

EVALUATING SEASONALITY AND PATHOGENICITY OF AEROMONAS IN KOREA USING ENVIRONMENTAL DNA

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Abstract– Fish are sensitive to environmental perturbations, and a common signal of an unbalanced ecosystem is fish disease and death. Recently in Inje County, Korea, finding dead fish has become a common situation. Our goal was to use environmental DNA (eDNA) approaches to look for geographic and seasonal patterns in the presence of *Aeromonas* in waterways. First, we cultured and identified bacteria from diseased fish and screened for virulence factors. Twelve of the 21 identified bacterial species are known fish pathogens, with *Aeromonas veronii* and *A. hydrophila* being most common (37/61 total strains). Focusing on *A. veronii* and *A. hydrophila*, we used an eDNA method to screen water samples from the major waterways. We discovered geographic and seasonal patterns—*Aeromonas* detection was highest in Inbuk Stream and lowest during the summer.

INTRODUCTION

Freshwater ecosystems play important roles transporting water and nutrients from land to sea, supporting ecological communities, and providing resources to humans (Bailey *et al.*, 2004). Humans depend on healthy water bodies for drinking water, food, and recreation, but human activity itself threatens water quality; these threats include overexploitation, water pollution, habitat destruction, flow modification, and invasive species (Dudgeon *et al.*, 2006). As poor water quality can lead to human disease (*e.g.*, Xiao *et al.*, 2013), it is important to monitor the health of freshwater ecosystems. Fish are sensitive to environmental perturbations, and a common signal of an unbalanced ecosystem is fish death. Fish deaths can disrupt ecosystems (disturbed food webs) and local economies (cleanup costs, reduced tourism) (Holmlund and Hammer, 1999). While fish deaths can be natural, many are caused by human activity (La and Cooke, 2011); the proximate cause can be a combination of biotic (biotoxins, pathogens) and abiotic (pollution, extreme temperature change) factors (Haslour, 1979; Thronson and Quigg, 2008).

Fish disease and death are commonly caused by

bacterial pathogens, especially by species in the genus *Aeromonas* (Austin and Austin, 2007). In *Aeromonas*, there are two major groups—psychrophilic, non-motile (*A. salmonicida* subspecies) and mesophilic, motile (*A. hydrophila* and *A. veronii*) (Janda and Abbott, 2010). Psychrophilic, non-motile *Aeromonas* are known to cause furunculosis (a highly contagious, lethal disease characterized by ulcers on the skin) and mass death in salmonid and other economically important fish (Austin and Austin, 2007). Mesophilic, motile *Aeromonas* cause stress-induced diseases (septicemia and red-sore disease) in freshwater fish (Beaz-Hidalgo and Figueras, 2012). Environmental DNA (eDNA) approaches are a powerful way to screen for *Aeromonas*. eDNA refers to DNA that can be extracted from environmental samples (air, soil, water) without first isolating the target organism (Taberlet *et al.*, 2012). Such methods require less time (sampling in the field and culturing in the laboratory), are non-invasive, and are more sensitive to detection.

In South Korea, freshwater for the residents of Seoul, the capital and largest city, comes from the confluence of several rivers. At the headwaters of one of these rivers (Soyang River) is Inje County.

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Inje's streams are known for their clean water and recreational fishing, but finding dead fish has become a common occurrence. In this study, we first use molecular methods to identify bacteria associated with diseased fish and test for pathogenicity. Next, we use eDNA approaches to look for geographic and seasonal patterns of *Aeromonas* in the waterways. As *Aeromonas* species are both fish and human pathogens (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013), these results are important to evaluate water quality for ecosystem and human health.

MATERIALS AND METHODS

Sample collection and preparation

We did preliminary sampling to identify bacterial species associated with diseased fish. In June 2013, many dead or moribund fish of two species (*Zacco platypus* [Temminck and Schlegel] and *Microphysogobio longidorsalis* Mori) were found at the upstream site of Inbuk Stream (site 1; Figure 1) in Inje County, South Korea. To eliminate the possibility of bacteria colonizing the fish after death, we only collected moribund fish. At the site, we found two moribund fish with abnormal swimming and external symptoms. We collected and transported the fish to the laboratory on ice (<5 hours) for immediate culturing. To prepare for culturing bacteria, fish were rinsed with distilled water to remove surface contaminants. From each area with visible infection (body, eye, head, or fin), a 5×5 mm piece of tissue was dissected and put in a microcentrifuge tube with 1 mL of distilled water. The tubes were vortexed (~1 min) and 100 µL of the liquid was spread on a trypticase soy agar (TSA, Difco) plate to culture bacteria; three culture plates were made for each tissue sample. Plates were incubated at 30°C for three days. To obtain pure cultures, all colonies from all plates were transferred to individual TSA plates and incubated at 30°C for three days. Genomic DNA was extracted from each strain using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Rogers and Bendich, 1994) by taking approximately 20 µL of bacterial material directly from the culture plate. Stocks of each strain were prepared for long-term storage by placing bacterial material in 1 mL of sterilized 20% glycerol and frozen at -80°C.

To investigate the geographic and seasonal patterns of *Aeromonas* in the environment, we

collected water samples from eight sites along the four major waterways—Inbuk Stream (sites 1, 2), Buk Stream (sites 3, 4), Naerin Stream (sites 5, 6), and Soyang River (sites 7,8)—at five times of the year (June 2013, August 2013, October 2013, January 2014, March 2014), for a total of 40 samples (Figure 2). The three streams (Inbuk, Buk, Naerin) flow into the Soyang River. Water was collected in sterile 2 L bottles specialized for water sampling (#WBE002; Y&K Healthcare, Korea) and transported on ice. For DNA extraction of each sample, 1 L of water was filtered using a 0.22 µm pore size, mixed cellulose membrane filter (#GSWP04700, Merck Millipore, USA). The filter was cut into smaller pieces with sterile scissors and placed in a 15 mL conical centrifuge tube (BD Falcon, USA) with 5 mL 2X CTAB. The filter was ground with a plastic pestle and genomic DNA was extracted using the modified CTAB protocol (Rogers and Bendich, 1994).

Strain identification

Three molecular markers (16S ribosomal RNA [*16S*], DNA gyrase subunit B [*gyrB*], and RNA polymerase sigma 70 factor [*rpoD*]) were amplified and sequenced to identify cultured strains to species. The three markers were amplified using primers and thermocycler conditions listed in Table 1. All PCR reactions were performed on a C1000™ thermal cycler (Bio-Rad, USA) using Maxime PCR premix i-StarTaq (Intron Biotechnology Inc., Korea) in a final volume of 20 mL containing 10 pmol of each primer and 1 mL of DNA (~10ng/mL). PCR products were visualized on a 1% agarose gel stained with Loading Star (DyneBio, Korea) and purified using the Expin™ PCR Purification Kit (GeneAll Biotechnology, Korea). DNA was sequenced at Macrogen (Seoul, Korea) on an ABI3700 automated DNA sequencer (Applied Biosystems, USA) using PCR primers. Sequences were assembled and proofread using Geneious v5.3.6 (Drummond *et al.*, 2010) and aligned using MUSCLE v.3.8.31 (Edgar 2004).

Strains were identified using a two-step process. First, all strains were identified by comparing 16S sequences to the EzTaxon database. EzTaxon is a curated database of 16S sequences from prokaryote type strains (<http://www.ezbioloud.net/eztaxon>; Kim *et al.*, 2012). Next, identification of *Aeromonas* strains was refined with a maximum likelihood (ML) phylogenetic analysis of 16S, *gyrB*, and *rpoD* using RAXML v.8.0.2 (Stamatakis, 2014). The analysis was partitioned by gene (to account for

different evolutionary rates between markers) and performed using the combined rapid bootstrap and ML search, GTR+G model of nucleotide substitution, and 1000 bootstrap replicates. Sequences for *Aeromonas* type strains were downloaded from GenBank and included in the analysis (Table 1); when multiple accession numbers were available for a type strain, sequences were assembled and the consensus sequence was used in the analysis.

Virulence profiles of strains

To evaluate pathogenicity, we used a rapid PCR-based method developed in Soler *et al.* (2002) to screen all strains for the presence of five *Aeromonas* virulence factors: lipase (*lip*), glycerophospholipid-cholesterol acyltransferase (*gcaT*), aerolysin/hemolysin (*aer*), serine protease (*ser*), and DNase (Table 1). These virulence factors may be involved in infection mechanisms and have been found in pathogenic species of *Aeromonas* (Pemberton *et al.*, 1997). After visualization, two PCR products for each virulence factor were sequenced to verify their identity; PCR purification and DNA sequencing followed the above protocols. We refer to the

combined result for virulence factors of a strain as its virulence profile.

eDNA detection of *Aeromonas* and virulence factors

For identifying geographic and seasonal patterns, we used the eDNA-based method of Dorsch *et al.* (1994) to screen water samples for the presence of *A. hydrophila* and *A. veronii* using species-specific primers (Table 1). Water samples were also screened for the five *Aeromonas* virulence factors following the previous protocol. Additionally, we measured the water temperature at the time samples were collected.

RESULTS

Strain identification

A total of 61 bacterial strains were recovered from cultures of diseased fish tissues. *16S* sequences identified the majority of strains as *Aeromonas* (37 strains), while the remaining 24 strains were identified as 16 species in 12 genera (Figure 1B). The resolution of *16S* for species identification in

Table 1. Information about primers used in this study.

Target gene	Primer name	Sequence (5'–3')	Amplicon size (bp)	Annealing temp (°C)	Reference
<i>16S</i>	27F	AGAGTTTGATCMTGGCTCAG	~1400	55	Lane 1991
	1492R	CGGTTACCTTGTTACGACTT			
	<i>hydrophila</i> ^a	GAAAGGTTGATGCCTAATACGTA	~600	64	Dorsch <i>et al.</i> 1994
	<i>veronii</i> ^a	GAGGAAAGGTTGGTAGCTAATAA	~600	64	
	reverse ^a	CGTGCTGGCAACAAAGGACAG			
<i>gyrB</i>	gyrB3F gyrB14R	TCCGGCGGTCTGCACGGCGT TTGTCCGGTTGTACTCGTC	1100	55	Yanez <i>et al.</i> 2003
<i>rpoD</i>	70 Fs 70 Rs	ACGACTGACCCGGTACGCATGTA ATAGAAATAACCAGACGTAAGTT	~820	58	Yamamoto <i>et al.</i> 2000
lipase	lip-F lip-R	CAYCTGGTKCCGCTCAAG GTRCCGAACCAGTCGGAGAA	247	56	Soler <i>et al.</i> 2002
<i>gcaT</i>	GCAT-f GCAT-r	CTCCTGGAATCCCAAGTATCAG GGCAGGTTGAACAGCAGTATCT	237	56	Soler <i>et al.</i> 2002
Aerolysin/ Hemolysin	aer-F aer-R	CCTATGGCCTGAGCGAGAAG CCAGTTCCAGTCCCACCACT	431	56	Soler <i>et al.</i> 2002
Serine protease	Serine-F Serine-R	CACCGAAGTATTGGGTCAGG GGCTCATGCGTAACTCTGGT	350	60	Soler <i>et al.</i> 2002
DNase	Exu-F Exu-R	RGACATGCACAACCTCTTCC GATTGGTATTGCCYTGCAAS	323	54	Soler <i>et al.</i> 2002

^aThese names were chosen in our study—no names for primers in original paper.

Aeromonas is low, so additional analyses were needed. The phylogenetic analysis of 16S, *gyrB*, and *rpoD* identified five *Aeromonas* species (Figure 3); data could not resolve the difference between the two *A. hydrophila* subspecies (*A. hydrophila* and *A. h. ranae*), so the name “*A. hydrophila* group” was used

for these strains. In total, we identified 21 species in 13 genera, with 12 species being known fish pathogens (Table 2). *Aeromonas veronii* was the most common species (23 strains). Strains of *A. hydrophila* group and *A. veronii* were the only species isolated from all infected tissues (Table 2). All sequences

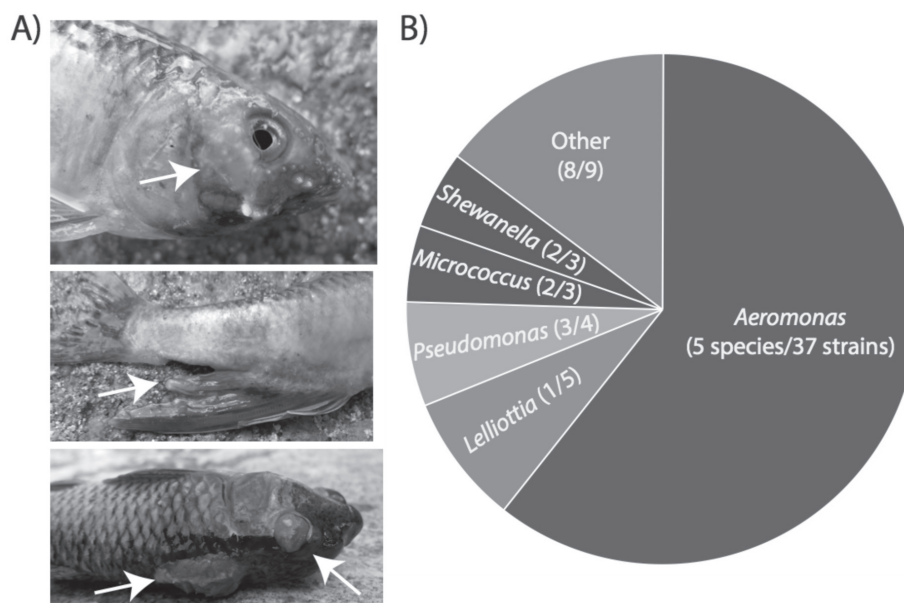


Fig. 1. A) Diseased fish used for culturing: (top and middle) *Zacco platypus* (Temminck and Schlegel) and (bottom) *Microphysogobio longidorsalis* Mori. White arrows point to areas of infection. B) Identification of the 61 bacterial strains to genus (# of species/ # of strains).

Table 2. Virulence profiles of *Aeromonas* strains isolated from diseased fish tissues. All non-*Aeromonas* strains were negative for all virulence factors. *lip*–lipase, *gcaT*–glycerophospholipid-cholesterol acyltransferase, *aer*–aerolysin, *ser*–serine protease.

Virulence Factors					Species	Isolate Nos. (SFCFD20130614-)
<i>lip</i>	<i>gcaT</i>	<i>aer</i>	<i>ser</i>	DNase		
+	+	+	+	+	<i>Aeromonas hydrophila</i> group	1, 2, 79
					<i>Aeromonas piscicola</i>	63
					<i>Aeromonas veronii</i>	16, 17, 28, 29, 30, 32, 33, 34, 47
+	+	+	+	-	<i>Aeromonas sobria</i>	67
					<i>Aeromonas veronii</i>	66
+	+	+	-	+	<i>Aeromonas hydrophila</i> group	104
					<i>Aeromonas veronii</i>	5, 12, 13, 21, 22, 56, 57, 60, 71
+	+	+	-	-	<i>Aeromonas sobria</i>	49
+	+	-	-	+	<i>Aeromonas allosaccharophila</i>	81
+	-	+	+	-	<i>Aeromonas sobria</i>	75
+	-	+	-	+	<i>Aeromonas veronii</i>	68
-	+	+	-	+	<i>Aeromonas hydrophila</i> group	96
					<i>Aeromonas veronii</i>	15
-	+	+	+	-	<i>Aeromonas sobria</i>	69
-	+	+	-	-	<i>Aeromonas sobria</i>	53
+	-	-	-	-	<i>Aeromonas veronii</i>	14
-	-	-	-	-	<i>Aeromonas hydrophila</i> group	102
					<i>Aeromonas sobria</i>	76
					<i>Aeromonas veronii</i>	99

were submitted to GenBank (KP115684–KP115781, KP939278–KP939307; Table 2).

Virulence profiles of strains

The five virulence factors were only found in *Aeromonas* strains (Table 2). Of the 37 *Aeromonas* strains, 34 screened positive for at least one virulence factor. Factors *lip*, *gcaT*, *aer*, and DNase were found in a majority of the strains (>70%), while *ser* was less common (~45%). Virulence profiles (the combination of virulence factors present) were not characteristic of a species— strains of different species had the same virulence profile, and multiple virulence profiles were found in a species. Single strains of *A. hydrophila* group, *A. sobria*, and *A. veronii* screened negative for all five virulence factors (Table 2). Representative sequences of virulence factors were submitted to GenBank

(KP939272–KP939277; Table 2).

eDNA detection of *Aeromonas* and virulence factors

Results of eDNA screening for *A. hydrophila*, *A. veronii*, and virulence factors are in Fig. 2. Geographically, *A. hydrophila* and *A. veronii* were present at all eight sites, but lowest in Naerin Stream (sites 5 and 6, 11/20 samples) and highest in Inbuk Stream (sites 1 and 2, 18/20 samples) (Fig. 2). Seasonally, *A. hydrophila* and *A. veronii* were both absent at a majority of sites (sites 1, 3, 4, 5, and 6) in summer (August). Overall, *A. veronii* (32/40 samples) was more common than *A. hydrophila* (26/40 samples) (Figure 2). All of the five virulence factors were found in water samples, but some more prevalent than others; *lip* was most common (30/40 samples), while DNase was least common (2/40

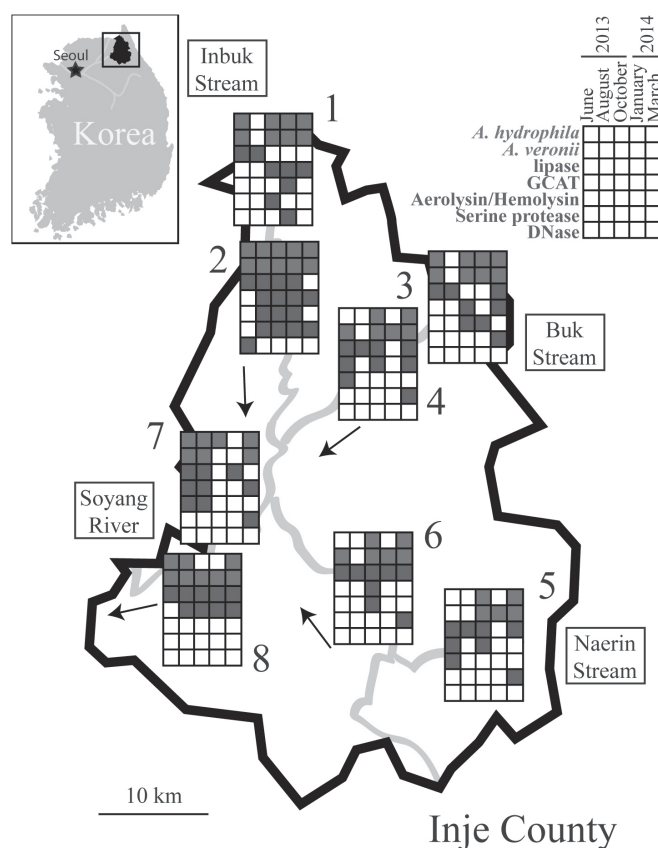


Fig. 2. Water sampling sites along the waterways of Inje County, South Korea (numbered 1-8). At each site, the results are shown for screening *Aeromonas hydrophila*, *A. veronii*, and five virulence factors. Arrows indicate the direction of water flow. The small, inset map of Korea shows the path of the major rivers flowing to the capital, Seoul.

samples; Figure 2). Generally, virulence factors were recovered at a lower rate than *Aeromonas* species. Average water temperature across sites showed the expected seasonal variation—high in the summer (June: 23.4°C, August: 23.2°C) and low in the autumn and winter (October: 10.6°C, January: 1.3°C, March: 4.5°C).

DISCUSSION

Identification of bacteria

From the 61 isolated strains, we identified 13 bacterial genera (Fig. 1). Eight of these genera contain species that are fish pathogens: *Aeromonas* (Janda and Abbott, 2010), *Bacillus* (Goodwin *et al.*, 1994), *Carnobacterium* (Leisner *et al.*, 2007), *Citrobacter* (Jeremic *et al.*, 2003), *Lelliottia* (previously *Enterobacter*; El-Sayyad *et al.*, 2010), *Pseudomonas* (Lopez *et al.*, 2012), *Shewanella* (Kozinaska and Pekala, 2004), and *Staphylococcus* (Kusuda and Sugiyama, 1981). The five *Aeromonas* species were most commonly isolated (37 total strains, Figure 1), of which *A. veronii* was dominant (23 strains). We focused on *Aeromonas* species because they were the most common, known fish pathogens, and isolated from all infected fish tissues (Table 2). One of the *Aeromonas* species identified, *A. piscicola*, is of particular interest because this is its first record since its description (Beaz-Hidalgo *et al.*, 2009). We isolated one strain (SFCFD20130614-63) from the infected fin of a freshwater minnow (*Z. platypus*). Previously, *A. piscicola* was only known from Spain, isolated from both wild (*Salmo salar* L., *Oncorhynchus mykiss* [Walbaum]) and farm-raised fish (*Carassius auratus* L.) that showed signs of disease (Beaz-Hidalgo *et al.*, 2009).

Virulence profiles of bacteria

The five virulence factors we screened for are mostly *Aeromonas*-specific (with *ser* and *gcaT* also present in some *Vibrio* species; Chacon *et al.*, 2003). Our results were the same, with virulence factors only screening positive in *Aeromonas* strains (Table 2). In the past, virulence factors have been used to identify *Aeromonas* strains; Beaz-Hidalgo *et al.* (2010) used *gcaT* to help identify *Aeromonas*, as it was present in 100% of *Aeromonas* strains and absent in non-*Aeromonas* strains. In our study, *gcaT* was absent in six *Aeromonas* strains (Table 2). Chacon *et al.* (2003) had similar findings, with *gcaT* absent in three environmental and one clinical *Aeromonas* samples.

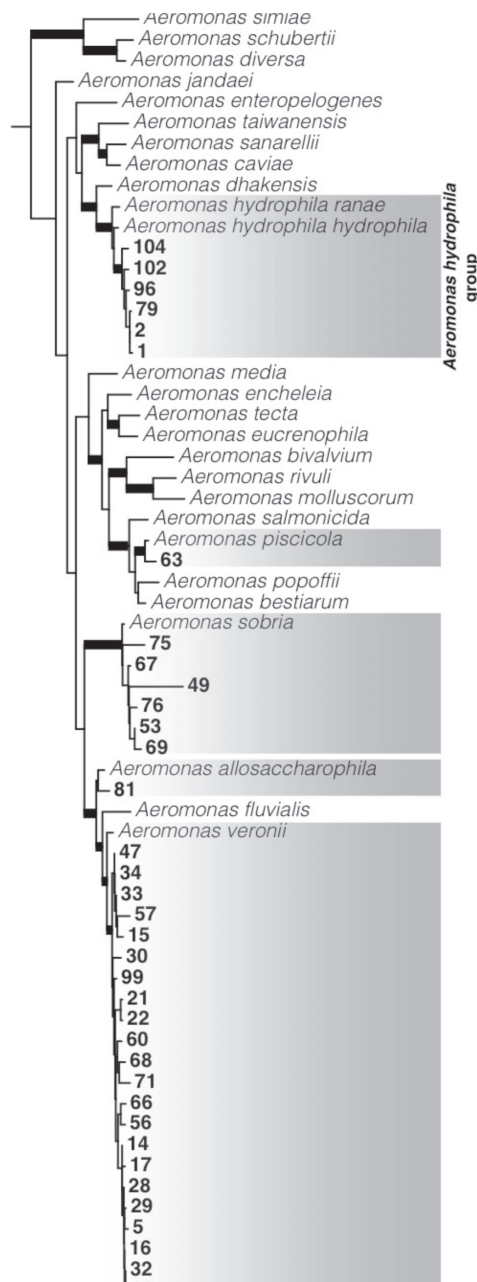


Fig. 3. Partitioned maximum likelihood phylogenetic analysis of the concatenated dataset of 16S ribosomal DNA (16S), DNA gyrase subunit B (*gyrB*), and RNA polymerase sigma 70 factor (*rpoD*) used to identify *Aeromonas* species. Thick branches indicate clades with bootstrap support greater than 70. Identified species are highlighted, and strains isolated in this study are in bold font (Complete strain numbers have “SFCFD20130614-” preceding the number). Subspecies of *A. hydrophila* could not be distinguished, so they are collectively referred to as “*Aeromonas hydrophila* group”. All other *Aeromonas* species in the phylogeny are type strains.

These results demonstrate that virulence factors can complement an identification based on other data, but may lead to misidentification if it is the only data used. As in other studies (e.g., Bin Kingombe *et al.*, 1999), virulence profiles varied between and within species. A total of 12 virulence profiles were found for *Aeromonas* strains, with different species having the same profile, and strains of the same species being different (Table 2). This includes strains of three species (*A. hydrophila* group, *A. sobria*, and *A. veronii*) that screened negative for all tested virulence factors (Table 2).

Patterns in *Aeromonas* detection

Aeromonas veronii was more common in waterways (32 samples) compared to *A. hydrophila* (26 samples). In all but one sample (site 7, October), whenever *A. hydrophila* was detected, *A. veronii* was also detected (Fig. 2). As this pattern matches the higher proportion of *A. veronii* strains isolated from the diseased fish, *A. veronii* is probably more common in the waterways of Inje County. PCR detection of *A. veronii* and *A. hydrophila* from eDNA was more sensitive than for virulence factors; many virulence factors were absent when *A. veronii* and *A. hydrophila* were present. Although this may be due to different virulence profiles of strains (*A. hydrophila* had four different profiles, *A. veronii* seven different profiles; Table 2), we believe gene copy number also influences this result—*Aeromonas* species have up to six copies of 16S (Morandi *et al.*, 2005), while (unknown for all genes and all species) *A. hydrophila* is known to have only one copy of *aer* (Yu *et al.*, 2008). Gene copy number should be considered when choosing a genetic marker for eDNA studies.

Using eDNA, we uncovered a geographic pattern in *Aeromonas* presence across sites. *Aeromonas* detection was lowest in Naerin Stream and highest in Inbuk Stream. Of the 20 PCR reactions performed to detect *A. hydrophila* and *A. veronii* along a waterway, only 11 were positive at Naerin Stream, compared to 18 at Inbuk Stream (Figure 2). High bacteria levels in aquatic habitats are often linked to organic or chemical compounds from fish farms (feed waste, feces; Gowen and Bradbury, 1987) or land-based farms (fertilizers; Alabaster, 1982). In the mountainous area around Inbuk Stream, there are several highland farms. Such farms use high amounts of chemical fertilizers (~450 kg ha⁻¹ year⁻¹; Kim *et al.*, 2001), which may impact the prevalence of bacteria in the water. *Aeromonas* detection decreased in summer (August)—at 5/8 of the sites,

neither *A. hydrophila* nor *A. veronii* were detected (Fig. 2). This pattern was found in other studies (Hazen, 1979; Hazen and Esch, 1983) and attributed to an increase in water temperature, which may increase mortality, predation by zooplankton, and/or competition for nutrients. This effect of higher temperatures on *Aeromonas* was not seen in our study, as the average water temperature in June (23.4°C) and August (23.2°C) were similar, but *Aeromonas* levels differed—high in June, low in August.

CONCLUSION

From diseased fish in natural waterways of Korea, we identified 21 bacteria species, 12 of which are known fish pathogens. Although additional histopathology data are needed to verify their role in fish disease, *Aeromonas* species are potential contributors, as they demonstrated potential pathogenicity (screening positive for virulence factors) and were dominant and present on the diseased fish. One of these species, *A. piscicola*, is of particular interest because this is the only the first identification of this species after its initial description. Using an eDNA approach, we detected geographic and temporal patterns in the presence of *A. hydrophila* and *A. veronii*. Geographically, both species were more common in Inbuk stream, which may be related to the presence of highland farms in the region. Temporally, *Aeromonas* detection decreased in the summer, although we could not identify any causes for this pattern. As *Aeromonas* species are both fish and human pathogens, these results demonstrate a way evaluate water quality for ecosystem and human health.

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