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Research article

Mesophilic *Aeromonas* spp. isolated from live Manila clam (*Ruditapes philippinarum*): virulence properties and multidrug resistance

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Abstract

This study aimed to evaluate the virulence determinants and multidrug resistance properties of mesophilic *Aeromonas* isolated from live Manila clams marketed in Korea. A total of 36 *Aeromonas* spp., comprising eleven *A. hydrophila*, eleven *A. salmonicida*, seven *A. media*, four *A. veronii*, two *A. allosaccharophila,* and one *A. caviae* isolate, were used in this study. The most common virulence traits observed were DNase and phospholipase activity, which were present in 100% of tested isolates, followed by gelatinase (94%), lipase (83%), hemolysis (78%), caseinase (75%), and slime production (31%). High prevalence of virulence genes; *ahyB*, *fla*, *hlyA*, *gcat*, *ast*, *lip*, *ser*, *aerA*, *ascV*, *act,* and *alt*, was identified in polymerase chain reaction (PCR) assay. Three *A. hydrophila* and seven *A. salmonicida* isolates were positive for all tested virulence genes. In contrast, three enterotoxin genes *(alt/ast/act*) were observed in *A. salmonicida*, *A. hydrophila,* and *A.veronii.* Alarmingly, in our study, 97% of *Aeromonas* isolates had MAR (multiple antibiotic resistance) values >0.2, indicating that they are from sources with a high risk of antibiotic-resistant contamination. Statistically significant differences were found between the frequency of virulence genes among *Aeromonas* spp. as well as the frequency of antibiotic resistance among *Aeromonas* spp. ($p < 0.05$). The incidence of potentially virulent and enterotoxigenic *Aeromonas* spp. showing multidrug resistance raises concern regarding the potential health risks of consuming raw or undercooked Manila clams.

Keywords: *Aeromonas* spp., Multidrug resistance, Public health, Shellfish, Seafood safety

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INTRODUCTION

In global aquaculture of marine bivalve mollusks, Manila clam (*Ruditapes philippinarum*), also known as the Japanese carpet shell or short-necked clam, is the second most important bivalve species, accounting for great commercial value and a worldwide distribution (Cordero et al., 2017; Hou et al., 2023). It is one of the most popular shellfish species owing to its smooth texture, sweet flavor, low fat, and high protein content (Venugopal and Gopakumar, 2017; Zhou et al., 2021). They are mostly eaten raw, steamed, stir-fried, grilled, or used in soup. Worldwide production of bivalve mollusks is around 17 million tons per year, with Manila clam comprising 20% of the total mollusks production (FIGIS, 2019: Bordignon et al., 2021). Manila clam is naturally dispersed along the Pacific coastlines of Japan, the Philippines, and China. China is the leading producer of Manila clam, with an annual production of 3 million tons, followed by Japan and Korea (Hou et al., 2023). Bivalves are suspension feeders that filter tiny feed particles from their surrounding water (Venugopal and Gopakumar, 2017). This can result in the concentration of pathogens in the bivalves, which can be a leading cause of foodborne illness upon consumption (Oliveira et al., 2011).

Aeromonas spp. is a key pathogenic organism associated with seafood-borne diseases (Venugopal and Gopakumar, 2017; Santajit et al., 2022). Members of the genus *Aeromonas* encompass Gram-negative, facultative anaerobic, rod-shaped, and non-spore-forming bacteria which are autochthonous and widely distributed in aquatic environments (Janda and Abbott, 2010; Miyagi et al., 2023). *Aeromonas* spp. are well recognized as etiological agents responsible for various illnesses in aquatic animals and humans (Janda and Abbott, 2010; El-Hossary et al., 2023). Among 36 *Aeromonas* species reported in the genus *Aeromonas, A. hydrophila*, *A. veronii* biovar sobria, *A. caviae*, and *A. schubertii* are more frequently involved in human infections (Teunis and Figueras, 2016; Pessoa et al., 2022). Human pathogenic *Aeromonas* spp. can cause enteric and extraintestinal infections, such as gastroenteritis, inflammatory bowel disease, cholangitis, wound infections, peritonitis, pneumonia, sepsis, bacteremia, and urinary tract infections (Lee et al., 2023a; Song et al., 2023). Studies have also shown the importance of some motile *Aeromonas* spp. emerging as food and water-borne pathogens (Ottaviani et al., 2011). In Korea, *Aeromonas* spp. are the most prevalent microorganism responsible for the occurrence of spontaneous bacterial peritonitis (Iyre et al., 2022). A fatal case of *A. aquariorum* infection was identified in a patient with liver cirrhosis, attributed to the consumption of raw fish in Korea (Shin et al., 2013).

The pathogenesis of aeromonads results from a complex network of pathogenic mechanisms attained by a multifactorial process (Guerra et al., 2022). These include the production of toxins, such as enterotoxins and cytotoxins; cell adherence and invasion; and the production of enzymes. Generally, three different enterotoxins identified as cytotoxic (act), heat-labile cytotonic (alt), and heat-stable cytotonic (ast) enterotoxins have been found to cause diarrhea, enteritis, and dysentery or colitis (Janda and Abbott, 2010; Lee et al., 2023). Furthermore, *Aeromonas* spp. produce a broad spectrum of extracellular enzymes, including amylase, elastase, nuclease, gelatinase, lipase, and protease, which may act alone or in combination to facilitate invasion, replication, and to compete with host immunity. These enzymes are also involved in meat spoilage (Tomás, 2012; Shao et al., 2024).

In Korean coastal areas, antimicrobials such as β-lactams, tetracycline, sulfonamides, and erythromycin, are found in abundance. The extensive use of antibiotics in humans, agriculture, animal husbandry, and aquaculture results in the introduction of antibiotics in aquatic environments through various channels (Jang et al., 2018; Le et al., 2023). This has resulted in high rates of antimicrobial

resistance in *Aeromonas* spp. and other bacteria (Germond and Kim, 2015; Jang et al., 2018; Le et al., 2023).

Most of the previous studies of Manila clam were conducted to assess mortality due to *Vibrio* infection (Paillard et al., 2004; Dubert et al., 2016). Detection, isolation, and characterization of *Aeromonas* spp. from Manila clam have not been studied well. To the best of our knowledge, the genetic characterization of the virulence and multidrug resistance of *Aeromonas* spp. in Manila clam has not been reported. Therefore, our approach in this study aims to evaluate the phenotypic pathogenicity, virulence traits, and antimicrobial resistance profiles of *Aeromonas* spp. isolated from live Manila clam marketed in Korea. The detection of virulence genes and antibiotic resistance in *Aeromonas* spp. isolated from live Manila clam will help to increase awareness about the potential health risks of seafood consumption.

MATERIALS AND METHODS

Phylogenetic analysis of *Aeromonas* spp.

Aeromonas spp. isolates [*A. hydrophilia* (n=11), *A. salmonicida* (n=11), *A. media* (n=7), *A. veronii* (n=4), *A. allosaccharophila* (n=2), and *A. caviae* (n=1)] which were previously isolated in our laboratory from 145 live Manila clams marketed in Korea, were used (Dahanayake et al., 2019). Further identification of *Aeromonas* spp. was performed by *gyrB* gene (1100 bp) sequencing. Gene amplification was performed using the primers gyrB3F (5′TCCGGCGGTCTGCACGGCGT3′) and gyrB14R (5′TTGTCCGGGTTGTACTCGTC3′) according to the report of Yanez et al. (2003). PCR products of the *gyrB* gene were purified using Exgene PCR SV kit (Gene All) and submitted to Cosmogenetech Co. Ltd. (Daejeon, Korea) for gene sequencing.

With the available sequences, a neighbor-joining phylogenetic tree was constructed by aligning multiple sequences using the ClustalW function in the MEGA7 sequence analysis software (Kumar et al., 2016a). The 1.2 kb-long *gyrB* sequences from all *Aeromonas* spp. isolates and their reference sequences were included in the phylogenetic analysis. The reference sequences were retrieved from the NCBI database: *A. hydrophila* (JN711787), *A. caviae* (GU573722), *A. allosaccharophila* (FJ940790), *A. veronii* (KJ747137), *A. salmonicida* subsp. *pectinolytica* (AM262158), *A. salmonicida* subsp. *masoucida* (AM262160), *A. media* (AF417627), and *Escherichia coli* strain KCTC 2441 (EU014649) were used as the outgroup taxa to increase the precision of the tree. A bootstrap value of one thousand was used to assess tree topology robustness in software analysis.

Phenotypic pathogenicity tests

All *Aeromonas* spp. isolates were sustained in tryptic soy agar (TSA) (MB Cell, LA, CA) during tests. Hemolysin production was observed using blood agar base (MB Cell, LA, CA) containing a sheep blood supplement. Colonies surrounded by clear zones were considered beta (β) hemolytic and zones with greenish discoloration demonstrated alpha (α) hemolysis (Schaufuss and Steller, 2003; Akhter et al., 2010). TSA supplemented with 0.5% (w/v) skim milk at 37°C for 48 h was used to assess caseinase production (Zhang and Austin, 2000). Lipase and phospholipase activities were examined by adding 1% (v/v) Tween 80 and 5% (v/v) egg yolk emulsion to TSA, respectively. Following incubation at 37 °C for 48 h, opaque halos around the inoculum site were considered positive for lipase and phospholipase (Liuxy et al., 1996). TSA supplemented with 0.08% (w/v) Congo red and 5% (w/v) sucrose were used to observe slime production. The black-colored colonies were recognized as slime producers (Freeman et al., 1989). DNase agar was used to grow colonies; then, grown colonies were treated with 1N HCl in DNase agar (MB Cell, LA, CA) plates. Bacterial colonies surrounded by halos effect

were distinguished as positive for DNase activity (Kitaura et al., 1983). Gelatinase activity was observed using a nutrient gelatin medium: gelatin (120 g/L), beef extract (3 g/L) and peptone special (5 g/L) in test tubes. Isolates were stab-inoculated into tubes containing nutrient gelatin medium and incubated for 48 h at 37°C. Following incubation, tubes were refrigerated at 4°C for 30 minutes, and the isolates that hydrolyzed gelatin medium were selected as positive (Elavarashi et al., 2017).

Detection of virulence-related genes

DNA extracted from *Aeromonas* spp. isolates were subjected to conventional PCR to reveal the presence of 11 virulence-related genes, using primers and conditions listed in Table 1. A total volume of 20 µL PCR mixture was prepared using 10 µL of Quick Taq HS die mix (Toyobo, Osaka, Japan), 0.2 µM of each forward and reverse primer, 20 ng of template DNA, and PCR water to a final volume of 20 µL. PCR products were checked by electrophoresis on 1.5% (w/v) agarose gels. PCR thermocycler conditions for each reaction were as follows: initial denaturation of 94°C for 2 min, followed by 35 amplification cycles. Each cycle consisted of 94°C denaturation for 30 s, annealing for 50 s, and 72°C extension for 10 min.

Table 1 Oligonucleotide primers and PCR conditions used to amplify virulence-related genes of *Aeromonas* spp.

Gene	Target	Nucleotide Sequence (5'-3')	Size (bp)	Annealing temperature (°C)	Reference
act	Cytotoxic enterotoxin	F: AGAAGGTGACCACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	232	64	(Nawaz et al., 2010)
alt	Heat-labile cytotonic enterotoxin	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	63	(Nawaz et al., 2010)
ast	Heat-stable cytotonic enterotoxin	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	331	63	(Nawaz et al., 2010)
aerA	Aerolysin	F: CCTATGGCCTGAGCGAGAAG R: CCAGTTCCAGTCCCACCACT	431	63	(Nawaz et al., 2010)
ser	Serine protease	F: CACCGAAGTATTGGGTCAGG R: GGCTCATGCGTAACTCTGGT	350	57	(Nawaz et al., 2010)
fla	Flagella	F: TCCAACCGTYTGACCTC R: GMYTGGTTGCGRATGGT	608	55	(Nawaz et al., 2010)
ahyB	Elastase	F: ACACGGTCAAGGAGATCAAC R: CGCTGGTGTTGGCCAGCAGG	513	57	(Nawaz et al., 2010)
lip	Lipase	F: ATCTTCTCCGACTGGTTCGG R: CCGTGCCAGGACTGGGTCTT	382	62	(Sen and Rodgers, 2004)
hlyA	Hemolysin A	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGG	597	62	(Wong et al., 1998)
gcaT	Glycerophospholipid -cholesterol acyltransferase	F: CTCCTGGAATCCCAAGTATCAG R: GGCAGGTTGAACAGCAGTATCT	237	65	(Nawaz et al., 2010)
ascV	Type III Secretion System	F: AGCAGATGAGTATCGACGG R: AGGCAT TCTCCTGTACCAG	891	58	(Carvalho-Castro et al., 2010)

Antibiotic susceptibility test and MAR index

In addition to the 19 antimicrobials that were tested previously (Dahanayake et al., 2019), we further tested five more antimicrobials [Macrolides; erythromycin (15 µg), Lipopeptides; colistin sulfate (10 µg), Glycopeptides; vancomycin (30 µg), Lincosamides; clindamycin (2 µg), and Ansamycins; rifampicin (5 µg)]. The disk diffusion method measured the antibiotic susceptibility on Muller-Hilton agar (Difco, Becton-Dickinson Co. Sparks, MD, USA). Antibiotic discs manufactured by Oxoid LTD, Basingstoke, Hampshire, England were used for antibiotic susceptibility tests. *Escherichia coli* ATCC 25922 was utilized as the quality control strain for this test.

The disk diffusion method was performed in triplicate according to the recommendations of the Clinical and Laboratory Standards Institute. The susceptibility of *Aeromonas* spp. isolates were categorized as susceptible (S), intermediately resistant (I), or resistant (R) according to CLSI 2020 guidelines (CLSI, 2020). The MAR index was calculated for each *Aeromonas* spp. isolate by following the method described by Krumperman (1983).

Statistical Analysis

Statistical analysis was performed in SPSS (IBM SPSS 29.0, USA) and the significance level was set at $p < 0.05$. The variation of virulence genes of all *Aeromonas* spp. isolates were tested by ANOVA. The post hoc Tukey HSD test and LSD test were used for statistical evaluation of the incidence of the virulence genes between the species and all isolates. Statistical assessment of the frequency of antibiotic resistance of group 1 (*A. hydrophila*) and group 2 (*A. salmonicida* subsp. *masoucida* and *A. salmonicida* subsp*. pectinolytica*) were also tested by ANOVA.

RESULTS

Phylogenetic analysis of *Aeromonas* spp.

The inferred phylogenetic tree (Figure 1) exhibited a distinct clustering pattern for each species. The tree depicted two main clades. One clade comprised only *A. media,* whereas the other clade included all the other species isolates. However, slight genetic divergences within species were also observed. For example, one *A. veronii* was grouped into the sub clade designated to *A. allosaccharophila,* and two of the *A. salmonicida* subsp. *pectinolytica* were grouped into the sub clade of *A. salmonicida* subsp. *masocida.*

Phenotypic pathogenicity tests

Results from seven pathogenicity tests demonstrating phenotypic virulence are presented in Table 2. *Aeromonas* spp. isolates showed 100% positivity for DNase, and phospholipase tests. Interestingly, all *A. hydrophila* and *A. salmonicida* were positive for all phenotypic tests except slime production. *A. allosaccharophila* showed the least positivity among all other species, being positive for only three tests out of seven. Gelatinase, lipase, hemolysis, caseinase, and slime production activities were observed in 94%, 83%, 78%, 75%, and 31% of the isolates, respectively. The majority of the *Aeromonas* spp. isolates (53%) expressed β-hemolysis on blood agar, while α-hemolysis and γ-hemolysis activities were found in 25% and 22% of isolates. Lipase and phospholipase activities were commonly observed among isolates in this study, except for negative lipase activity for *A. veronii* and *A. allosaccharophila* isolates. All *A. hydrophila, A. allosaccharophila,* and *A. salmonicida* isolates were negative for slime production. However, slime production was observed in *A. media*, *A. caviae* and *A. veronii* isolates.

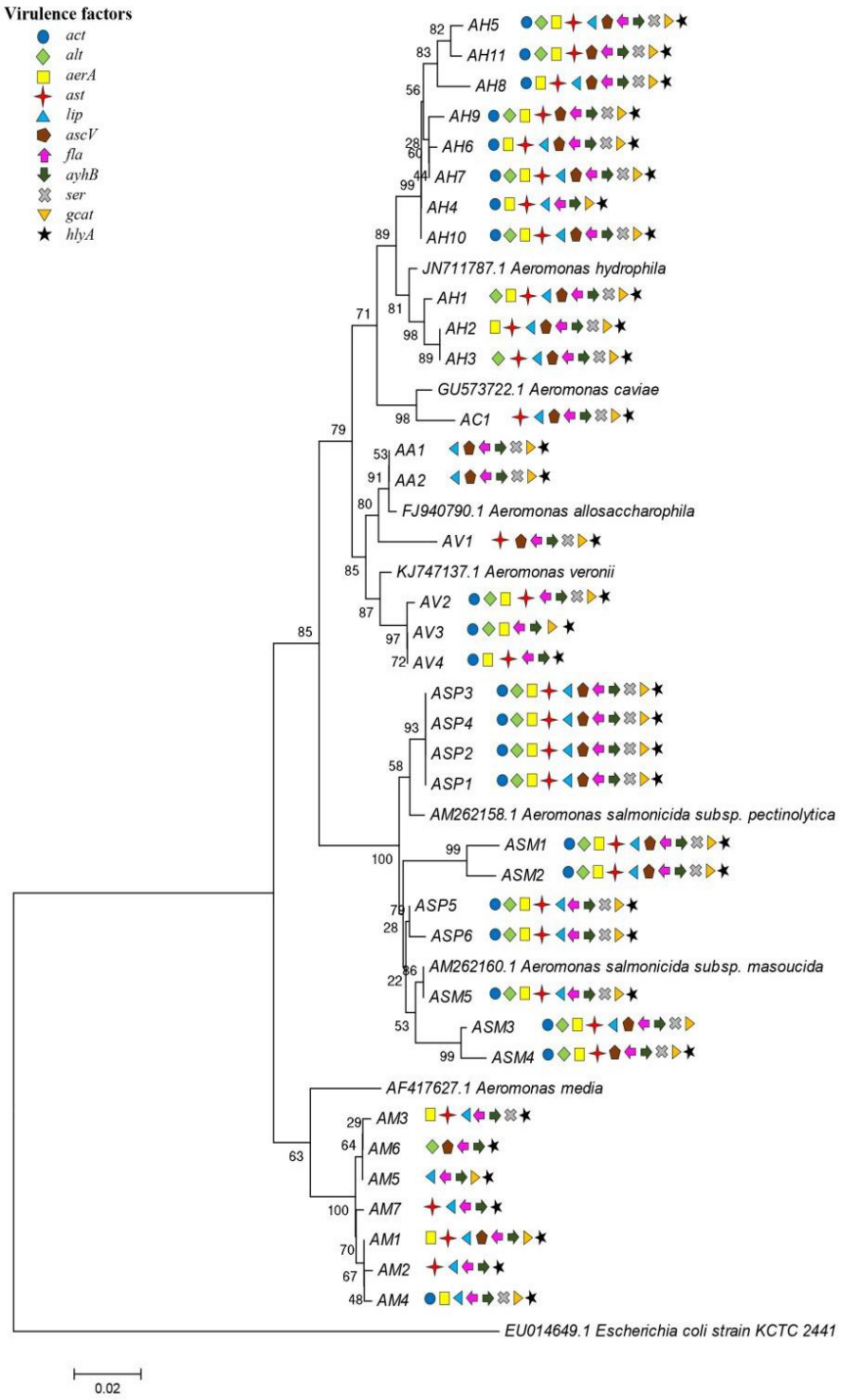

Figure 1 Neighbor-joining phylogenetic tree of *Aeromonas* strains and the distribution of virulence genes of isolated *Aeromonas* spp. Taxa labels; AH = *A. hydrophila*, AC = *A. caviae*, AV = *A. veronii* ASM = *A. salmonicida* subsp*. masocida*, ASP = *A. salmonicida* subsp. *pectinolytica* AM = *A.media*. AA = *A. allosaccharophila*. Other taxa have been obtained from the National Center for Biotechnology Information (NCBI) database as reference sequences. Colored shapes indicate only the positive expression of virulence genes and do not represent their sequential organization in the genome analyzed in this study.

Table 2 Phenotypic virulence traits of *Aeromonas* spp. isolated from live Manila clam

Species	Number of positive isolates								
	DNase	Caseinase	Gelatinase	Lipase	Phospholipase	Slime production	Haemolysis		
							α	β	γ
A. hydrophila (*n* = 11)	11	11	11	11	11	0	3	8	-
A. media (*n* = 7)	7	1	7	7	7	7	1	-	6
A. veronii (*n* = 4)	4	3	4	0	4	3	3	-	1
A. caviae (*n* = 1)	1	1	1	1	1	1	-	-	1
A. allosaccharopila (*n = 2*)	2	0	0	0	2	0	2	-	-
A. salmonicida subsp. *masoucida* (*n* = 5)	5	5	5	5	5	0	-	5	-
A. salmonicida subsp. *pectinolytica* *(n=6)*	6	6	6	6	6	0	-	6	-
Total (%) (*n* = 36)	36 (100%)	27 (75%)	34 (94%)	30 (83%)	36 (100%)	11 (31%)	9 (25%)	19 (53%)	8 (22%)

Detection of virulence-related genes

The presence of eleven virulence-associated genes was investigated by polymerase chain reaction (PCR). Results obtained from the tests are shown in Figure 2, and types of gene combinations are illustrated in Figure 3.

A high frequency of positive isolates was observed for most of the tested genes. Three *A. hydrophila* isolates and seven *A. salmonicida* isolates harbored all the tested virulence genes. All except one *A. salmonicida* isolate contained the *hlyA* gene. The *ahyB* and *fla* genes were detected in all isolates (Figure 2). Particularly, a high occurrence of enterotoxin genes (*alt, ast, act*) was observed among all *A. hydrophila*, *A. salmonicida* and *A. veronii* isolates. Six different combinations of the enterotoxins encoding genes *alt/ast/act* were observed. The most common enterotoxin gene combination (*alt/ast/act*) was found in 17 *Aeromonas* isolates, including *A. hydrophila, A. salmonicida,* and *A. veronii.* The *act/ast* gene combination was detected in eight isolates, including six *A. hydrophila* and two of *A. veronii*. In contrast, *alt/ast* combination was detected in seven isolates of *A. hydrophila*. *A. caviae* harbored only *ast* gene among those toxin genes. The *A. allosaccharophila* isolates did not harbor any enterotoxin genes (*alt, ast, act*) (Figure 3). Statistical evaluation of variation frequency of the virulence genes of all *Aeromonas* spp. showed no significant difference between group 1 (*A. hydrophila*) and group 2 (*A. salmonicida* subsp. *masoucida* and *A. salmonicida* subsp. *pectinolytica*). However, significant differences were observed in the rate of frequency of the virulence genes between group 1 and group 3 (*A. media, A. veronii, A. caviae, A. allosaccharophila*) ($p < 0.05$); and group 2 and group 3 ($p < 0.05$).

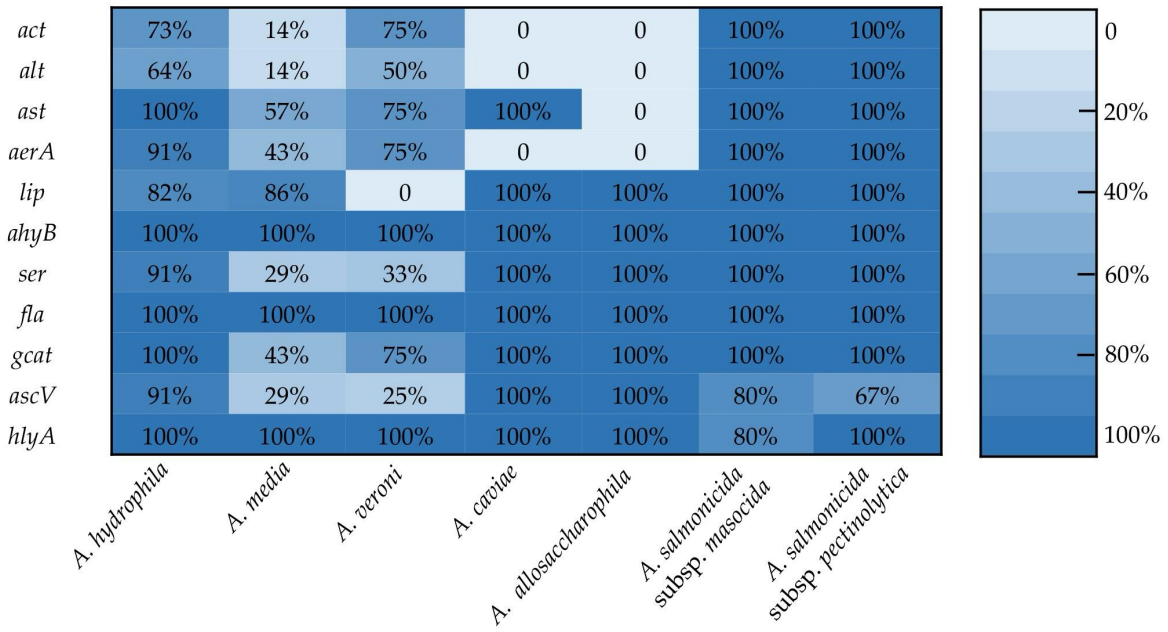

Figure 2 The distribution of the virulence genes among *Aeromonas* spp. isolated from live Manila clam

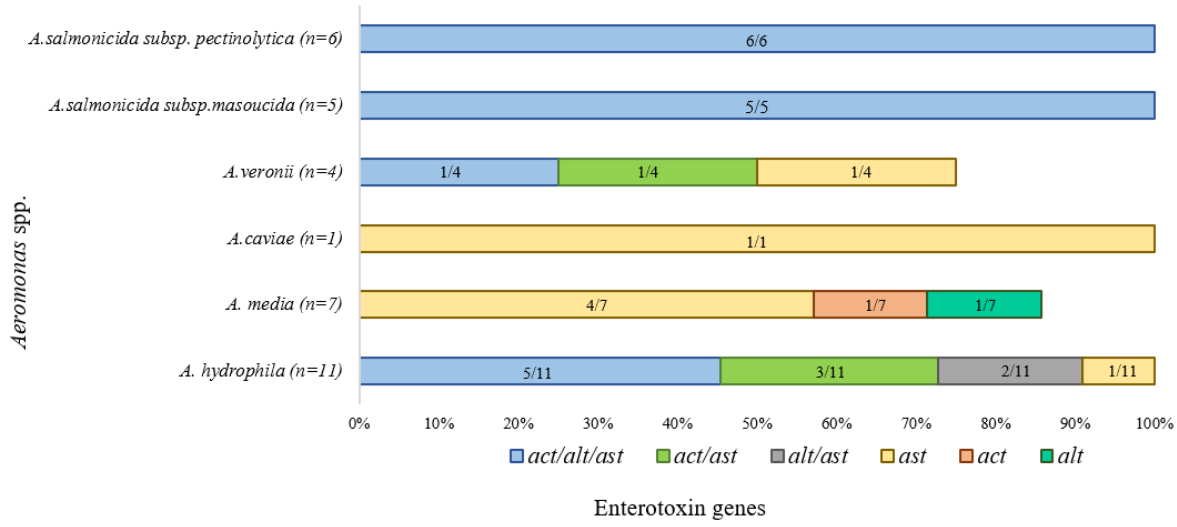

Figure 3 Relative distribution of enterotoxin gene (*act*, *alt* and *ast*) combinations among *Aeromonas* spp. isolated from live Manila clam

Antibiotic susceptibility test and MAR index

In the present study, susceptibility patterns were found to be heterogeneous among the *Aeromonas* spp. isolates (Table 3 and Figure 4). The calculated MAR index values showed that three *A. hydrophila* isolates (AH5, AH6, AH7) were resistant to nine tested antimicrobials. Specifically, isolate AH5 was resistant to piperacillin (PRL), while isolates AH6 and AH7 were resistant to colistin (COL). Similar resistance patterns were also observed in other *Aeromonas* spp. isolates. Distinctly, except for one *A. veronii* isolate, all others showed MAR index values of 0.2 or higher. The calculated MAR index values showed that all *A. hydrophila*

isolates were resistant to ≥8 of the 24 tested antibiotics. All isolates were resistant to vancomycin. Additionally, resistance was observed for clindamycin (94%), colistin (58%), rifampicin (47%), and imipenem (38%). Statistical evaluation of the frequency of antibiotic resistance in *A. hydrophila* and *A. salmonicida* revealed a significant difference between these two species ($p < 0.05$).

Table 3 MAR index values of *Aeromonas* spp. isolated from live Manila clams

Aeromonas spp.	Isolate	Resisted antimicrobials[a]	MAR index
A. hydrophila	AH1	AMP, TET, OT, COL, VA, DA, NAL, KF	0.33
	AH2	AMP, TET, OT, COL, VA, DA, NAL, KF	0.33
	AH3	AMP, TET, OT, COL, VA, DA, NAL, KF	0.33
	AH4	AMP, TET, OT, COL, VA, DA, NAL, KF	0.33
	AH5	AMP, PRL, TET, OT, VA, DA, NAL, IMI, KF	0.37
	AH6	AMP, TET, OT, COL, VA, DA, NAL, IMI, KF	0.37
	AH7	AMP, TET, OT, COL, VA, DA, NAL, IMI, KF	0.37
	AH8	AMP, TET, OT, VA, DA, NAL, IMI, KF	0.33
	AH9	AMP, TET, OT, VA, DA, NAL, IMI, KF	0.33
	AH10	AMP, TET, OT, COL, VA, DA, NAL, KF	0.33
	AH11	AMP, TET, OT, COL, VA, DA, NAL, KF	0.33
A. media	AM1	AMP, VA, DA, ERY, MRP, RD, CRO, KF	0.33
	AM2	AMP, COL, VA, RD, KF	0.20
	AM3	AMP, COL, VA, DA, ERY, RD, KF	0.29
	AM4	AMP, TET, OT, VA, DA, KF	0.25
	AM5	AMP, VA, DA, ERY, KF	0.20
	AM6	AMP, COL, VA, DA, MRP, RD, CRO, KF	0.33
	AM7	AMP, COL, VA, DA, KF	0.20
A. veronii	AV1	AMP, TET, OT, VA, DA	0.20
	AV2	AMP, VA, DA, STR, IMI	0.20
	AV3	AMP, VA, DA, IMI	0.16
	AV4	AMP, COL, VA, DA, STR, IMI	0.25
A. salmonicida subsp. *masoucida*	ASM1	AMP, COL, VA, DA, RD, KF	0.25
	ASM2	AMP, COL, VA, DA, RD, KF	0.25
	ASM3	AMP, OT, VA, DA, NAL, RD, KF	0.29
	ASM4	AMP, OT, VA, DA, NAL, RD, KF	0.29
	ASM5	AMP, VA, DA, IMI, RD, KF	0.25
A. salmonicida subsp. *pectinolytica*	ASP1	AMP, COL, VA, DA, RD, KF	0.25
	ASP2	AMP, COL, VA, DA, RD, KF	0.25
	ASP3	AMP, COL, VA, DA, RD, KF	0.25
	ASP4	AMP, COL, VA, DA, IMI, RD, KF	0.29
	ASP5	AMP, VA, DA, IMI, RD, KF	0.25
	ASP6	AMP, VA, DA, IMI, RD, KF	0.25
A. allosaccharophila	AA1	AMP, VA, DA, IMI, RD, KF	0.25
	AA2	AMP, PRL, COL, VA, DA, IMI, RD, KF	0.33
A. caviae	AC1	AMP, PRL, COL, VA, STR, SXT, KF	0.29

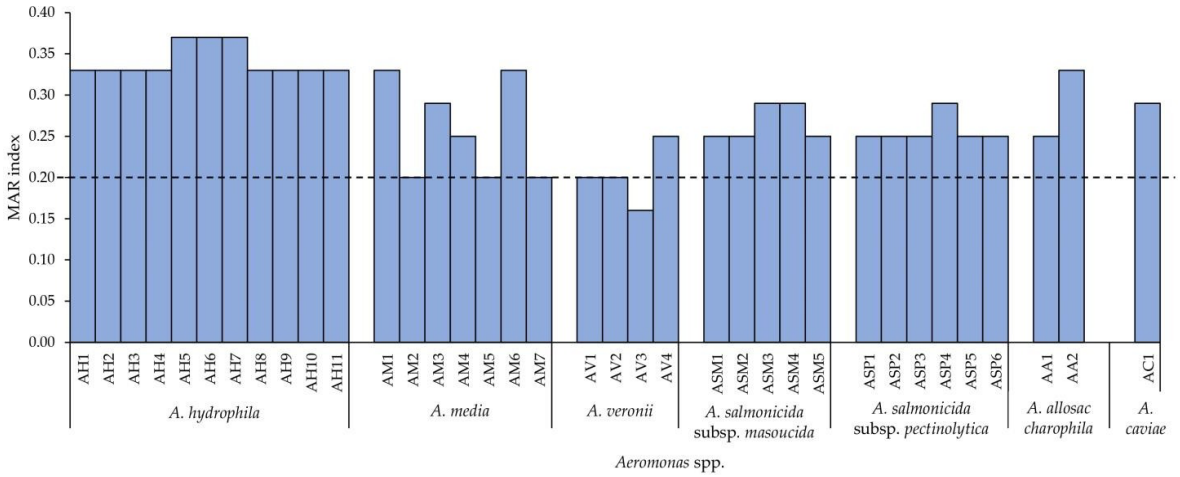

Figure 4 Distribution of MAR index values among *Aeromonas* spp. isolated from live Manila clam.

DISCUSSION

Aeromonas spp. have emerged as important pathogens in aquaculture and opportunistic human pathogens that cause serious diseases, especially in immunocompromised patients (Chenia, 2016). A previous study has reported that *Aeromonas* exhibited a much higher risk of illness, even at moderate and low densities in natural exposure conditions (Teunis and Figueras, 2016). *A. hydrophila* was reported as the most prevalent *Aeromonas* sp. isolated from scallops and shrimps marketed in Korea (De Silva et al., 2018; De Silva et al., 2019). The psychrophilic non-motile *A. salmonicida* is usually related to fish infections causing furunculosis (Austin et al., 1998). However, *A. salmonicida* isolates in our study can be categorized in the group of non-typical mesophilic *A. salmonicida* as they were grown under 37˚C incubated conditions as previously reported (Austin, 1993). The presence of *A. salmonicida* subsp. *masoucida* has been reported in various fish species (Goldschmidt-Clermont et al., 2009). Although *A. salmonicida* is well recognized as a fish pathogen, the occurrence of *A. salmonicida* in human blood and a case report of *A. salmonicida* bacteremia in a patient with diabetes have also been reported. This emphasizes its importance as a zoonotic species (Moore et al., 2017). One *A. veronii* isolate was grouped into the sub-clade designated as *A. allosaccharophila* in the constructed phylogenetic tree. This grouping of *A. veronii* isolate (AV1) into the sub clade of *A. allosaccharophila* could be due to the close taxonomic relationship between *A. veronii* and *A. allosaccharophila.* Several studies have considered *A. allosaccharophila* as a synonym for *A. veronii* (Huys et al., 2001). The reliable identification of *Aeromonas* spp. is necessary to treat patients effectively, prevent infections, and establish outbreak management and threat analyses (Hu et al., 2012).

The pathogenicity of *Aeromonas* spp. is complex and involves several factors, such as extracellular proteins, exotoxins, enzymes, and different virulence genes (Lee et al., 2021). Seasonal prevalence of *A. hydrophila* with several putative virulence genes was reported in sushi and retail seafood in Korea (Park et al., 2021). In this study, seven pathogenicity tests and eleven virulence-associated genes were used. A high occurrence of toxin-related genes was observed among *A. hydrophila*, *A. veronii,* and *A. salmonicida* isolates. Previous studies have reported the aeromonads' potential to cause illnesses with the presence of *act, alt,* and *ast* virulence determinants in clinical, food, and environmental samples (Ottaviani et al., 2011). Lee et al. (2021) reported the presence of *alt* and *act* genes in *Aeromonas* spp. isolated from seafood. In addition, 70% of the *Aeromonas* spp. isolates, including *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. veronii* bv sorbia, and *A.*

enchelia were found to carry the *act* gene in aeromonads isolated from drinking water in the USA (Sen and Rodgers, 2004). Furthermore, the *act* gene was detected in *A. veronii* (100%) and *A. aquariorum* (25%) from cultured shrimps in Thailand (Yano et al., 2015).

Hemolysins produced by *A. hydrophila* and *A. sobria* have been shown to induce fluid accumulation in a mouse intestinal loop test. Therefore, hemolysin is considered one of the primary etiological agents of diarrhea with enterotoxic activity (Takahashi et al., 2014). In the current study, *Aeromonas* spp. isolates (53%) expressed β-hemolysis on blood agar, while 25% showed α-hemolysis and 22% exhibited γ-hemolysis activities. Furthermore, most of the Manila clam-borne *Aeromonas* spp. isolates harbored both *aerA* and *hlyA* genes. A. *hydrophila* associated with recent disease outbreaks in freshwater aquaculture in Vietnam were found positive for *aerA* and *hlyA* genes (Nhinh et al., 2021). Most hemolysin-positive isolates carried both *aerA* and *hlyA* genes; three *A. media* isolates were found to be hemolysin-negative even though they carried both genes. As similarly reported in a previous study, *A. caviae* and *A. media* strains isolated from retailed sushi were found negative for β-hemolysis, but they harbored both genes. The lack of hemolysis could be related to the absence of the *act* gene (Hoel et al., 2017). Virulence genes (*alt, ast, act, hlyA*, and *aerA*) exist in *Aeromonas* spp. isolates posed high potential pathogenicity to fish mortality and human infections (Janda, 1985).

Extracellular proteases (*ser*) in *Aeromonas* spp. facilitate the invasion of pathogens by degrading multifaceted biological proteins present in the host (Janda, 1985; Han et al., 2008). Additionally, in the process of quorum sensing, serine protease has been identified as an activator for toxins, such as aerolysin (*aer*) and glycerophospholipid-cholesterol acyltransferase (*gcat*) (Janda and Abbott, 2010; Tomás, 2012). Similar to our study, *ser* gene was reported in *Aeromonas* spp. including *A. hydrophila, A. salmonicida*, and *A. veronii*; isolated from fish and water (Onuk et al., 2013). Moreover, the *ahyB* gene was detected in all our isolates. The *ahyB* gene is an important virulence factor determining elastase (metalloprotease) production (Guerra et al., 2007).

Phospholipases in bacteria are related to intestinal damage and are involved in different pathogenic processes. Furthermore, secreted phospholipases act as hemolysins and glycerophospholipid cholesterol acyl-transferases (Onuk et al., 2013). Lipases interact with human leukocytes to release free fatty acids that distress several immune system functions through lipolytic activity (Tomás, 2012). In the present study, all *Aeromonas* spp. isolates showed positivity for phospholipase. In contrast, lipase was detected in 83% of the isolates. Scoaris et al. (2008) reported phospholipase activity in *Aeromonas* spp. isolated from water sources. Furthermore, similar to our study, the *gcat* gene, which causes erythrocyte lysis by degrading erythrocyte membrane, and *lip* gene have been found in *A. hydrophila* and *A. salmonicida* (Thornton et al., 1988; Austin et al., 1998). Martino et al. (2011) have suggested that these discrepancies can occur due to aeromonads' genetic variability.

Slime production is essential in pathogenesis. It inhibits neutrophil function, chemotaxis, phagocytosis, and antimicrobial drugs and supports biofilm formation (Merino et al., 1995). Furthermore, it reflects the pathogen's capacity to adhere to host tissue and cause severe illness (Sreedharan et al., 2012). A previous study detected high slime production in *A. hydrophila* (78.1%) from fish and shrimp isolates (Illanchezian et al., 2010). Nevertheless, Sechi et al. (2002) reported that the frequency of slime-producing *A. hydrophila* in clinical isolates (50%) was higher than that in environmental isolates (35.3%). Flagella increase adhesion and biofilm formation (Onuk et al., 2013). All the *Aeromonas* isolates displayed the *fla* gene. In agreement with our study, the *fla* gene was reported in *Aeromonas* spp. isolated from three different ponds in Bangladesh (Sadique et al., 2021). The presence of DNase in *Aeromonas* may play an essential role in the spoilage of fishery products.

Also, gelatinase production has been identified as a cause of human bacteremia (Vergis et al., 2002). In the present study, higher production of DNase and gelatinase was observed. Similar results were reported for *Aeromonas* spp. isolated from shrimps (Yanez et al., 2003). The display of the enzymatic activity of different extracellular proteins and the presence of various virulence genes indicate the virulence potential of *Aeromonas* spp. (Lee et al., 2021).

The extensive use of antibiotics as prophylactics, growth promoters, and for the treatment of bacterial infections in aquaculture, agriculture, and animal husbandry has increased the number of resistant bacteria in aquatic environments, which can increase the risk of antibiotic-resistant bacteria in seafood (Rahman et al., 2009; Kumar et al., 2016b). In the current study, except for one *A. veronii* isolate, all other isolates showed MAR index values of 0.2 or higher, indicating that they originated from a high-risk source of contamination where antibiotics are widely used (Krumperman, 1983). Additionally, it has been reported that increased use of antimicrobials has led to a rise in the number of resistant *Aeromonas* spp. and other pathogenic bacteria in coastal areas in South Korea (Germond and Kim, 2015). In previous literature, *Aeromonas* spp. isolates showed resistance to erythromycin, colistin sulfate, and clindamycin (Scarano et al., 2018; Zdanowicz et al., 2020; Mulia et al., 2021). Therefore, in the present study, we examined an additional five antibiotics (erythromycin, colistin sulfate, vancomycin, clindamycin, and rifampicin) to enhance our understanding of the multidrug resistance in *Aeromona*s spp. Similar to our study, Kusdarwati et al. (2018) reported that *A. hydrophila* exhibited resistance to colistin sulfate, vancomycin, and rifampicin. Furthermore, *A. hydrophila* isolated from diseased fish that were resistant to multiple antibiotics also showed resistance to colistin sulfate, erythromycin, and vancomycin in different studies (Mazumder et al., 2021; Preena et al., 2021). Bacteria modify the outer membrane structure of lipopolysaccharides, weakening the binding of colistin with the lipopolysaccharide wall, thus developing resistance colistin sulfate. A *vanA* gene facilitates resistance to vancomycin. *VanA* changes the target terminal D-alanil-D-serine or D-alanil-D-alanil, which causes poor bonding of vancomycin, resulting in decreased sensitivity (Leclere et al., 2006). Moreover, in this study, a high percentage of *Aeromonas* spp. isolates (97%) were found to be resistant to clindamycin. Similarly, Zdanowicz et al. (2020) reported that *Aeromonas* spp. isolates from the water of three carp ponds were resistant to clindamycin and ampicillin. Resistance to clindamycin was also observed in *A. hydrophila* isolated from aquatic animals in Sichuan, China (Peng et al., 2024). Resistance to tetracycline and oxytetracycline was observed in all *A. hydrophila* isolates. *Aeromonas* spp. especially *A. veronii* isolated from common carp showed resistance to tetracycline (Syrova et al., 2018). A study conducted on fish, shellfish, and water confirmed that tetracycline resistance has been linked to various plasmids inside the *A. hydrophila* (Borrego et al., 1991). Similar to our study, *A. hydrophila* isolated from rainbow trout showed resistance to piperacillin (Saavedra et al., 2004).

Parallel to our results, another study reported that *Aeromonas* spp. isolated from oysters collected from a natural oyster bed at the Cocó River estuary in Brazil, were sensitive to ciprofloxacin and chloramphenicol antimicrobials. Additionally, most of the isolates from the same study showed sensitivity to nalidixic acid (97%), ceftriaxone (93%) and cephalothin (92%) (Evangelista-Barreto et al., 2006). In contrast to our study, *A. hydrophila* and *A. caviae* isolates from food and environmental samples were sensitive to imipenem (Kong et al., 2020). Resistance to third-generation imipenem occurs due to the depression of the chromosomal enzymes (Dhanapala et al., 2021). Similarly, *A. hydrophila* isolated from fish farms having hemorrhagic septicemia cases were resistant to imipenem (Taha et al., 2021). Many previous studies have reported *Aeromonas* resistance to erythromycin (Sicuro et al., 2020; Saengsitthisak et al., 2020; Morshdy et al., 2022). However, our isolates were susceptible to erythromycin. Selective pressures in these areas,

where antibiotics are used in aquatic and human treatment and prevention strategies, may have contributed to the development of resistance in these strains (Al-Mashhadany, 2020). The wide use of antibiotics to prevent and treat bacterial disease and subtherapeutic dose application in fish farming has led to increase antibiotic resistance among bacterial pathogens in fish (Zdanowicz et al., 2020). Treating infections caused by resistant *Aeromonas* spp. can be challenging because of its antimicrobial resistance mechanism (Dhanapala et al., 2021). It implies that the overuse of antimicrobials has led to the rise of multi-drug-resistant bacteria, posing a health risk for consumers.

No significant difference was observed between the frequency of the virulence genes of *A. hydrophila* and *A. salmonicida.* However, a significant difference in antibiotic resistance frequency between these two species was observed. Moreover, a significant difference was detected in the frequency of the virulence genes of *A. hydrophila* (group 1) and *A. media, A. veronii, A. caviae, A. allosaccharophila* (group 3). Similarly, *A. salmonicida* subsp. *masoucida, A. salmonicida* subsp*. pectinolytica* (group 2) and *A. media, A. veronii, A. caviae, A. allosaccharophila* (group 3) also showed significant differences in the frequency of virulence genes. The detection of various virulence factors, including extracellular enzymes, toxins, and virulence genes, as well as multidrug resistance in *Aeromonas* spp. isolates, underscores the necessity for regular identification of *Aeromonas* spp. and continuous monitoring of their virulence potential and antibiotic resistance patterns.

CONCLUSIONS

Our results reveal the presence of potentially pathogenic *Aeromonas* spp. in marketed live Manila clam. Notably, we identified mesophilic strains of *A. salmonicida* subspecies for the first time in Manila clam sold in Korea. The frequency of virulence-related factors observed among *A. hydrophila* and *A. Salmonicida* subsp. isolates cannot be underestimated. The observed distribution of a combination of three enterotoxin genes in all *A. salmonicida* strains suggests a potential infection risk for raw Manila clam consumers. Therefore, this study contributes to enhancing consumer awareness of the potential public health risk hazard associated with the consuming raw or undercooked Manila clams marketed in Korea. However, a further clinical inspection of *Aeromonas* spp. infection and continuous monitoring of *Aeromonas* spp. in Manila clams is required for health risks assessment.

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AUTHOR CONTRIBUTIONS

Pasan Sepala Dahanayake, Sana Majeed and Gang-Joon Heo contributed to the conception and design of the study. Pasan Sepala Dahanayake, Sana Majeed, P.M. Kumarage, and Gang-Joon Heo performed material preparation, data collection, and analysis. Pasan Sepala Dahanayake and Sana Majeed wrote the first draft of the manuscript, and all authors contributed to reviewing and editing the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

There is no conflict of interest to report on the part of the authors.

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