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Antibiotic and heavy-metal resistance of *Vibrio parahaemolyticus* isolated from oysters in Korea

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ABSTRACT

Vibrio parahaemolyticus, found frequently in oysters and other seafoods, is the most prevalent gastroenteritis-causing pathogen in Korea and other Asian countries. It is associated exclusively with the consumption of raw or improperly cooked contaminated seafood, especially oysters. In this study, we isolated and characterized 59 *V. parahaemolyticus* strains (toxR-positive) from May to October 2016 in shellfish-harvesting areas off the west coast of Korea. The results revealed that none of the isolates contained the *tdh* and *trh* toxicity genes. The multiple antibiotic resistance (MAR) value of most isolates was 0.32, but it was as high as 0.69 in one isolate strain. Moreover, when resistance to heavy metals was examined, the majority of the isolates displayed resistance to Ba^{2+} (98.3%), Co^{3+} (28.8%), Cd^{2+} (16.9%), and Cu^{2+} (13.6%). Interestingly our data revealed that tolerance to heavy metals was prevalent in the *V. parahaemolyticus* strains with more than two antibiotic resistance phenotypes.

1. Introduction

Vibrio parahaemolyticus is a gram-negative halophilic and mesophilic bacterium that is commonly found in estuarine and marine environments. Since it was discovered in the 1950s in Japan, this organism has been isolated from a variety of seafoods, including fish and shellfish, in many countries, including the US, China, and Korea (Fujino et al., 1953; Cook et al., 2002; Lee et al., 2008; Zhao et al., 2011). *V. parahaemolyticus* is a zoonotic pathogen that not only can cause disease in marine animals (Heuer et al., 2009) but also is one of the most important foodborne pathogens, causing gastroenteritis, wound infections, and septicemia (Fujino et al., 1953). *V. parahaemolyticus* is considered the major causative agent of seafood poisoning outbreaks in Asia (Lee et al., 2009; Zhao et al., 2011) and the most important enteric bacterium associated with gastroenteritis after the consumption of fresh seafood in Korea, especially during the summer, accounting for more than 30% of gastroenteritis cases (Ham et al., 2002).

Antibiotics and other chemotherapeutic agents are used on fish farms as feed additives and/or immersion baths to treat and prevent the spread of disease (Heuer et al., 2009), and the risks posed by *Vibrio* species that are major fish and shellfish pathogens have led to the widespread use of such antibiotics. For example, high incidences of resistance to antibiotics such as ampicillin, rifampicin, and streptomycin have been reported in *V. parahaemolyticus* isolates originating from some aquatic products in Asian and European countries, e.g., southern China (Xie et al., 2015), Korea (Kang et al., 2016), Poland (Lopatek et al., 2015), and Italy (Ottaviani et al., 2013). On the other hand, because of increasing industrialization, environmental pollution has become one of the most challenging issues in developing countries. The frequent occurrence of heavy metal-resistant bacteria has been detected in various environments, e.g., marine environments, rivers, and agricultural soil (Ansari et al., 2008; Malik and Aleem, 2011). Water contaminated with industrial pollutants (e.g., heavy metals) is thought to enhance the selection for antibiotic resistance and vice versa. Indeed, a direct link between antibiotic resistance and heavy metal resistance has been well established in a number of studies (Zhao et al., 2012; Kang et al., 2016).

The overuse of antibiotics in veterinary medicine, however, has led to the emergence of single- and multiple-resistant bacterial strains (Levy, 2001) and to the selection of antibiotic-resistant bacteria (Ferrini et al., 2008; Kang et al., 2016). Therefore, it is necessary to establish a monitoring system for the objective evaluation of antibiotic resistance in aquaculture at the local and national levels. Because *Vibrio* strains are common gut flora in farmed fish, they can be used as indicators of the profiles of resistance to antibiotics. However, there are currently few data on the antibiotic resistance patterns of *Vibrio* strains in farmed fish in Korea.

In this study, we aimed to determine antibiotic and heavy metal

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resistance patterns of 59 *V. parahaemolyticus* strains isolated from farmed oysters in Korea. In addition, we investigated the virulence properties of the isolates based on detection of two virulence genes, *tdh* and *trh*.

2. Materials and methods

2.1. Sample collection and bacterial isolation

From May to October 2016, we collected oysters (*Crassostrea gigas*) from four commercial shellfish-harvesting areas off the west coast of Korea. Site 1 was located on Seungbong Island (latitude 37° 09′ 20″ N; longitude 126° 20′ 00″ E); site 2 was on Daeijak Island (37° 10′ 04″ N; 126° 15′ 50″ E); site 3 was on Soijak Island (37° 11′ 10″ N; 126° 13′ 27″ E); and site 4 was on Soya Island (37° 13′ 00″ N; 126° 10′ 55″ E). The shellfish meat (200 g) was transferred to an autoclaved beaker and mixed with 200 mL of phosphate-buffered saline (PBS; 2.5 mM KH_2PO_4; pH 7.2). The mixture was ground in an autoclaved stainless-steel blender (7011S; Waring, Torrington, CT, USA) for 90 s (30 s at low speed and 60 s at high speed), and the shellfish homogenate was then serially diluted to 10^{-1}–10^{-2} in PBS. Thiosulfate citrate bile salt sucrose (TCBS; Difco, Detroit, MI, USA) agar plates were used to select for *Vibrio* species, whereas tryptic soy agar (TSA; Difco) containing 3% NaCl was used as a control. A 100-μL aliquot of the PBS-diluted shellfish homogenates was spread on the TCBS plates, and 100 μL of another set of serial 10-fold dilutions (in 0.1% peptone water) of the homogenates was spread on the TSA control plates, after which they were incubated at 35 °C for 24 h. Thereafter, three to five green colonies presumed to be *Vibrio* spp. on the basis of their color, size, and shape were picked from the TCBS agar plates. The presumed *V. parahaemolyticus* colonies were characterized using microscopic tests (Gram staining and morphology) and biochemical tests (triple sugar iron (TSI) test) and then confirmed using an API 20E system (bioMerieux, Marcy-l'Étoile, France) before being subjected to PCR analysis.

2.2. Physical and chemical water quality measurements

The physical parameters of the sampling sites were measured once a month from May to October 2016. The water column depth and surface water salinity, temperature, dissolved oxygen (DO), conductivity, and pH were measured on every sampling date at each location with an YSI 556 Multiprobe system (YSI Incorporated, Yellow Spring, OH, USA) with multiparameter display, in accordance with the manufacturer's instructions.

2.3. Molecular identification of V. parahaemolyticus and detection of the tdh and trh virulence genes by PCR

All 69 of the biochemically identified *V. parahaemolyticus* isolates were further verified by molecular characterization with PCR to detect the presence of a highly conserved species-specific marker gene (*toxR*) of this bacterium (Han et al., 2007) as well as the *trh* and *tdh* virulence genes. Oligonucleotide primer sets were constructed for the amplification of these three genes; the primer sequences and the expected sizes of the amplified DNAs are listed in Table 1. Again, *V. parahaemolyticus* KCTC2729 was used as a positive control. PCR amplification was performed under the following conditions: 35 cycles at 95 °C for 2 min, 60 °C for 30 s, and 72 °C for 30 s, followed by a final extension step at 72 °C for 7 min. All amplified products were separated and visualized on a 2.0% agarose gel containing 0.5% ethidium bromide.

2.4. Antibiotic susceptibility testing of V. parahaemolyticus isolates

The susceptibility of the isolates to several antibiotics was determined using the disk diffusion technique (NCCLS, 2003). The 59 isolated strains were spread onto Mueller-Hinton agar (Difco) plates, onto which antibiotic disks (Oxoid, Basingstoke, Hampshire, UK) were then placed. One disk of each of the following antibiotics was tested per isolate: ampicillin (AM; 10 μg), cefotaxime (CTX; 30 μg), cefotetan (CTT; 30 μg), cephalothin (CF; 30 μg), chloramphenicol (C; 30 μg), ciprofloxacin (CIP; 5 μg), cefepime (CEP; 30 μg), erythromycin (E; 15 μg), gentamicin (GM; 10 μg), kanamycin (K; 30 μg), nalidixic acid (NA; 30 μg), rifampicin (RA; 5 μg), streptomycin (S; 10 μg), tetracycline (TE; 30 μg), sulfamethoxazole/trimethoprim (SXT; 1.25 μg and 23.75 μg, respectively), and vancomycin (VA; 30 μg). The plates were incubated at 35 °C for 18–24 h under aerobic conditions, after which the diameter of the zone of inhibition around each disk was measured and recorded. An individual bacterial strain was classified as resistant (R), intermediately resistant (I), or susceptible (S) according to the guidelines of the Clinical and Laboratory Standards Institute (NCCLS, 2003). The multiple antibiotic resistance (MAR) index of the isolates was defined as *x*/*y*, where *x* represents the number of antibiotics to which a particular isolate was resistant and *y* represents the number of antibiotics to which the isolate was susceptible (Krumperman, 1983).

2.5. Tolerance of V. parahaemolyticus isolates to heavy metals

Tolerance of the isolates to heavy metals was determined according to a method described previously (Malik and Aleem, 2011; Song et al., 2013; Kang et al., 2016). The minimal inhibitory concentration (MIC) of the tested heavy metals against the isolates was measured quantitatively using broth dilution testing (CLSI, 2006). The heavy metals used in this study were $BaCl_2$, $CdCl_2$, $CoCl_2$, and $CuCl_2$. The assays were performed in triplicate, and *Escherichia coli* K12 was used as a control strain (Malik and Aleem, 2011; Song et al., 2013).

3. Results and discussion

3.1. Characteristics of sea water from the sampling sites

Measurements of water temperature, salinity, pH, and DO were similar across the four sampling sites (Table 2). The water temperature at the sampling sites varied from 12.31 °C to 24.03 °C (average, 20.38 ± 3.49 °C), and the salinity ranged from 32.91 to 34.18 psu (average, 33.26 ± 0.36 psu). The pH varied from 7.91 to 8.15 (average, 8.05 ± 0.06), and DO values ranged from 5.80 to 8.30 mg/L (average, 6.84 ± 0.84 mg/L). Overall, statistically significant correlations between the environmental parameters and *V. parahaemolyticus* abundance were identified because there was little change in the environmental parameters among the four sites during the study period.

Table 1
List of primers, target genes, and amplicon sizes for PCR analysis.

	Primers and sequences (5′ to 3′)	Amplicon size (bp)	Reference
Vp-*toxR*1	CTC TTC TGA CGC AAT CGT TG	370	Han et al., 2012
Vp-*toxR*2	ATA CGA GTG GTT GCT GTC ATG		
tdh 1	GCA CCG GTC AAT GTA GAG G	200	Han et al., 2012
tdh 2	CAC AGC AGA ATG ACC GTG C		
trh 1	GGC TCA AAA TGG TTA AGC G	250	Tada et al., 1992
trh 2	CAT TTC CGC TCT CAT ATG C		