperature was low. In addition, the *V. parahaemolyticus* in bivalve shellfish samples had the highest detection rate in August (90.9%), followed by July (90.0%), September (81.8%), and June (77.8%) (Fig. 5A). In contrast, the detection rate of the strain in bivalves decreased to below 10.0% between December and April of the following year; in particular, the species was not detected from February to April. Importantly, among the oyster samples consumed raw, *V. parahaemolyticus* was also detected at a very high level from June to September, ranging from 75.0% to 87.5% (Fig. 5A). Notably, the pathogen strain in oysters was detected at a rate ranging from 12.5% to 50.0% during the oyster-harvesting season in Korea, October to January of the following year.

V. parahaemolyticus strains at each seawater sampling station were detected from 0% to 50.0% throughout the survey period (Fig. 4B and Supplementary Table A). Higher detection rates (> 40%) in seawater samples were found at stations 1, 16, and 17, areas that are strongly affected by large rivers such as the Nam River and the Nackdong River, which transport abundant nutrients from inland wastewater. In addition, the detection rates of *V. parahaemolyticus* at each bivalve shellfish sampling station ranged from 18.2% to 66.7% (Fig. 5B and Supplementary Table A). The highest detection rate was at station 1 for oysters, in an area also strongly affected by the Nam River. Among the bivalve samples, the detection rates of *V. parahaemolyticus* in oyster samples (41.1%) collected at sites 1 and 5–12 were slightly higher than those in mussels (37.5%) collected at sites 3 and 4, and ark shells (25.0%) collected at site 2 (Fig. 3B and Table 2). Among the different types of bivalve shellfish, the highest level of *V. parahaemolyticus* were detected also in oysters, ranging from < 30 to 4600 MPN/100 g with the mean level of 145 MPN/100 g (Table 2).

3.3. Virulence genes of V. parahaemolyticus isolates

The virulence genes (*tdh* and *trh*) in *V. parahaemolyticus* isolates from the seawater samples and bivalve shellfish along the Gyeongnam coast in 2017 are summarized in Table 2. We analyzed *V. parahaemolyticus* isolates for the presence of virulence genes using PCR. *V. parahaemolyticus* strains were positive for the *tdh* and *trh* virulence genes in 0 (0%) and 18 (9.5%) isolates, respectively, among the 190 isolates from bivalve shellfish. In contrast, of the 104 seawater isolates, only 1.9% (n = 2) were positive for the *trh* gene, and all seawater isolates were negative for the *tdh* gene. Interestingly, all the strains positive for the *trh* gene were isolated from only oysters and oyster-culture water samples.

Table 3
Antimicrobial resistance of *Vibrio parahaemolyticus* isolates (n = 181) from seawater and bivalve shellfish samples collected along the Gyeongnam coast in 2017.

Antimicrobials	Number (%) of isolates		
	Susceptible	Intermediate	Resistant
Penicillins			
Ampicillin (AM)	13 (7.2)	10 (5.5)	158 (87.3)
Piperacillin (PIP)	173 (95.6)	5 (2.7)	3 (1.7)
β-lactams			
Amoxicillin/clavulanic acid (AMC)	180 (99.4)	1 (0.6)	0 (0)
Piperacillin-tazobactam (TZP)	180 (99.4)	1 (0.6)	0 (0)
Cephems			
Cefotaxime (CTX)[a]	181 (100)	0 (0)	0 (0)
Cefotetan (CTT)	181 (100)	0 (0)	0 (0)
Cefazolin (CZ)	40 (22.1)	0 (0)	141 (77.9)
Cefixime (CFM)[a]	180 (99.4)	1 (0.6)	0 (0)
Carbapenems			
Imipenem (IPM)	181 (100)	0 (0)	0 (0)
Meropenem (MEM)	181 (100)	0 (0)	0 (0)
Monobactams			
Aztreonam (ATM)	130 (71.8)	49 (27.1)	2 (1.1)
Aminoglycosides			
Amikacin (AN)	140 (77.3)	24 (13.3)	17 (9.4)
Gentamicin (GM)	170 (93.9)	9 (5.0)	2 (1.1)
Kanamycin (K)	120 (66.3)	56 (30.9)	5 (2.8)
Streptomycin (S)	13 (7.2)	61 (33.7)	107 (59.1)
Tetracyclines			
Tetracyclin (TE)[a]	181 (100)	0 (0)	0 (0)
Quinolones and fluoroquinolones			
Ciprofloxacin (CIP)[a]	180 (99.4)	1 (0.6)	0 (0)
Ofloxacin (OFX)[a]	181 (100)	0 (0)	0 (0)
Norfloxacin (NOR)	181 (100)	0 (0)	0 (0)
Nalidixic acid (NA)	181 (100)	0 (0)	0 (0)
Sulfonamides			
Trimethoprim-Sulphamethoxazole (SXT)[a]	179 (98.9)	0 (0)	2 (1.1)
Trimethoprim (TMP)	46 (25.4)	80 (44.2)	55 (30.4)
Phenicols			
Chloramphenicol (C)[a]	181 (100)	0 (0)	0 (0)

[a] The antibiotics were recommended by the Center for Disease Control and Prevention (CDC) for the treatment of *Vibrio* species infections (CDC, 2019b, c).

3.4. Antimicrobial resistance profiles of V. parahaemolyticus isolates

The antimicrobial resistance profiles of *V. parahaemolyticus* isolates from seawater samples (71 isolates) and bivalve shellfish (110 isolates) on the Gyeongnam coast in 2017 are shown in Table 3. Among the 181 *V. parahaemolyticus* isolates from seawater and bivalve shellfish samples, a large percentage exhibited high resistance to AM (87.3%), CZ (77.9%), and S (59.1%). The 17 (9.4%) and 55 (30.4%) isolates were also resistant to AN and TMP, respectively, and a small number of isolates (ranging from 1.1% to 2.8%) exhibited resistance to 5

Table 2
Distribution and virulence genes of *Vibrio parahaemolyticus* strains from seawater samples and bivalve shellfish collected along the Gyeongnam coast in 2017.

Samples			Concentration (Mean) (MPM/100 mL or 100 g)	Isolates	
Type	Total number	Positive number (%)		Total number	Positive number for virulence genes
Seawater	173	40 (23.1)	< 3.0–240 (5.5)	104[a]	*tdh* (0), *trh* (2)[b]
Bivalves [c]					
Oyster	95	39 (41.1)	< 30–4600 (145)	144	*tdh* (0), *trh* (18)
Mussel	24	9 (37.5)	< 30–930 (132)	38	*tdh* (0), *trh* (0)
Ark shell	8	2 (25.0)	< 30–430 (85)	8	*tdh* (0), *trh* (0)
Subtotal	127	50 (39.4)	< 30–4600 (139)	190	*tdh* (0), *trh* (18)

tdh, thermostable direct hemolysin; *trh*, tdh-related hemolysin.
[a] The 36 isolates were collected from oyster culture water samples.
[b] Isolates positive for the *trh* gene were collected only from oyster culture water samples.
[c] Bivalve shellfish, such as oysters (stations 1, 5, 6, 7, 8, 9, 10, 11, and 12), mussels (stations 3 and 4), and ark shells (station 2), were collected from each fixed sampling station.

Table 4
Multiple antimicrobial resistance (MAR) index values for *Vibrio parahaemolyticus* isolates (n = 181) from seawater and bivalve shellfish samples collected along the Gyeongnam coast in 2017.

Resistance pattern	Number of antimicrobials	Number (%) of isolates		MAR index
–	0	3	3 (1.6)	0.00
AM or others (patterns)	1	23	23 (12.7)	0.04
AM, PIP	2	1	55 (30.4)	0.09
AM, CZ		33		
AM, S		10		
AM,TMP		5		
CZ, S		3		
CZ, TMP		3		
AM, CZ, AN	3	2	61 (33.7)	0.13
AM, CZ, S		48		
AM, CZ, TMP		5		
AM, S, TMP		4		
CZ, S, TMP		2		
AM, CZ, AN, S	4	3	26 (14.4)	0.17
AM, CZ, S, TMP		21		
AM, CZ, STX, TMP		1		
CZ, S, STX, TMP		1		
AM, CZ, AN, S, TMP	5	5	6 (3.3)	0.22
AM, CZ, GM, S, TMP		1		
AM, PIP, CZ, AN, K, S	6	1	7 (3.9)	0.26
AM, PIP, CZ, AN, S, TMP		1		
AM, CZ, ATM, AN, S, TMP		1		
AM, CZ, ATM, AN, K, S		1		
AM, CZ, AN, GM, K, S		1		
AM, CZ, AN,K, S, TMP		2		

AM, ampicillin; PIP, piperacillin; CZ, cefazolin; ATM, Aztreonam; AN, amikacin; GM, Gentamicin; K, Kanamycin; S, Streptomycin; TMP, trimethoprim.

antimicrobials (PIP, ATM, GM, K, and SXT). In contrast, all *V. parahaemolyticus* isolates were sensitive to 9 of the 23 antimicrobial agents tested (CTX, CTT, IPM, MEM, TE, OFX, NOR, NA, and C), and more than 90.0% of the isolates were also sensitive to 7 antimicrobials (PIP, AMC, TZP, CFM, GM, CIP, and STX). The antimicrobial resistance profiles did not differ significantly between seawater and bivalve samples.

The MAR index values for *V. parahaemolyticus* isolates from the seawater samples and bivalves are shown in Table 4. The MAR index, first suggested by Krumperman (1983), reflects the extent of environmental contamination by antimicrobials and is used to evaluate potential human health risk. MAR index values greater than 0.2 indicate that the marine sources have a high risk of antimicrobial contamination. The MAR index values ranged from 0.00 to 0.26, and the highest MAR index was found in seven isolates that exhibited resistance to six antimicrobials tested. Most *V. parahaemolyticus* isolates (64.1%) had MAR index values between 0.09 and 0.13, indicating that the isolates were resistant to two or three types of antibiotic tested. In contrast, 7.2% of the isolates had a MAR value higher than 0.2, indicating resistance to more than five antimicrobials tested. In addition, of the 181 *V. parahaemolyticus* isolates examined in this study, 55.3% (116 isolates) had multiple-antibiotic resistance to at least three antimicrobials.

4. Discussion

Three major pathogenic *Vibrio* species, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, have emerged as a severe threat to human health worldwide, because they are linked to infections associated with the consumption of raw or undercooked seafood (Robert-Pillot et al., 2014). In the present study, *V. parahaemolyticus* was the most abundant of the three pathogenic *Vibrio* strains. These results are similar to published data from other parts of the world (Robert-Pillot et al., 2014; Rosec, Causse, Cruz, Rauzier, & Carnat, 2012; Serracca et al., 2011; Yang et al., 2017). Most previous studies focused on determining the presence of *V. cholerae* strains with O1 and O139 serogroups, and few studies have reported the prevalence of strains of the non-O1/non-O139 serogroups in seafood (Rosec et al., 2012; Schärer, Savioz, Cernela, Saegesser, & Stephan, 2011). Notably, the *V. cholerae* strains detected in this study were non-O1/non-O139 strains.

In addition, the detection rate of *V. parahaemolyticus* in our study was higher in bivalve shellfish (39.4%) than in the surrounding seawater (23.1%). In previous studies, bivalve shellfish have been found to accumulate different microorganisms at different levels in marine environments, ranging from very low levels to levels more than 100-fold higher than the surrounding seawater (Kim et al., 2017; Mok et al., 2016, 2018; Park et al., 2018b). We previously reported the detection of *V. parahaemolyticus* at levels approximately 2-fold higher in shellfish than in the surrounding seawater (Park et al., 2018b), and the bioaccumulation of fecal coliform bacteria in oysters and mussels from the Korean coast was also reported to be in the range of 6.9–13.4 fold (Mok et al., 2016) and 11.7–30.5 fold (Mok et al., 2018) higher than the surrounding seawater, respectively. In addition, Silva et al. (2018) reported higher detection rates of *V. parahaemolyticus* in oyster samples (83%) than in seawater samples (44%) along the coast of Brazil, whereas the strain was slightly higher in water samples (77.5%) than in oyster samples (70.8%) collected along the coast of Taiwan (Yu et al., 2013). These studies demonstrate that bivalve shellfish accumulate *V. parahaemolyticus* strains at relatively lower levels than fecal coliform bacteria, which are useful indicators of fecal contamination, in marine environments. Additionally, we detected *V. parahaemolyticus* strains along the Gyeongnam coast at relatively high levels at stations strongly affected by large rivers transporting abundant nutrients from inland wastewater. Our findings indicated an abundance of *V. parahaemolyticus* in marine environments likely to be exposed to high-turbidity sources with abundant nutrients from inland wastewater effluent.

In Korea, seafood-borne outbreaks associated with *V. parahaemolyticus* increase dramatically during summer (KMFDS, 2018). Also, the numbers of *V. parahaemolyticus* in seawater samples and seafood (including shellfish) from the Korean coast markedly increase in summer (Han, Yoon, & Kim, 2012; Kang et al., 2016; Park et al., 2016b; Park et al., 2018a, b). In particular, Na, Hong and Chung (2016) reported that *V. parahaemolyticus* strains (isolated from 2220 seawater samples from 11 stations along the Korean coast during 2013–2015) showed the highest correlation coefficient (0.90) with seawater temperature among the environmental factors (temperature of seawater and atmosphere, salinity, and pH). In contrast, the strains exhibited the lowest correlation (0.23) with pH ranging from 7.48 ± 0.08 to 7.67 ± 0.08. Our previous studies also reported that monthly mean pH values varied from 7.92 to 8.32 in surface seawater samples collected from the southern coast of Korea in 2014 (Jung et al., 2017; Park et al., 2016a), which is within this study area. These results indicate that pH values in seawater on the Korean coast showed slightly alkalinity without significant differences all around year, and had relatively lower correlation with *Vibrio* species. Based on the previous studies in Korea, we investigated the seawater temperature and salinity to compare with the occurrence of *Vibrio* species. Johnson et al. (2012) also reported that the abundance and distribution of these species have been linked to environmental factors, most notably temperature and salinity. In this study, *V. parahaemolyticus* in seawater and bivalve samples collected along the Korean coast were present at relatively high levels during summer to early autumn, but decreased in winter. These results indicate that both the occurrence in marine environments and seafood-borne outbreaks of *V. parahaemolyticus* strongly increase during the summer in Korea, and that the detection levels of the strains closely correlate with water temperature along the Korean coast. Moreover, various studies described a positive correlation between the presence of *V. parahaemolyticus* and water temperature (DePaola et al., 2003; Park et al., 2018b; Sudha, Mridula, Silvester, & Hatha, 2014). Additionally, we determined the differences between the detection rates of *V. parahaemolyticus* and the environmental parameters (water temperature and

salinity) at a 95% confidence level in this study. In seawater samples, the detection rate of *V. parahaemolyticus* at each water sampling station showed a significant difference with the salinity only; higher detection rates in seawater samples were found at stations with relatively low salinity (data not shown). A study in Brazil also reported that *V. parahaemolyticus* was largely present in water samples collected the region with relatively low salinity (Silva et al., 2018). Similarly, among various environmental parameters (dissolved oxygen, temperature, pH, and salinity), *V. parahaemolyticus* density in water samples along the coast of Taiwan was significantly correlated with salinity and dissolved oxygen using Pearson coefficients, but surface water temperature and pH were not significantly related to the prevalence of this pathogen (Yu et al., 2013). Meanwhile, a *V. parahaemolyticus* strain isolated from the rhizosphere of the ecosystem dominant estuarine grass was shown to fix N_2, indicating that the capability of some *V. parahaemolyticus* strains to fix N_2 may support their maintenance in nitrogen-limited coastal marine environments, contributing to a broader distribution of pathogenic strains (Criminger, Hazen, Sobecky, & Lovell, 2007).

Additionally, many foodborne outbreaks of *V. parahaemolyticus* in various countries have occurred through the consumption of raw or under-cooked bivalves including oysters, which can accumulate *V. parahaemolyticus* and are an important source of transmission of this pathogen (Kang et al., 2016; Turner, Malayil, Guadagnoli, Cole, & Lipp, 2014; Yamamoto et al., 2008; Zhao, Zhou, Cao, Ma, & Jiang, 2011). Notably, *V. parahaemolyticus*, one of the major seafood-borne gastroenteritis-causing bacteria, is frequently isolated from oysters in Korea (Kang et al., 2017; Lee et al., 2008; Park et al., 2018b). *V. parahaemolyticus* was isolated in more than 75.0% of the oyster samples tested in this study during June to September. Moreover, this pathogen was detected in oysters with a range of 12.5%–50.0% from October to January of the following year, during the oyster-harvesting season in Korea. Similarly, *V. parahaemolyticus* is prevalent in raw oysters collected from the coast in other countries, including 83% of oysters in Brazil (Silva et al., 2018) and 70.8% of oysters in Taiwan (Yu et al., 2013).

The virulence of *V. parahaemolyticus* is attributed to the presence of the *tdh* and *trh* genes (Nishibuchi, Ishibashi, Takeda, & Kaper, 1985; Shimohata & Takahashi, 2010; Su & Liu, 2007; Xie et al., 2017). These genes are considered predominant indicators of virulence in *V. parahaemolyticus*, and their monitoring in seafood, including bivalve shellfish, is critical due to the hazard to human health (Park et al., 2018b; Yang et al., 2017). Gutierrez West et al. (2013) reported that *tdh*-positive *V. parahaemolyticus* strains are more virulent than *trh*-positive strains. In Taiwan, *tdh*-positive *V. parahaemolyticus* (9.34%) strains are detected more often than *trh*-positive strains (3.70%) in oyster-growing environments (Chang, Chen, Su, Pai, & Chiu, 2011). Fortunately, in our study, all the isolates were negative for the *tdh* virulence gene. Of bivalve shellfish tested, all the strains positive for the *trh* gene were isolated from only oysters, which are commonly consumed raw in many countries, including Korea. Oysters are frequently consumed in Korea and are cultured extensively along the southern coast, including the present study area. Similarly, recent studies in oysters from along the Korean coast reported that 9.1%–53.5% of *V. parahaemolyticus* isolates were positive for the *trh* genes; however, all isolates tested were negative for the *tdh* gene (Kang et al., 2016, 2017; Kim et al., 2016a). In our previous study, only 1.7% and 3.5% of the *V. parahaemolyticus* isolates from bivalve shellfish including oysters along the Gyeongnam coast during 2013–2016 were positive for the *tdh* and *trh* genes, respectively (Park et al., 2018b). In this study, the prevalence of virulent *V. parahaemolyticus* was 11.1% (20/180) among the isolates from both oyster and oyster culture water samples, results similar to the prevalence of 10.8% (94/867) and 9.7% (30/308) of strain isolates from oysters and oyster culture environments reported in Taiwan (Chang et al., 2011) and the USA (Chiu, Duan, & Su, 2007), respectively. Interestingly, none of the 252 *V. parahaemolyticus* strains, isolated from 94 oyster samples collected between June 2005 and September 2006 in the USA, possessed the *tdh* or *trh* gene (Han, Walker, Janes, Prinyawiwatkul, & Ge, 2007).

The occurrence and prevalence of antimicrobial-resistant *V. parahaemolyticus* in seafood and marine environments is a major concern regarding human health and veterinary medicine worldwide (Xie et al., 2017; Yang et al., 2017; Park et al., 2018a; Silva et al., 2018). Fortunately, more than 98.0% of *V. parahaemolyticus* isolates from both seawater and bivalve shellfish samples were susceptible to 7 of the 23 antibiotics tested in this study and recommended by the CDC (2019b, c) for the clinical treatment of *Vibrio* species infections; these antibiotics were fluoroquinolones (CIP and OFX), cephalosporins (CTX and CFM), tetracyclines (TE), folate pathway inhibitors (SXT), and phenicols (C). Importantly, all isolates (n = 181) tested in this study were susceptible to TE, which has long been the most commonly used antibiotic in Korean fisheries, particularly for the treatment of heavy *Vibrio* infection (Morris & Tenny, 1985; Oh et al., 2011). In addition, TE can be used to treat severe or prolonged human illnesses due to *V. parahaemolyticus* (Elmahdi et al., 2016). Our results are in agreement with those from many other studies reporting that *V. parahaemolyticus* isolates from marine sources in Korea exhibit very low TE resistance (Kang et al., 2017; Kim et al., 2016b; Oh et al., 2011; Park et al., 2016b; Yang et al., 2017). These results indicate that TE is still very useful for the treatment of *V. parahaemolyticus* infections in Korea.

Generally, *Vibrio* species are highly susceptible to most clinically used antibiotics (Shaw et al., 2014; Letchumanan et al., 2015). Nonetheless, the prevalence of AM and S resistance in *V. parahaemolyticus* stains is very common (Han et al., 2007; Wong, Liu, Wan, & Chen, 2012; Xie et al., 2017; Silva et al., 2018). Consistent with this trend, the strains isolated from both seawater and bivalve samples in this study were very highly resistant to these antibiotics, particularly AM. In Korea, various studies confirmed that *V. parahaemolyticus* strains isolated from marine environments also have high prevalence of resistance to AM (86.4%–100% of isolates) (Son et al., 2005; Kim et al., 2016a, b; Kang et al., 2017; Yang et al., 2017). Additionally, Letchumanan et al. (2015) reported that first-generation antibiotics, including AM, are extensively used in aquaculture, thus decreasing the susceptibility to AM and resulting in a low efficacy of AM for *Vibrio* species treatment. Moreover, Xie et al. (2017) confirmed that *V. parahaemolyticus* clinical isolates are resistant to first-generation antibiotics, such as AM (87.1%) and CZ (64.5%). Similarly, *V. parahaemolyticus* isolates in the present study were highly resistant to a first-generation cephalosporin (CZ, 77.9%); however, more than 99% of the isolates were susceptible to second-and third-generation cephalosporins (CTT, CTX, and CFM). Our findings are in agreement with those of Shaw et al. (2014) in the USA, who reported that a very high percentage (97%) of environmental *V. parahaemolyticus* isolates (n = 77) are susceptible to CTX.

We determined a MAR value of more than 0.2 in 7.2% of the isolates in this study. MAR index values higher than 0.2 indicate high-risk sources for antimicrobial contamination that may poses potential human health risk (Letchumanan et al., 2015). Many studies report that some *V. parahaemolyticus* isolates from marine or clinical sources show multiple-antibiotic resistance, which has become an increasingly critical public health and economic concern (Al-Othrubi et al., 2014; Kang et al., 2017; Kim et al., 2016b; Lesmana et al., 2001; Ottaviani et al., 2013; Shaw et al., 2014). We commonly observed multidrug-resistant isolates in this study. Indeed, more than half of the isolates were resistant to three or more antimicrobial agents, consistent with recent reports in Korea (Kang et al., 2017; Yang et al., 2017).

5. Conclusions

The three major pathogenic *Vibrio* species causing human illness are *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, which have emerged as a major threat to human health worldwide. *V. parahaemolyticus* was found at the highest level among the pathogenic *Vibrio* strains tested in this study. The detection rates of *V. parahaemolyticus* strains closely

correlated with water temperatures along the Korean coast. Notably, *V. parahaemolyticus* strains were detected at high prevalence (> 75.0% of oysters) during June to September. Moreover, this pathogen was detected frequently in oysters during the oyster-harvesting season, and all strains positive for the virulence genes were isolated from oysters, which are commonly consumed raw in many countries, and the oyster growing water. More than 90.0% of the *V. parahaemolyticus* strains (n = 181) isolated in the present study were susceptible to 16 antimicrobials, including 7 agents recommended by the CDC for the clinical treatment of *Vibrio* species infections. In addition, more than 50% of the isolates showed resistance to three antibiotics (AM, CZ, and S), particularly AM. Moreover, more than half of the isolates exhibited multiple-antibiotic resistance to three or more antimicrobial agents. Therefore, more research on *V. parahaemolyticus* strains is needed to reveal the relationship between their occurrence and presence of virulence genes in oysters, particularly during the harvesting season, given the common consumption of raw oysters. In addition, the high multiple-antibiotic resistance of *V. parahaemolyticus* isolates is concerning and warrants ongoing surveillance to protect human health.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.06.023.

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