Characterisation of pathogenic *Vibrio* spp. isolated from live Pacific abalone (*Haliotis discus hannai* Ino, 1953) marketed in South Korea

M. V. K. S. WICKRAMANAYAKE, P. S. DAHANAYAKE, S. HOSSAIN, B. C. J. DE SILVA AND G. J. HEO

Laboratory of Aquatic Animal Medicine, Veterinary Medical Center and College of Veterinary Medicine
Chungbuk National University, Chungdae-ro 1, Seowon-gu, Cheongju 28644, Republic of Korea

e-mail: gheo@cbu.ac.kr

ABSTRACT

This study aimed to evaluate the profile of virulence factors and antimicrobial resistance in *Vibrio* spp. isolated from live Pacific abalone (*Haliotis discus hannai* Ino, 1953) marketed in South Korea. A total of 32 *Vibrio* isolates comprising *V. alginolyticus* (n=15), *V. diabolicus* (n=14), *V. antiquarius* (n=2) and *V. parahaemolyticus* (n=1) were analysed. All isolates demonstrated DNase, lipase, phospholipase and amylase activities. Additionally, production of slime (97%), gelatinase (94%), α-haemolysin (22%), β-haemolysin (78%) and protease (53%) were detected. Virulence genes *viz., toxR, tih, tdh, VAC, VPI, ctxAB* and *hupO* were recorded in 56, 59, 13, 100, 41, 9 and 9% of the isolates, respectively. All isolates were resistant to ampicillin and 88% of the isolates were resistant to cephalothin and colistin sulphate. Twenty-one isolates (66%) showed multiple antimicrobial resistance (MAR) index ≥0.2. Antimicrobial resistance genes *blaCTX* (85%), *blaTEM* (10%), *blaSHV* (10%), *strAB* (13%) and *aphA-IAB* (22%) and class 1 integrons (19%) were detected. *V. diabolicus* and *V. antiquarius* were identified and characterised for the first time in the Pacific abalone. Our findings imply the significance of integrated monitoring and surveillance programmes for the occurrence, virulence and antimicrobial resistance patterns of vibrios in Pacific abalone.

Keywords: *Haliotis discus hannai*, Pacific abalone, Pathogenicity, South Korea, *Vibrio* spp., virulence

Introduction

Abalone (*Haliotis* spp.) is recognised as one of the most valuable seafood in the world. South Korea is the world’s second largest abalone producer with over 10,000 t of annual production after China, the leading producer of farmed abalone in the world. Also, South Korea is considered one of the world’s top seafood consuming countries where a variety of species including abalones are in abundance (FAO, 2017; Yonhap, 2017).

The statistics and studies related to food borne diseases reveal bacteria as the major causative agents. Among the pathogenic bacteria, *Vibrio* spp. have been identified as the major group of microbes which can cause severe food borne illnesses (Lee et al., 2001). In South Korea, ten cases of *Vibrio* gastritis have been reported in 2017, which occurred due to the consumption of seafood in a restaurant (KCDC, 2018). *Vibrio* spp. are Gram negative bacteria that are ubiquitous in marine environment (Thompson et al., 2004). Among the large number of *Vibrio* spp. identified, *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae*, *V. vulnificus* and *V. fluvialis* have been found to be frequently associated with seafood related disease outbreaks (Elhadi et al., 2004).

Thermostable direct haemolysin (TDH) encoded *tdh* gene, TDH related haemolysin (TRH) encoded *trh* gene and thermolabile haemolysin (TLH) encoded *tlh* gene are considered as important virulence factors in food borne *Vibrio* infections (Zhang and Austin, 2005; Wang et al., 2015). Moreover, production of transmembrane regulatory protein (*toxR*), cholera toxin (CTX) and secretions in *V. cholerae* pathogenicity island (VPI) also have a significant contribution to the pathogenicity of *Vibrio* spp. (Sarkar et al., 2002). Besides the toxin production and haemolysins, there are many other virulence related extracellular enzymes such as protease, amylase, DNase, lipase and gelatinase (Bunpa et al., 2016). Also, *Vibrio* spp. have been noted for their emerging antimicrobial resistance patterns (Harbottle et al., 2006).

Though abalone is considered as valuable seafood all over the world, only a few studies have been conducted to assess the potential health risk of abalone consumption. Lee et al. (2016) reported the prevalence of food poisoning microbiota in abalone. However, to the best of our knowledge, there have been no studies conducted to characterise virulence factors and antimicrobial resistance properties of *Vibrio* spp. isolated from Pacific abalone marketed live in south Korea. Therefore, the current
study intended to characterise the virulence factors, antimicrobial resistance patterns, antimicrobial resistance gene profiles and class 1 integrons of Vibrio spp. isolated from marketed Pacific abalone (Haliotis discus hannai Ino, 1953) in South Korea.

Materials and methods

Abalone sampling

A total of 120 live Pacific abalone samples were procured from retail markets from February to June 2018. Samples were transported to the laboratory and processed immediately. Each abalone was shucked and a homogenised composite was prepared by blending each sample in a sterile blender. The entire sampling procedure was conducted under aseptic conditions.

Isolation and biochemical identification of Vibrio spp.

One gram each of homogenised tissue sample was incubated in alkaline peptone water (APW) at 37°C for 24 h. One loopful of each enrichment was streaked onto thiosulphate citrate bile salts sucrose (TCBS) agar (MB cell, California, USA) plates and incubated for 24 h at 37°C. All colonies that appeared yellow or green in colour on TCBS agar were subcultured on tryptic soy agar (TSA) supplemented with 1% (w/v) NaCl to obtain pure colonies. Triple sugar iron agar (TSI) test, oxidase test and sensitivity to vibriostatic discs were employed for phenotypic confirmation of Vibrio spp. status.

DNA extraction, polymerase chain reaction and phylogenetic analysis

Each Vibrio sp. identified based on biochemical tests was incubated in APW at 37°C for 24 h and genomic DNA was extracted using Exgene Cell SV extraction kit (Geneall, Seoul, Korea) according to the manufacturer’s protocol. Polymerase chain reaction (PCR) and sequencing of gyrB housekeeping gene amplified with gyrB-F and gyrB-R primers were employed for species identification. Amplified PCR products were purified using Exgene PCR SV (Geneall, Seoul, Korea) kit and submitted for gene sequencing at Cosmogenetech Co. Ltd. (Daejeon, Korea). Sequencing data were checked for BLAST compatibility with available gene sequences in the GenBank database (NCBI). Neighbor joining phylogenetic tree was constructed according to Kumar et al. (2016). The following sequences were downloaded from GenBank database (NCBI) for analysis: V. diabolicus strain LMG 23867, V. alginolyticus strain DX 0406, V. antiquarius strain EX 25, V. parahaemolyticus strain GQ426112 and Escherichia coli strain KCTC 2441 (EU014649) as the outgroup taxa.

Phenotypic pathogenicity tests

A total of 32 Vibrio strains isolated were subjected to eight phenotypic pathogenicity tests. Tryptic soy agar (TSA) supplemented with 1% (w/v) NaCl was used to maintain test strains. Additionally, 1% (w/v) NaCl was supplemented in each test medium. Protease production was examined by incubating the isolates on TSA added with 0.5% (w/v) skim milk (MB Cell, Seoul, Korea) for 48 h at 37°C (Zhang and Austin, 2000). To detect DNase activity, 1N HCl was added to the well grown colonies on DNase agar (MB Cell, California, USA) plates and the presence of halo effect around colonies was noted as positive (Twedt et al., 1970). Slime production was detected by Congo red uptake method according Freeman and Falkine (1989). Production of lipase and phospholipase were examined employing TSA added with 1% (v/v) Tween 80 (Samchun, Pyeongtaek, Korea) and 5% (v/v) egg yolk emulsion (MB Cell, Seoul, Korea), respectively. The presence of opaque halos around the colonies was considered as positive (Liuxy et al., 1996). Gelatinase activity was tested according to Elavarashi et al. (2017). Amylase production was examined by adding Gram’s iodine onto well grown colonies in TSA supplemented with 0.2% (w/v) soluble starch. The presence of clear halos around the colony after overnight incubation at 37°C was considered as positive. Haemolysin activity was assessed by streaking the strains on blood agar base (MB Cell, California, USA) supplemented with 5% (v/v) sheep blood. Greenish discolouration and clear zone around the colonies were considered as (α) and (β) haemolysis, respectively.

Antibacterial susceptibility testing

Antimicrobial susceptibility was examined against 20 antimicrobials belonging to 11 antimicrobial classes by the disc diffusion test. The antimicrobials used included Penicillins: ampicillin (10 µg) and piperacillin (100 µg); Lipopeptide: colistin sulfate (10 µg); Tetracyclines: tetracycline (30 µg) and oxytetracycline (30 µg); Phenicol: chloramphenicol (30 µg); Aminoglycosides: streptomycin (10 µg), gentamycin (10 µg) and kanamycin (30 µg); Quinolones: nalidixic acid (30 µg), ofloxacin (5 µg) and ciprofloxacin (5 µg); Macrolide: erythromycin (15 µg); Ansamycins: rifampicin (5 µg); Carbapenems: imipenem (10 µg) and meropenem (10 µg); Cephalosporins: cephalothin (30 µg), ceftriaxone (30 µg) and cefotaxime (30 µg) and Folate pathway inhibitor: trimethoprim-sulfamethoxazole (25 µg). The testing procedure was carried out according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2014). The multiple antimicrobial resistance (MAR) index was calculated according to the formula described by Krupperman (1983).
Screening of virulence related and antimicrobial resistance genes

The presence of eleven virulence related genes, 6 antimicrobial resistance genes and integrons as detailed in Table 1 were examined by conventional PCR method. Details of primer pairs and conditions are also given in Table 1. Each PCR reaction mixture was 30 µl in final volume comprising 0.3 µl AmpOne Taq DNA polymerase (GeneAll, Seoul, Korea), 3 µl dNTP mix, 3 µl 10×Taq polymerase buffer, 1 µl of each forward and reverse primers, 1 µl of template DNA and 21.7 µl of PCR water. PCR cycling conditions for each reaction included: Initial denaturation at 94°C for 2 min followed by a total of 35 cycles, each cycle comprising denaturation at 94°C for 30s, annealing at respective temperature for 50s and extension at 72°C for 1 min. Amplified PCR products were visualised by electrophoresis on 1.5% (w/v) agarose gels.

Results and discussion

Abalone samples were collected during the period of 7 months from February to September, when the food borne Vibrio outbreaks was frequently reported in South Korea as reported in Park et al. (2018). Vibrio spp were isolated from 28 samples (23%) out of the 120 abalone samples tested. From the 28 samples, a total of 32 Vibrio spp. comprising V. alginolyticus (n=15), V. diabolicus (n=14), V. antiquarius (n=2) and V. parahaemolyticus (n=1) species were identified by sequencing the gyrB housekeeping gene. V. alginolyticus and V. diabolicus were the dominant species. Pang (2006) reported V. alginolyticus as one of the dominant bacteria species in abalone aquaculture systems.

Neighbor-joining phylogenetic tree was constructed using the gyrB gene sequence data and the isolates were sorted into two major clades (Fig. 1). V. parahaemolyticus strain belonged to the first major clade with V. parahaemolyticus reference strain that has been deposited in NCBI database under reference number GQ426112 as food and waterborne pathogen. In the second major clade, two V. antiquarius isolates grouped in the same subclade along with V. alginolyticus isolates and the reference V. antiquarius strain. Also, V. diabolicus and V. alginolyticus isolates exhibited a scattered distribution pattern in the second major clade. V. alginolyticus, V. diabolicus and V. antiquarius isolated from Pacific oysters (Crassostrea gigas) have demonstrated similar phylogenetic distribution in the study by Turner et al. (2018).

All Vibrio spp. isolated in this study demonstrated DNase, lipase, phospholipase and amylase production (Table 2). DNase involves pathogenicity by the degradation of neutrophil extracellular traps and innate immune structures composed of chromatin and granule proteins (Buchanan et al., 2006). In line with the current study, Dahanayake et al. (2018) reported DNase production among 100% of Vibrio spp. isolated from oyster (Crassostrea gigas) in Korea. Lipolytic activity may be important for the dissemination and/or nutrition of bacteria in infections. In vitro studies have shown that lipases influenced the actions of various immune cells, such as the chemotaxis of neutrophils (Stehr et al., 2003). In this study, protease and gelatinase were observed in 57 and 94% of the isolates, respectively. Gelatinase enzymes were reported to hydrolyse collagen, haemoglobin and some other peptides. Protease secretion ensures establishment of the pathogen in contact with the host cell (Frees et al., 2013). These extracellular secretions of Vibrio spp. have been studied and reported as virulent related exoenzymes due to their involvement in virulence activities.

Haemolysis (α and β) is considered as one of the major virulence activity which facilitate the intercellular growth of pathogenic bacteria (Jia et al., 2010). α-haemolysis can reduce the host cell resistance by inhibition of phagocytosis (Cavalieri and Snyder, 1982). In our study, we observed a high rate of β-haemolysis activity and a low rate of α-haemolysis. β-haemolysis plays a role in bacterial infection by releasing iron from red blood cells and making it available for bacterial growth (Janda and Abbott, 1993). However, α-haemolysis causes only an incomplete degradation of haemoglobin (Zhang and Austin, 2005).

Slime production (97%) was observed among all isolates except V. parahaemolyticus strain. Slime is an important factor required for biofilm production which can increase colonisation and contamination by pathogenic microflora (Abdallah et al., 2009). Snoussi et al. (2008) observed strong adhesive properties of slime producing V. alginolyticus which can cause contamination easily. Thus, the Vibrio spp. isolated from the Pacific abalone can easily colonise and can cause contaminations by sticking to surfaces, which would also help preventing from being washed away.

Virulence related gene profiles of Vibrio spp. isolated are presented in Table 2. Among the tested virulence genes, V. alginolyticus specific collagenase gene (VAC) was recorded as the most prevalent virulence gene (100%). Furthermore, we detected positive isolates for tdh gene among V. alginolyticus (6%) and V. diabolicus (21%) strains. tdh gene is often associated with pathogenicity of V. parahaemolyticus. However, Gargoui et al. (2015) reported the presence of tdh gene in V. alginolyticus strains isolated from shrimp. V. alginolyticus species specific tllh gene was detected in 59% of the isolates. Haemolysin production stimulating hupO gene was detected in 9% of
Table 1. Oligo sequences and PCR conditions employed in the study

| Gene          | Target action                    | Nucleotide sequence 5’-3’                      | Size (bp) | Annealing temperature (ºC) | Reference    |
|---------------|----------------------------------|------------------------------------------------|-----------|----------------------------|--------------|
| toxR (V. alginolyticus) | Intestinal colonisation and toxin production | F: GATTAGGAAGCAACGAAAG R: GCAATCACCCTCCACTGGTAAC | 658       | 54                         | Xie et al. (2005) |
| toxR (V. parahaemolyticus) | Intestinal colonisation and toxin production | F: GTCTTCTGACGCAATCTGGTG R: ATACGAGTTGTTGCTTCACTG | 368       | 56                         | Kim et al. (1999) |
| tdh           | Thermostable direct haemolysin    | F: CCACACATTCACTATGC R: GGTACTAAATGGCTGACATC | 251       | 55                         | Gargouli et al. (2015) |
| trh           | TDH-related haemolysin           | F: TTGGCTTCGATATTTTCAGTATCT R: CATAAACAAACATATGGCCATTTCCG | 500       | 58                         | Bej et al. (1999) |
| tlh (V. alginolyticus) | Thermolabile haemolysin      | F: AGCGGATTATGCAGAAGCAC R: GCTACTTTCTAGCATTTTCTCTGC | 448       | 54                         | Woodring et al. (2012) |
| Collagenase (VAC) | Collagenase activity          | F: CGAGTACAGTCACTTGAAGCC R: GCCATCAATTTGCGCAATGCATG | 737       | 58                         | Di Pinto et al. (2005) |
| ctxAB         | Gene encoding cholera toxin     | F: GCCGGGTGTCTGGAATCCTACAAAG R: GGGCTACATTGGAATGC | 536       | 59                         | Miller et al. (1987) |
| hupO          | Hemin-binding outer membrane protein | F: ATTACGGCAACGAGTCGAAC R: ATGGAGATGGTAAACAGCGCC | 600       | 56                         | Liang et al. (2013) |
| vfh           | Extracellular haemolysin        | F: GCGCCTCAGTGTTGTTGTAAG R: TGGGTCGGTACCCTTGCCTT | 800       | 61                         | Liang et al. (2013) |

Pathogenicity Island (VPI) of V. cholerae

|    | VP specific | VA specific | VF specific | No. of positive isolates for virulence test |
|----|-------------|-------------|-------------|------------------------------------------|
|    | toxR        | tlh         | toxR        | intI1                                    | protease     |
| V. parahaemolyticus (n=1) | 1 | 1 | - | - | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| V. alginolyticus (n=15) | - | - | 14 | 9 | 1 | - | 15 | 3 | - | 7 | 2 | 15 | 7 | 14 | 15 | 15 | 15 | 15 | 2 | 13 |
| V. diabolicus (n=14) | - | - | 3 | 8 | 3 | - | 14 | - | - | 5 | - | 14 | 7 | 13 | 14 | 14 | 14 | 5 | 9 |
| V. antiquarius (n=2) | - | - | - | 1 | 1 | - | 2 | - | - | - | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 |
| Total (%) (n = 32) | 1(3%) | 1(3%) | 18(56%) | 4(12%) | 3(9%) | 13(41%) | 3(9%) | 17(53%) | 30(94%) | 32(100%) | 32(100%) | 32(100%) | 32(100%) | 31(97%) | 31(97%) | 25(78%) |

Table 2. Virulence associated genes and phenotypic pathogenicity profile of Vibrio spp. isolated from live Pacific abalone

| Virulence genes | Species |
|-----------------|---------|
| VP specific     | VA specific | VF specific |
| toxR            | tlh      | toxR | intI1 | protease | gelatinase | lipase | phospholipase | amylase | slime | α-Haemolysis | β-Haemolysis |
| V. parahaemolyticus (n=1) | 1 | 1 | - | - | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| V. alginolyticus (n=15) | - | - | 14 | 9 | 1 | - | 15 | 3 | - | 7 | 2 | 15 | 7 | 14 | 15 | 15 | 15 | 15 | 2 | 13 |
| V. diabolicus (n=14) | - | - | 3 | 8 | 3 | - | 14 | - | - | 5 | - | 14 | 7 | 13 | 14 | 14 | 14 | 5 | 9 |
| V. antiquarius (n=2) | - | - | - | 1 | 1 | - | 2 | - | - | - | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 |
| Total (%) (n = 32) | 1(3%) | 1(3%) | 18(56%) | 4(12%) | 3(9%) | 13(41%) | 3(9%) | 17(53%) | 30(94%) | 32(100%) | 32(100%) | 32(100%) | 32(100%) | 31(97%) | 31(97%) | 25(78%) |

*VP = V. parahaemolyticus, VA = V. alginolyticus, VF = V. fluvialis*
Fig. 1. Neighbor joining phylogenetic tree based on gyrB gene sequences showing the relationship between Vibrio spp. isolated from Pacific abalone and and reference sequences from the GenBank database. VA= V. alginolyticus, VD= V. diabolicus, VP= V. parahaemolyticus, VN= V. antiquarius discloses the risk of infection of Vibrio spp. isolated from the Pacific abalone.

The gene ctxAB, encoding cholera toxin (CT) and the gene representing V. cholerae pathogenicity island (VPI) are associated with the epidemic strains of V. cholerae (Waldor and Mekalanos, 1996). Xie et al. (2005) reported the presence of V. cholerae originated virulence determinants among V. parahaemolyticus and their close genetic relatives. In agreement with this statement, we detected VPI (41%) and ctxAB (9%) positive isolates among all species. Though these genes are often linked with the virulence of V. cholerae, there could be a specific virulence mechanism in V. alginolyticus which is activated with the presence of these genes (Ren et al., 2013).

Antimicrobial resistance patterns and the MAR indices recorded from disc diffusion assay results are shown in Table 3. All Vibrio spp. isolates in this study were identified as ampicillin resistant (Fig. 2). First generation antimicrobials including ampicillin have been extensively used in aquaculture leading to occurrence of ampicillin resistant Vibrio spp. in aquatic environment (Sudha et al., 2014). Similar to our study, Dahanayake et al. (2018) detected antimicrobial resistant Vibrio spp.
from live oysters marketed in Korea and the majority of them were reported as ampicillin resistant. In this study, 66% of isolates showed multidrug resistance by demonstrating resistance against four or more antimicrobial agents. Highest MAR index value of our study was scored as 0.3 by a \textit{V. alginolyticus} (VA13) isolate. Bacteria having MAR index ≥0.2 originate from a high-risk source of contamination where several antimicrobials are used (Paul \textit{et al.}, 1997).

Majority of the \textit{Vibrio} isolates in this study were resistant against \textit{β}-lactam antimicrobials (ampicillin, piperacillin and cephalothin). The production of extended spectrum \textit{β}-lactamases (ESBLs) is described as the major reason of being resistant to \textit{β}-lactam antimicrobials (Shaikh \textit{et al.}, 2015). In the present study, \textit{blaCTX} (85%) gene was recorded as the most prevalent gene coding for \textit{β}-lactamase and \textit{blaSHV} (10%) as well as \textit{blaTEM} (10%) genes were also recorded. These have been identified as emerging \textit{β}-lactamase producing genes (Overdevest \textit{et al.}, 2011).

\textit{strAB} and \textit{aphA-IAB} genes regulate streptomycin and kanamycin resistance mechanisms, respectively (Frana \textit{et al.}, 2001; Bush and Fisher, 2011). Although we detected 22% of the isolates harbouring \textit{α-IAB} gene, only one isolate (3%) showed kanamycin resistance in the disc diffusion test. Also, the number of isolates that

| Species | Isolate | Resisted antimicrobials | Antimicrobial resistance gene | Class 1 integron and gene cassette | MAR index |
|---------|---------|-------------------------|------------------------------|-----------------------------------|-----------|
| \textit{V. parahaemolyticus} | VP1 | AMP, COL, CPL, PRL | \textit{blaSHV} | - | 0.2 |
| \textit{V. alginolyticus} | VA1 | AMP, COL, PRL | \textit{blaCTX} | - | 0.15 |
| | VA2 | AMP, COL, CPL, PRL | \textit{blaCTX} | - | 0.2 |
| | VA3 | AMP, COL, CPL | \textit{blaCTX} | - | 0.15 |
| | VA4 | AMP, CPL, COL | - | \textit{Intl 1, qacE2} | 0.15 |
| | VA5 | AMP, STR, CPL, PRL, COL | \textit{blaCTX}, \textit{blaTEM}, \textit{aphA-IAB} | - | 0.25 |
| | VA6 | AMP, COL, CPL, PRL | \textit{blaCTX} | - | 0.2 |
| | VA7 | AMP, COL, CPL, PRL | \textit{blaCTX}, \textit{strAB} | - | 0.2 |
| | VA8 | AMP, PRL, COL, CPL | - | \textit{Intl 1, qacE2} | 0.2 |
| | VA9 | AMP, CPL, COL | \textit{blaCTX} | - | 0.15 |
| | VA10 | AMP, STR, CPL, COL, PRL | \textit{blaCTX}, \textit{strAB} | - | 0.25 |
| | VA11 | AMP, CPL, COL | \textit{blaCTX} | \textit{Intl 1, qacE2} | 0.15 |
| | VA12 | AMP, COL, PRL | \textit{aphA-IAB} | \textit{Intl 1, qacE2} | 0.15 |
| | VA13 | AMP, CPL, COL, PRL, STR, RD | \textit{blaCTX} | - | 0.3 |
| | VA14 | AMP, COL, PRL, CPL | \textit{blaCTX} | \textit{Intl 1, qacE2} | 0.2 |
| | VA15 | AMP, COL, PRL, CPL | \textit{blaCTX}, \textit{blaSHV} | \textit{Intl 1, qacE2} | 0.2 |
| \textit{V. diabolicus} | VD1 | AMP, COL | \textit{blaCTX} | - | 0.1 |
| | VD2 | AMP, CPL, COL, PRL | \textit{blaCTX} | - | 0.2 |
| | VD3 | AMP, STR, CPL, COL, KAN | \textit{blaCTX}, \textit{aphA-IAB} | - | 0.25 |
| | VD4 | AMP, CPL, COL | \textit{blaCTX} | - | 0.15 |
| | VD5 | AMP, COL, PRL, STR | \textit{blaCTX}, \textit{strAB} | - | 0.2 |
| | VD6 | AMP, CPL, COL, RD | \textit{blaCTX}, \textit{blaTEM} | - | 0.2 |
| | VD7 | AMP, STR, COL, RD, CPL | - | - | 0.25 |
| | VD8 | AMP, COL, CPL | \textit{blaCTX} | - | 0.25 |
| | VD9 | AMP, CPL, COL | \textit{blaCTX}, \textit{aphA-IAB} | - | 0.15 |
| | VD10 | AMP, CPL, COL, STR | \textit{blaCTX}, \textit{blaTEM}, \textit{strAB}, \textit{aphA-IAB} | - | 0.2 |
| | VD11 | AMP, CPL, COL, PRL | \textit{blaCTX} | - | 0.2 |
| | VD12 | AMP, CPL, COL, CRO | \textit{blaCTX} | - | 0.2 |
| | VD13 | AMP, STR, ERY, RD, CPL | \textit{blaCTX}, \textit{aphA-IAB} | - | 0.25 |
| | VD14 | AMP, CPL, PRL, RD | \textit{blaCTX}, \textit{aphA-IAB} | - | 0.2 |
| \textit{V. antiquarius} | VA1 | AMP, STR, CPL, PRL | \textit{blaCTX}, \textit{blaSHV} | - | 0.2 |
| | VA2 | AMP, CPL, COL, RD, PRL | \textit{blaCTX} | - | 0.25 |

$^a$Antibiotics: AMP = ampicillin, PRL = piperacillin, COL = colistin, STR = streptomycin, KAN = kanamycin, ERY = erythromycin, IMI = imipenem, CPL = cephalothin, CRO = ceftriaxone, OT = oxytetracycline, RD = rifampicin
showed streptomycin resistance in the disc diffusion test was higher than the number of isolates that showed strAB gene in molecular analysis. Similarly, Randall et al. (2004) detected different ratios between phenotypic and genotypic expressions of antimicrobial resistance characteristics.

Integrons that contain a site specific recombination system called gene cassettes are considered as the elements that can facilitate the horizontal transfer of antimicrobial resistance genes among bacteria (Stokes and Gillings, 2011). Class 1 integron-related integrase gene, intI1 has long evolution history initiated from the environmental contaminants and this gene aid to accumulate antimicrobial resistance determinants in the bacterial genome (Bahl et al., 2006; Davies 2007). The qacE2 gene cassette is an efflux pump for the cationic compounds which causes resistance to disinfectants (Ghaly et al., 2017). Moreover, qac genes in class 1 integrons are known as the genetic marker derived from clinical ancestors (Gillings et al., 2009). Hence, six V. alginolyticus isolates that were identified as harbouring qacE2 gene cassette and intI1 gene in this study could have been derived from the clinical ancestors.

Results of the present study indicate occurrence of pathogenic Vibrio spp. in marketed Pacific abalone in South Korea. V. alginolyticus and V. diabolicus were the most prevalent species among the Vibrio isolates and also, we detected virulence and antimicrobial resistance properties in higher percentages. V. diabolicus and V. antiquarius were identified and characterised for the first time in Pacific abalone. The presence of virulence related determinants as well as the presence of multidrug resistance properties among the abalone borne Vibrio isolates is a real concern and warrants ongoing surveillance.  

### Acknowledgment

This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sector.

### References

Abdallah, F. B., Chaieb, K., Zmantar, T., Kallel, H. and Bakhrouf, A. 2009. Adherence assays and slime production of Vibrio alginolyticus and Vibrio parahaemolyticus. Braz. J. Microbiol., 40(2): 394-398. doi: 10.1590/S1517-838220090002000033.

Bahl, M. I., Boucher, Y., Nesbo, C. L., Holley, M. and Stokes, H. W. 2006. Class 1 integrons potentially predating the association with Tn402-Like transposition genes are present in a sediment microbial community. J. Bacteriol., 188(16): 5722-5730. DOI:10.1128/JB.01950-05.

Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Koth, M. and Nizet, V. 2006. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr. Biol., 16(4): 396-400. DOI:10.1016/j.cub.2005.12.039.

Bunpa, S., Sermwittayawong, N. and Vuddhakul, V. 2016. Extracellular enzymes produced by Vibrio alginolyticus isolated from environments and diseased aquatic animals. Procedia Chem., 18(18): 12-17. https://doi.org/10.1016/j.proche.2016.01.002.

Bush, K. and Fisher, J. F. 2011. Epidemiological expansion, structural studies and clinical challenges of new β-Lactamases from Gram negative bacteria. Ann. Rev. Microbiol., 65(1): 455-478. DOI:10.1146/annurev-micro-090110-102911.

Cavalieri, S. J. and Snyder, I. S. 1982. Effect of Escherichia coli alpha-Haemolysin on human peripheral leukocyte viability in vitro. Infect. Immun., 36(2): 455-461.

CLSI 2014. Performance standards for antimicrobial susceptibility testing; Twenty-fourth informational supplement. CLSI Document M100-S24. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Dahanayake, P. S., De Silva, B. C. J., Hossain, S., Shin, G. W. and Heo, G. J. 2018. Occurrence, virulence factors and antimicrobial susceptibility patterns of Vibrio spp. isolated from live oyster (Crassostrea gigas) in Korea. J. Food. Saf., 38:e12490.
Davies, J. 2007. Microbes have the last word; A drastic re-evaluation of antimicrobial treatment is needed to overcome the threat of antibiotic-resistant bacteria. EMBO reports, 8(7): 616-621. doi: 10.1038/sj.embor.7401022.

Diaz, M. A., Cooper, R. K., Clococke, T. A. and Siebeling, R. J. 2006. Plasmid-mediated high-level gentamicin resistance among enteric bacteria isolated from pet turtles in Louisiana. Appl. Environ. Microbiol., 72(1): 306-312.

Elavarashi, E., Kindo, A. J. and Rangarajan, S. 2017. Enzymatic and non-enzymatic virulence activities of dermatophytes on solid media. J. Clin. Diagn. Res., 11(2): DC23-D025. DOI:10.7860/JCDR/2017/23147.9410.

Elhadi, N., Radu, S., Chen, C. H. and Nishibuchi, M. 2004. Prevalence of potentially pathogenic Vibrio species in the seafood marketed in Malaysia. J. Food. Prot., 67(7): 1469-1475. DOI:10.4315/0362-026x-67.7.1469.

FAO 2017. GLOBEFISH Analysis and information on world fish trade. Food and Agricultural Organization, Rome, Italy. http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/902597/. (Accessed 20 December 2018).

Frana, T. S., Carlson, S. A. and Griffith, R. W. 2001. Relative distribution and conservation of genes encoding aminoglycoside-modifying enzymes in Salmonella enterica serotype typhimurium phage type DT104. Appl. Environ. Microbiol., 67: 445-448. DOI:10.1128/AEM.67.1.445-448.2001.

Freeman, D. J. and Falkiner, F. R. K. C. 1989. New method for detecting slime production by coagulase negative staphylococci. J. Clin. Pathol., 42: 872-874. DOI:10.1136/jcp.42.8.872.

Frees, D., Brondsted, L. and Ingmer, H. 2013. Bacterial proteases and virulence. In: Dougan, D. (Eds), Regulated proteolysis in microorganisms: Subcellular biochemistry. Springer, Dordrecht, Netherlands, p. 161-192.

Gargouti, A. S., Ab-Rashid, M. N. K., Ghazali, M. F., Mitsuaki, H., Haresh, K. K. and Radu, S. 2015. Detection of sdh and trh toxic genes in Vibrio alginolyticus strain from mantis shrimp (Oratosquilla oratoria). J. Nutr. Food. Sci., 5(5): 100405. DOI: 10.4172/2155-9600.1000405.

Ghaly, T. M., Chow, L., Asher, A. J., Waldron, L. S. and Gillingers, M. R. 2017. Evolution of class 1 integrons: Mobilisation and dispersal via food-borne bacteria. PloS ONE, 12(6): e0179169.

Gillingers, M. R., Xuejun, D., Hardwick, S. A., Holley, M. P. and Stokes, H. W. 2009. Gene cassettes encoding resistance to quaternary ammonium compounds: A role in the origin of classic class 1 integrons. ISME J., 3: 209-215. DOI:10.1038/ismej.2008.98.

Harbottle, H., Thakur, S., Zhao, S. and White, D. G. 2006. Genetics of antimicrobial resistance. Anim. Biotechnol., 17: 111-124. DOI:10.1080/10495390600957092.

Janda, J. M. and Abbott, S. L. 1993. Expression of an iron-regulated haemolysin by Edwardsiella tarda. FEMS Microbiol. Lett., 111(1993): 275-280.

Jia, A., Woo, N. Y. S. and Zhang, X. H. 2010. Expression, purification and characterisation of thermolabile haemolysin (TLH) from Vibrio alginolyticus. Dis. Aquat. Organ., 90(2): 121-127. doi:10.1354/dao02225.

KCDC 2018. National infectious disease surveillance system. Korea Centers for Disease Control and Prevention, South Korea, http://www.cdc.go.kr/npt/biz/npp/portal/nppPbldtDtaMain.do. (Accessed 25 December 2018).

Krumperman, P. H. 1983. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of faecal contamination of foods. Appl. Environ. Microbiol., 46: 165-170.

Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol., 33(7): 1870-1874. doi: 10.1093/molbev/msw054.

Lee, M. F., Peng, C. H., Lin, Y. H. and Lin, S.R. 2008. Molecular diversity of class 1 integrons in human isolates of Aeromonas spp. from southern Taiwan. Jpn. J. Infect. Dis. 61(5): 343-349.

Lee, M. J., Lee, J. J., Han, Y. C., Sang, H. C. and Kim, B. S. 2016. Analysis of microbiota on abalone (Haliotis discus hannai) in South Korea for improved product management. Int. J. Food. Microbiol., 234: 45-52. DOI: 10.1016/j.ijfoodmicro.2016.06.032.

Lee, W. C., Lee, M. J., Kim, J. S. and Park, S. Y. 2001. Foodborne illness outbreaks in Korea and Japan studied retrospectively. J. Food. Prot., 64(6): 899-902. DOI: 10.4315/0362-028x-64.6.899.

Lixuy, P. C., Lee, K. K. and Chen, S. N. 1996. Pathogenicity of different isolates of Vibrio harveyi in tiger prawn, Penaeus monodon. Lett. Appl. Microbiol., 22: 413-416.

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P. and Kluytmans, J. 2011. Extended spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerg. Infect. Dis., 17(7): 1216-1222. doi: 10.3201/eid1707.110209.

Pang, J. T. Y. 2006. Yield efficiency in progeny trials with cocoa. Exp. Agr., 42(3): 289-299.

Park, K., Mok, J. S. and Kwon, J. Y. 2018. Food borne outbreaks, distributions, virulence and antibiotic resistance profiles of Vibrio parahaemolyticus in Korea from 2003 to 2016: a review. Fish. Aquat. Sci., 21: 3. DOI 10.1186/s41240-018-0081-4.

Paul, S., Bezbearuah, R. L., Roy, M. K. and Ghosh, A. C. 1997. Multiple antibiotic resistance (MAR) index and its reversion in Pseudomonas aeruginosa. Lett. Appl. Microbiol., 24: 169-171. DOI:10.1046/j.1472-765x.1997.00364.x.

Randall, L. P., Coles, S. W., Osborn, M. K., Piddock, L. J. V. and Woodward, M. J. 2004. Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of Salmonella enterica isolated from humans and animals in the UK. J. Antimicrob. Chemother., 532(2): 08-216.
Ren, C., Hu, C., Jiang, X., Sun, H., Zha, Z., Chen, C. and Luo, P. 2013. Distribution and pathogenic relationship of virulence associated genes among Vibrio alginolyticus from the mariculture systems. Mol. Cell. Probes, 27: 164-168.

Sarkar, A., Nandy, R. K., Nair, G. B. and Ghose, A. C. 2002. Vibrio pathogenicity island and cholera toxin genetic element-associated virulence genes and their expression in non-O1 non-O139 strains of Vibrio cholerae. Infect. Immun., 70(8): 4735-4742. DOI:10.1128/iai.70.8.4735-4742.2002.

Shaikh, S., Fatima, J., Shakil, S., Rizvi, S. M. D. and Kamal, M. A. 2015. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J. Biol. Sci., 22: 90-101. DOI:10.1016/j.sjbs.2014.08.002.

Snoussi, M., Noumi, E., Cheriaa, J., Usai, D., Sechi, L. A., Zanetti, S. and Bakhrouf, A. 2008. Adhesive properties of environmental Vibrio alginolyticus strains to biotic and abiotic surfaces. New Microbiol., 31: 489-500.

Stehr, F., Kretschmar, M., Kroger, C., Hubea, B. and Schafer, W. 2003. Microbial lipases as virulence factors. J. Mol. Catal. B Enzym., 22: 347-355.

Stokes, H. W. and Gillings, M. R. 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol. Rev., 35(5): 790-819. doi:10.1111/j.1574-6976.2011.00273.x.

Sudha, S., Mridula, C., Silvester, R. and Hatha, A. A. M. 2014. Prevalence and antibiotic resistance of pathogenic Vibrios in shellfishes from Cochin market. Indian J. Mar. Sci., 43(5): 815-824.

Thompson, F. L., Iida, T. and Swings, J. 2004. Biodiversity of vibrios. Microb. Mol. Biol. Rev., 68(3): 403-431. DOI:10.1128/MMBR.68.3.403-431.2004.

Turner, J. W., Tallman, J. J., Macias, A., Pinnell, L. J., Elledge, N. C., Nasr, A. D. and Strom, M. S. 2018. Comparative genomic analysis of Vibrio diabolicus and six taxonomic synonyms: A first look at the distribution and diversity of the expanded species. Front. Microbiol., 9: 1893. doi: 10.3389/fmicb.2018.01893.

Twedt, R. M., Novelli, R. E., Spaulding, P. L. and Hall, H. E. 1970. Comparative haemolytic activity of Vibrio parahaemolyticus and related vibrios. Infect. Immun., 1(4): 394-399.

Waldor, M. K. and Mekalanos, J. J. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science., 272(5270): 1910-1914. DOI:10.1126/science.272.5270.1910.

Wang, R., Zhong, Y., Gu, X., Yuan, J., Saeed, A. F. and Wang, S. 2015. The pathogenesis, detection, and prevention of Vibrio parahaemolyticus. Front. Microbiol., 6: 144. doi: 10.3389/fmicb.2015.00144.

Xie, Z. Y., Hu, C. Q., Chen, C., Zhang, L. P. and Ren, C. H. 2005. Investigation of seven Vibrio virulence genes among Vibrio alginolyticus and Vibrio parahaemolyticus strains from the coastal mariculture systems in Guangdong, China. Lett. Appl. Microbiol., 41(2): 202-207. DOI:10.1111/j.1472-765X.2005.01688.x.

Yonhap 2017. Yonhap news. https://en.yna.co.kr/view/AEN2017021308033020 (Accessed 26 December 2018).

Zhang, X. H. and Austin, B. 2000. Pathogenicity of Vibrio harveyi to salmonids. J. Fish. Dis., 23(2): 93-102.

Zhang, X. H. and Austin, B. 2005. Haemolysins in Vibrio species. J. Appl. Microbiol., 98(5): 1011-1019. DOI:10.1111/j.1365-2672.2005.02583.x.