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Rapid Detection of Virulence Factors of *Aeromonas* Isolated from a Trout Farm by Hexaplex-PCR

In-Young Nam and Kiseong Joh*

Departments of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yong-In 449-791, Republic of Korea

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The detection of virulence factors of *Aeromonas* is a key component in determining potential pathogenicity because these factors act multifunctionally and multifactorially. In this study water samples were collected from a trout farm on a seasonal basis, and diseased fish and *Aeromonas* species were isolated and identified. For rapid detection of six virulence factors of isolated *Aeromonas*, a hexaplex-polymerase chain reaction (hexaplex-PCR) assay was used. The detected virulence factors include aerolysin (*aer*), GCAT (*gcat*), serine protease (*ser*), nuclease (*nuc*) lipase (*lip*) and lateral flagella (*laf*). The dominant strain found in our isolates was *Aeromonas sobria*, and the dominant virulence factors were *aer* and *nuc* for all seasons. We confirmed that *A. sobria* and two of the virulence genes (*aer* and *nuc*) are related. We proposed a method by which one can identify the major strains of *Aeromonas*: *A. hydrophila*, *A. sobria*, *A. caviae*, and *A. veronii*, using hexaplex-PCR.

Keywords: *Aeromonas*, virulence factor, 16S rDNA RFLP, multiplex-PCR

Aeromonas species are facultatively anaerobic Gram-negative bacteria that belong to the family *Aeromonadaceae*. These bacteria have a broad host spectrum, with both cold-and warm-blooded animals, including humans, and are known as psychrophilic and mesophilic (Nerland, 1996). In fish, these bacteria cause hemorrhagic septicemia, fin rot, soft tissue rot and furunculosis. It was recently reported that epizootic ulcerative syndrome (EUS) caused by *Aeromonas sobria* resulted in great damage to fish farms in parts of southeast Asia such as Bangladesh and India (Rahman *et al.*, 2002). *A. sobria* was also the causative agent of fish disease in a farm of perch, *Perca fluviatilis* L, in Switzerland (Wahli *et al.*, 2005). In humans, *Aeromonas* causes diarrhea, gastroenteritis, and extraenteritic conditions such as septicemia, wound infection, endocarditis, meningitis, and pneumonia (Buckely and Howard, 1999).

Aeromonas species secretes many extracellular proteins, including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase and protease (Pemberton *et al.*, 1997). These proteins are known as virulence factors that cause disease in fish and humans. Aerolysin is a representative virulence factor of *Aeromonas* and was reported to function as hemolysins and cytolytic enterotoxins (Chang *et al.*, 1992; Hirono and Aoki, 1993; Buckley and Howard, 1999). The detection method of *aerA* was recently proposed as a reliable approach by which to identify a potential pathogenic *Aeromonas* strain (Heuzenroeder *et al.*, 1999). Glycerophospholipid cholesterol acyltransferase (GCAT) is a lipase that is secreted by *A. salmonicida* and causes furunculosis in fish (Nerland *et al.*, 1996; Thornton *et al.*, 1998).

Serine protease is known to activate toxins such as aerolysin and GCAT, which were controlled by virulence mechanisms; this process is referred to as quorum sensing (Whitby *et al.*, 1992; Cascon *et al.*, 2000). Aerolysin also forms channels by heptamerization to the host cell membrane after proaerolysin is activated by the proteolytic activity of furin (Hirono *et al.*, 1992; Dodd and Pemberton 1996; Abrami *et al.*, 1998, 2000; Fivaz *et al.*, 2001; Lafont *et al.*, 2004). The virulence of aerolysin was confirmed in *A. hydrophila* when Tn-induced protease-deficient mutants were used (Heuzenroeder *et al.*, 1999). Nuclease has not yet been confirmed in terms of its association with pathogenicity, but reports have indicated that it participates in the development of host infection. It has also been found as a virulence factor in clinical samples more than in environmental samples (Chang *et al.*, 1992; Dodd and Pemberton, 1996). Nucleases were shown to be important virulence factors in other genera such as genus *Streptococcus* (Gavin *et al.*, 2003; Kirov *et al.*, 2004), and lipases have been found to change the host plasma membrane (Husslein *et al.*, 1992; Anguita *et al.*, 1993; Chuang *et al.*, 1997). Flagella have recently been examined as virulence factors by many researchers (Gavin *et al.*, 2003; Kirov *et al.*, 2004). Polar flagella offer swimming motility in liquid. Lateral flagella give swarming motility in a solid matrix and have been reported as virulence factors, as they are associated with adsorption to the host cell membrane (Gavin *et al.*, 2003). Additionally, it was reported that lateral flagella may act as adhesin in human intestinal epithelial cells (Kirov *et al.*, 2004). The detection of *Aeromonas* is a key component in the determination of potential pathogenicity, because more than two virulence factors act multifunctionally and multifactorially.

A multiplex-PCR (m-PCR) assay has been used to identify viruses and bacteria in clinical samples (Anguita *et al.*,

* To whom correspondence should be addressed.
(Tel) 82-31-330-4271; (Fax) 82-31-330-4566
(E-mail) ksjoh@hufs.ac.kr

1993). Because of its speed and sensitivity, it has also been used to detect in the distribution of virulent genes (Chuang *et al.*, 1997; Hirono *et al.*, 1993; Wang *et al.*, 2003; Yu *et al.*, 2005). Sen and Rodgers (2004) reported the distribution of six virulence factors in *Aeromonas* from drinking water utilities in the United States, achieving this experiment through three sets of duplex-PCR. The detection of virulence factors by m-PCR assay indicated changes of species compositions and virulence factors in water systems. Continuous monitoring of fish farms is needed because *Aeromonas* is a ubiquitous, autochthonous, and opportunistic pathogen. Six representative virulence factors were detected simultaneously by hexaplex-PCR (h-PCR) in this study.

Materials and Methods

Sampling

In February, May, August and November, 2004, water samples were collected at four sites; influent (Site 1), farms (Site 2 and 3), and effluent (Site 4) in the trout farms of Pyungchang, Kangwondo, Republic of Korea. They were collected aseptically in individual sterile bottles to prevent contamination. Samples were stored on ice, and were immediately transported to the laboratory and processed within 8 h after collection. Diseased trout that showed no lesions or serious wounds on the body and fin were caught at a trout farm (Fig. 1). The diseased trout were kept in an ice box, and were immediately transported to the laboratory and processed within 4 h after collection.

Fig. 1. Rainbow trout with lesions on the body and tail fin.

Bacterial strains

Reference strains used in this experiment were *A. hydrophila* ATCC 7966, ATCC 14715, and *A. caviae* ATCC 15468, all purchased from the Korean Collection for Type Cultures (KCTC, Republic of Korea), and *A. sobria* ATCC 43979, and *A. salmonicida* ATCC 33658, both purchased from the Korean Culture Center of Microorganisms (KCCM, Republic of Korea).

Isolation of Aeromonas from fish farm water and trout

To isolate *Aeromonas*, water samples were cultured on ampicillin-dextrin agar base (Alpha Biosciences, USA) supplemented with ampicillin (10 μg/ml; Sigma, USA) and vancomycin (2 μg/ml; Sigma, USA) (EPA method 1605) at 30°C for 24-48 h. Yellow colonies were considered presumptive *Aeromonas* colonies, and were counted. Yellow colonies that were easily distinguished from the other colonies were picked from each plate and subcultured on DNase test agar (Difco Laboratories, USA) plates at 30°C for 12 h for subsequent experiments. The surfaces of the trout were washed for 30 sec with ethanol (70%, v/v). The intestines were obtained aseptically, washed for 1 min with 10 ml sterilized phosphate-buffered saline (PBS: 130 mmol/L NaCl, 10 mmol/L, NaH_2PO_4; pH 7.2), and then homogenized for 1 min after the addition of 100 ml sterilized PBS. Presumptive *Aeromon*as spp. in the homogenate were isolated as described above (Rahman *et al.*, 2002; Chacon *et al.*, 2003).

Design of primers

Primers reported by Borrell *et al.* (1997) were used to amplify 16S-rDNA genes. The forward primer was 16SA-F; 5'-AGA GTTTGATCATGGCTCAG-3' and the reverse primer was 16SA-R; 5'-GGTTACCTTGTTACGACTT-3'. The size of the expected PCR product was 1,502 base pairs. For hexaplex-PCR, primers were designed by using a web-based tool,

Table 1. Primer sequences and sizes of PCR-amplified gene targets of 16S rDNA and six gene types

Target gene	Primer	Sequences (5'→3')	Expected product size (bp)	Accession number
16S rDNA	16S-rDNA-F 16S-rDNA-R	AGAGTTTGATCATGGCTCAG GGTTACCTTGTTACGACTT	1,502	Borrell *et al.* (1997)
Lateral flagella B	LafB-F LafB-R	GACCAGCAAGGATAGTGGGTTGGAG AAGCACCATCGCGTTGGTATAAGG	624	AF348135
Nuclease	Nuc-F Nuc-R	CAGGATCTGAACCGCCTCTATCAGG GTCCCAAGCTTCGAACAGTTTACGC	504	AF004392
Aerolysin	Aero-F Aero-R	GAGCGAGAAGGTGACCACCAAGAAC TTCCAGTCCCACCACTTCACTTCAC	417	M16495 X65044
Serine protease	Ser-F Ser-P	ACGGAGTGCGTTCTTCCTACTCCAG CCGTTCATCACACCGTTGTAGTCG	211	X67043 AF159142
GCAT	GCAT-F GCAT-R	CATGTCTCCGCCTATCACAACAAGC CCAGAACATCTTGCCCTCACAGTTG	339	X70686 AF268080
Lipase	Lip-F Lip-R	GACCCCCTACCTGAACCTGAGCTAC AGTGACCCAGGAAGTGCACCTTGAG	155	U63543

Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), after the homology was investigated using the GenBank BLAST network service. GenBank accession numbers and expected sizes are presented in Table 1.

PCR and multiplex-PCR (m-PCR) assay

A total of 450 isolated colonies were examined. To extract DNA, cultures were centrifuged and cell pellets were resuspended in Tris-Cl (pH 6.8), lysed by the freezing-thawing method in dry ice-ethanol bath, and boiled for 10 min to halt extracellular DNase activity (Nam *et al.*, 2004). The resulting DNAs were stored at -20°C until use. In total, 431 amplified PCR products were identified by 16S rDNA restriction fragment length polymorphism (RFLP), as described by Borrell *et al.* (1997). Hexaplex-PCR assays were performed with reaction mixtures (final 20 μl) containing six primers sets (*laf, nuc, aer, gcat, ser,* and *lip* ; each 2.0 pmol/μl), 1× PCR buffer, 0.2 mM each dNTPs (Takara, Japan), 4.0 mM $MgCl_2$, and Super Taq 5 U/μl. PCR was performed with a thermal cycler (MiniCycler™, USA) according to the following protocol: 30 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 30 sec, and extension at 72°C for 45 sec in one tube. PCR products were electrophoresed in a 1.7% agarose gel and stained with EtBr. Pictures were taken using UV illumination and a Gel Documentation System.

Sequence analysis

Among *Aeromonas* spp. identified by 16S rDNA RFLP, twenty strains were chosen for further analysis of DNA sequencing (Macrogen, Republic of Korea). The resulting sequences were used to identify species, and similarity was analyzed using the GenBank BLAST network service. The sequences were submitted to the GenBank database.

Adhesion assay

Isolates and type strain (*A. sobria* ATCC 43979) were grown in Luria-Bertani (LB) medium at 30°C for 19 h. After growth, the bacterial pellet was collected by centrifugation, washed with phosphate buffered saline (PBS) and dissolved in PBS. Caco-2 cells were incubated for 14 days in Dulbecco's modified Eagle's Medium (DMEM, Sigma, USA) on a 24-well plate with a cover slip. Media was replaced with fresh media prior to bacterial infection. For the adhesion assay, bacterial cells were added to the monolayer culture of Caco-2 cells at a final concentration of 10^7 CFU/ml. PBS was added as a negative control. The infected cells were further incubated for 60 or 120 min. Each well was washed three times with PBS and fixed with methanol for 5 min. Cells were then stained with Giemsa staining solution for 40 min, followed by rinsing with dH_2O prior to microscopic analysis at 200×.

Table 2. The number of presumed *Aeromonas* spp. grown on ADV plates from a trout farm (in 2004)

	Feb.	May	Aug.	Nov.
	Presumed *Aeromonas* spp. count (CFU/ml)			
Site 1	1.0×10	4.0×10	1.5×10	2.3×10
Site 2	1.2×10^2	2.0×10^2	8.1×10^2	5.8×10^2
Site 3	1.4×10^2	3.9×10^2	3.4×10^2	3.3×10^3
Site 4	8.5×10^2	3.6×10^2	7.3×10^2	6.2×10^3

Results and Discussion

Seasonal and regional distribution of presumed Aeromonas spp.

The temperature of the fish farm was maintained at 10°C, except in the summer (data not shown). The population size of presumed *Aeromonas* spp. isolated on an ADV plate was the smallest from site 1 (influent), and was increased in samples from sites 2, 3 (trout farm), and 4 (effluent), respectively (Table 2). Surprisingly, the density was ten times higher in November than it was in August (Table 2). Because *Aeromonas* is bothe psychrophilic as well as a mesophilic, *Aeromonas* tends to grow at low temperature.

Distribution of isolates by PCR-amplified 16S rDNA restriction fragment length polymorphisms (RFLP)

The four hundred thirty-four out of 450 amplified 16S

Table 3. Distribution of isolates by amplified 16S rDNA RFLP

	Water						Diseased trout		
	Feb.	May	Aug.			Nov.	Intestine	Lesions on body	Lesions on fin
			F	P	K				
A. sobria	72[a]	66	65			63	100	89	63
A. popoffii	28	16	19		70	14			
A. encheleia		16	8		3	5			28
A. bestiarum		2	1			16			9
A. hydrophila			4	82	10	2			
A. salmonicida			3					11	
A. caviae				13					
A. veronii				5	10				
A. media					7				

[a]Percentage of isolates in each season.
F, trout farm; P, Pyungchang stream in Kangwondo, Republic of Korea; K, Kyungan stream in Kyunggido, Republic of Korea.

Table 4. Distribution of six virulence factors and *Aeromonas* spp. samples in August, 2004

	No. of isolates	Laf	Nuc	Aer	GCAT	Ser	Lip
Fish farm							
Site 1							
A. hydrophila	2*	–	+	+	–	+	+
	1	–	–	+	–	+	+
A. encheleia	1	–	–	+	+	+	+
	1	–	+	–	+	+	+
A. popoffii	1	–	+	–	+	–	+
A. sobria	1	–	+	–	–	–	–
Site 2							
A. sobria	9	–	+	+	–	–	–
	2	–	+	–	–	–	–
A. salmonicida	2	+	+	+	–	+	+
A. popoffii	1	+	+	+	+	+	+
A. encheleia	2	–	+	+	+	+	+
	1	+	+	+	–	+	+
Site 3							
A. sobria	11	–	+	+	–	–	–
	3	–	–	+	–	–	–
A. popoffii	5	–	+	+	+	+	+
A. encheleia	1	+	+	+	+	+	+
Site 4							
A. sobria	12	–	+	+	–	–	–
	6	–	+	–	–	–	–
	6	–	–	+	–	–	–
A. popoffii	7	+	+	+	–	+	+
A. bestiarum	1	+	+	+	–	+	+
Pyungchang Stream							
A. hydrophila	17	–	+	+	–	+	+
	2	–	+	+	–	–	+
A. caviae	3	–	–	–	–	–	+
A. veronii	1	–	–	+	–	–	–
Kyungan Stream							
A. popoffii	18	–	+	+	+	+	+
	2	–	+	+	+	–	+
	1	–	+	–	+	–	+
A. hydrophila	2	–	+	+	–	+	+
	1	–	+	+	–	–	+
A. veronii	3	–	–	+	–	–	–
A. media	2	–	–	–	–	–	+
A. encheleia	1	+	+	–	–	+	+
Diseased fish lesions on body							
A. sobria	5	–	+	+	–	–	–
	2	–	+	–	–	–	–
	1	–	–	+	–	–	–
A. salmonicida	1	–	+	+	+	+	+
Diseased fish intestine							
A. sobria	16	–	+	+	–	–	–
	5	–	–	+	–	–	–
	4	–	+	+	–	+	–
	1	–	+	–	–	–	–
Lesions on tail fin							
A. sobria	10	–	+	+	–	–	–
	10	–	–	+	–	–	–
	2	–	+	–	–	–	–
A. encheleia	10	+	+	+	–	+	+
A. bestiarum	3	+	+	+	–	+	+

Number of isolates with a given combination of virulence factor genes. Laf, lateral flagella; Nuc, nuclease; Aer, aerolysin; GCAT, glycerophospholipid cholesterol acyltransferase; Ser, serine protease; Lip, lipase.

rDNA PCR products were identified by RFLP, as described by Borrell *et al.* (1997). Twenty candidates were sequenced and confirmed for similarity using the GenBank BLAST network service. The sequences were submitted to the GenBank database. The corresponding accession numbers range from DQ133170 to DQ133183.

The dominant strain at this farm during all four seasons was *A. sobria* (Tables 4 and 5). *A. sobria* was detected in 100% of intestinal samples from diseased trout. In addition, *A. sobria* (89%) and *A. salmonicida* (11%) were detected in lesions on the body. *A. sobria* (63%), *A. encheleia* (28%), and *A. bestiarum* (9%) were detected in lesions on the tail fin. However, *A. sobria* was not dominant at site 1 (influent), where *A. popoffii* was dominant in February and *A. hydrophila* was dominant in August (data not shown; Table 4). In May and November, *A. sobria* was the dominant isolated found in influent (data not shown). It was recently reported that epizootic ulcerative syndrome (EUS) caused by *A. sobria* resulted in great damage to fish farms in southeast Asian countries such as Bangladesh and India (Chacon *et al.*, 2003). And *A. sobria* was also found to be the causative agent of fish disease in a farm of perch, *Perca fluviatilis* L, in Switzerland (Wahli *et al.*, 2005). Notably, the sample of origin in the trout farm did not contain any *Aeromonas* (data not shown).

The diversity of *Aeromonas* was at the highest level in August. For control experiments, we collected samples from two sites within the Republic of Korea; Kangwondo (Pyungchang stream), which was located close to our farm and Kyunggido (Kyungan stream), which was located far away from the farm. The results differed: *A. hydrophila* (82%), *A. caviae* (14%), and *A. veronii* (4%) were the predominant isolates in Kangwondo, and *A. popoffii* (70%), *A. hydrophila* (10%), *A. caviae* (10%), *A. veronii* (6%), and *A. encheleia* (4%) were the main isolates found in Kyunggido. Sugita *et al.* (1995) reported the distribution: *A. veronii* (22%), *A. caviae* (18%), *A. hydrophila* (13%), *A. sobria* (8%), *A. jandaei* (7%), and other *Aeromonas* spp. (33%), using

Table 5. Distribution of six virulence factors in fish farms and diseased fish

		Laf	Nuc	Aer	GCAT	Ser	Lip
Feb.		4[a]	88	88	28	20	28
May		4	60	84	27	33	33
	I	5	86	86	26	29	30
Aug.	P	0	83	87	0	74	96
	K	3	83	90	70	70	90
	Farm	20	61	100	14	37	37
	I	0	65	85	0	0	0
Nov.	L. B.	0	55	100	0	0	0
	L. F.	37	71	94	0	37	37

[a]Overlapped percentage.
I, intestine of diseased trout; P, Pyungchang stream in Kangwondo, Republic of Korea; K, Kyungan stream in Kyunggido, Republic of Korea; L.B., lesions on the body; L.F., lesions on the tail fin in trout; Laf, lateral flagella; Nuc, nuclease; Aer, aerolysin; GCAT, glycerophospholipid cholesterol acyltransferase; Ser, serine protease; Lip, lipase.

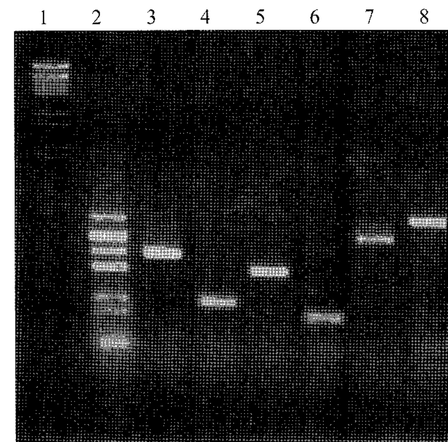

Fig. 2. Multiplex and monoplex-PCR. Lane 1, λ DNA-*Bst*E II size marker; 2, hexaplex-PCR of *A. bestiarum*; 3, 417 bp-aerolysin; 4, 211 bp-serine protease; 5, 339 bp-GCAT; 6, 155 bp-lipase; 7, 504 bp-nuclease, and lane 8, 624 bp-lateral flagella B.

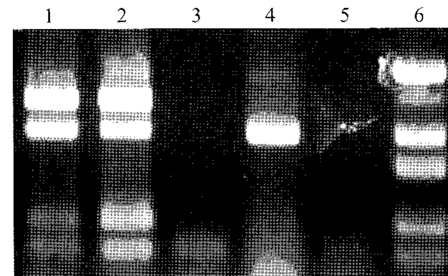

Fig. 3. Multiplex-PCR of *Aeromonas* reference strains. Lane 1, *A. hydrophila* ATCC 7966; 2, *A. hydrophila* ATCC 14715; 3, *A. caviae* ATCC1 5468; 4, *A. sobria* ATCC 43979; 5, *E. coli* (negative control), and 6, *A. salmonicida* ssp. *salmonicida*.

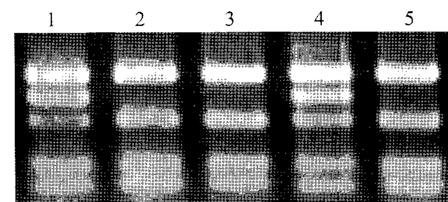

Fig. 4. Detection of virulence factors. Lanes 1-5, *A. popoffii* isolated from influent water on the trout farm, as detected by hexaplex- PCR.

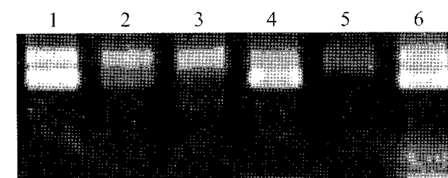

Fig. 5. Detection of virulence factors. Lanes 1-6, *A. sobria* isolated from diseased fish intestines, as detected by hexaplex-PCR.

the DNA-DNA hybridization method in the intestinal tracts of diseased fish and in water from a fish farm (Sugita *et al.*, 1995).

The emergence frequency of *A. bestiarum* (16%) was high. In November, sampling indicated that the density of *Aaromonas* increased and the overall composition changed. Because *Aeromonas* is both a mesophile and a psychrophile, *Aeromonas* can grow at low temperatures. The second most dominant species was *A. bestiarum* (e.g., a psychrophile), not *A. popoffii*. Lee *et al.* (2002) reported the distribution of *Aeromonas* by using 16S rDNA RFLP in water sediment and in the intestinal tracts of diseased fish at a trout fish farm. The results of this experiment showed that levels of *Aeromonas* from samples collected in May and August were higher than those in January and November at all sampling sites. *A. salmonicida* was the dominant species in January and November, and the proportion of pathogenic species (*A. hydrophila, A. caviae,* and *A. veronii*) increased in May and August. *A. bestiarum* was the dominant species isolated in November. Fish disease is normally related to water temperature, as fish that are exposed to stress are more susceptible to infection when water temperature is low (Kingombe *et al.*, 1999). The distribution of species observed in this report differed from the results of other studies (in most results, the dominant strain was *A. hydrophila* and not *A. sobria*). The distribution of *Aeromonas* may be more closely related to epidemic disease than to water quality.

Hexaplex-PCR (h-PCR)

Each primer used in this experiment was designed after the sequence analysis by BLAST, and the melting temperature (T_m) was determined using the Primer 3 web-based tool. Primer sets were designed not to polymerize with each other. Target genes could be detected by simultaneously using six primer sets in one tube; this process is referred to as hexaplex-PCR (h-PCR). Each monoplex-PCR product identified by an individual primer set was shown to be in the expected size range (Fig. 2). Virulence factors were detected by hexaplex-PCR using the *Aermonas* reference strain (Fig. 3). Four genes (*lip, ser, aer,* and *nuc*) were detected in *A. hydrophila* subsp. (ATCC 7966 and 14715). Only one gene (*lip*) was detected in *A. caviae*, while two genes (*aer* and *nuc*) and six genes (*lip, ser, gcat, aer, nuc*, and *laf*) were detected in *A. salmonicida* (Fig. 3). None of these genes was detected in *E. coli* (Fig. 3; lane 6). Using the results of by using h-PCR, we proposed an identification method for four genotypes in major species of Aeromonads. Of course, although only the lipase gene was detected in *A. caviae*, the strain showed cytotoxic and hemolytic activity in an animal passage test (Yu *et al.*, 2005). Wang *et al.* (2003) proposed five genotypes containing the hemolysin gene using the results of multiplex-PCR (m-PCR) in *Aeromonas* spp., while *A. caviae* was reported not to contain the hemolysin gene. Additionally, Kingombe *et al.* (1999) reported a common primer for *A. hydrophila* SSU cytolytic enterotoxin gene (AHCYTOEN), but this gene could not be detected in *A. caviae*. Therefore, the virulence phenotypes in species such as *A. caviae* did not yield any information related to toxins. In other cases, hexaplex-PCR could be used to rapidly screen and detect virulence factors from major *Aeromonas* species.

Distribution of six virulence factors

In all samples, aerolysin (*aer*) and nuclease (*nuc*) were found to be dominant (Tables 4 and 5). As a control, we performed h-PCR assay with water samples collected from Pyungchang and Kyungan streams, Republic of Korea, during the month of August. Four virulence factors (excluding *laf* and *gcat*) were detected in the Pyungchang samples, and five virulence factors (excluding *laf*) were detected in the Kyungan samples. The *lip* (96% and 90%) gene was found to be dominant in the water from both sites (Table 5).

In September, the *aer* gene was detected in 100% of the lesions found on trout bodies (Table 5). Unlike on the lesions of the tail fin, *laf, ser,* and *lip* genes were detected in 37% of the intestinal sessions of diseased fish and in lesions found on other portions of the body (Table 5). Thus, the identification of *Aeromonas* species can be performed using a profile of virulence factors.

In all samples, *aer* and *nuc* genes were detected at dominant levels. In November, the *aer* gene was detected in 100% of samples. Heuzenroeder *et al.* (1999) proposed that the detection of *aer*A was a reliable approach that could be used to identify potential pathogenic *Aeromonas* strains. The role of the *nuc* gene product as a virulence factor was not reported. This gene product was detected more often in clinical samples than in environmental samples, and has been proposed as an important virulence factor (Krovacec *et al.*, 1994). In other genera such as *Streptococcus*, the *nuc* gene has been reported as a key virulence factor. The results of h-PCR assay are correlated with the fact that *A. sobria* was found to be the dominant species in a 16S rDNA RFLP assay (Table 3). In Fig. 3, the *aer* gene was detected in *A. sobria* ATCC 43979. Therefore, we assumed that the *A. sobria* isolates from this farm could be a pathogenic bacterium that harbor virulence factors such as aerolysin and nuclease. h-PCR can be used for detection of *A. sobria*.

Chacon *et al.* (2003) identified five virulence factors [aerolysin/hemolysin gene (*aer*), serine protease gene (*ser*), GCAT gene (*gcat*), lipase gene (*lip*), and DNase gene (*dns*)] using PCR. Four genes (*aer, dns, gcat,* and *lip*) were detected in *A. hydrophila*, four genes (*aer, gcat, ser,* and *lip*) were detected in *A. sobria*, and five genes were detected in *A. caviae* and *A. salmonicida*. At this farm, the *nuc, aer, ser,* and *lip* genes were detected in *A. hydrophila*. *A. sobria* possessed *nuc* and *aer* or only *nuc* or only *aer*. *A. veronii* only contained the *aer* gene, while *A. caviae* and *A. media* contained only the *lip* gene. *A. salmonicida, A. popoffii, A. bestiarum,* and *A. encheleia* each contained all six virulence genes or overlapping virulence genes. Therefore, we could not identify or distinguish these species using h-PCR. Figuras *et al.* (2000) reported that the common pattern examined by 16S rDNA RFLP was shown in four *Aeromonas* strains. It is possible that the four strains were related phylogenetically. We detected virulence factors of *Aeromonas* isolates by hexaplex-PCR assay: this process allowed for rapid, sample detection (<2 h). In the case of *A. sobria*, the strains could be identified by detection of the aerolysin and nuclease genes using h-PCR.

Adhesion assay

To investigate the pathogenicity of *Aeromonas* in humans, we observed the surfaces of Caco-2 cells after infection

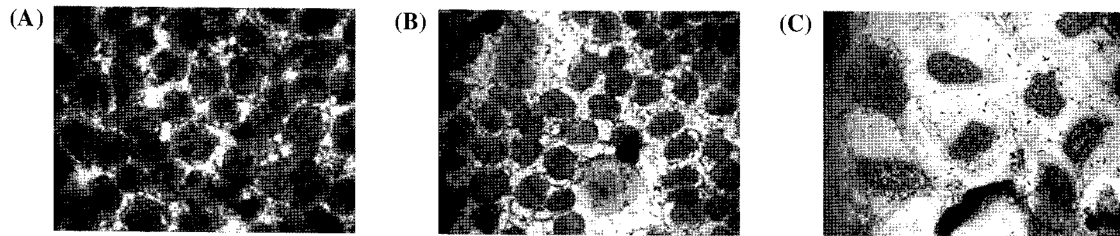

Fig. 6. Micrographs of Caco-2 cells infection with: (A) PBS only, (B) *A. sobria* ATCC 43979 for 60 min, (C) *A. sobria* ATCC 43979 for 120 min. Magnification 200×.

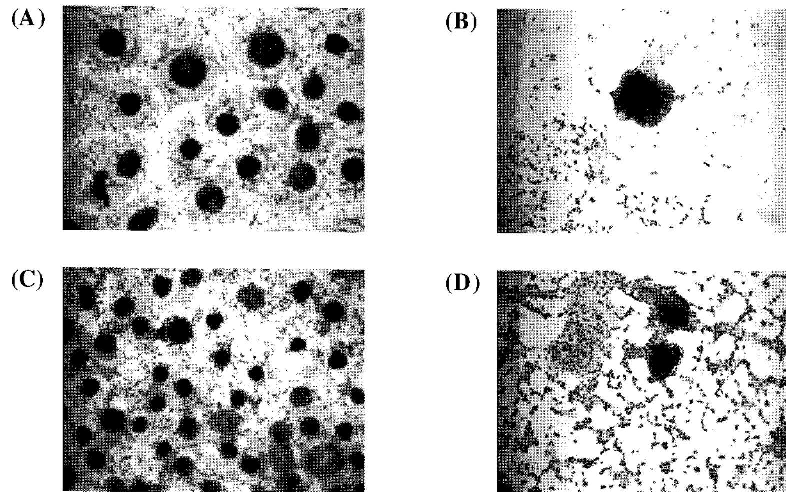

Fig. 7. Micrographs of Caco-2 cells infection with: (A) *A. sobria* isolated from diseased fish intestine after 60 min infection, (B) *A. sobria* isolated from diseased fish intestine after 120 min infection, (C) *A. bestiarum* isolated from lesions on the tail fin after 60 min infection, (D) *A. bestiarum* isolated from lesions on the tail fin after 120 min infection. Magnification 200×.

with isolates and reference strains *A. sobria* ATCC 43979, DI-7 (*A. sobria*), and FL-15 (*A. bestiarum*); cells were observed 60 and 120 min post-infection. The bacteria were shown to induce morphological changes of the host cell in 60 min (Figs. 6 and 7). Nearly complete lysis of the host cell was observed by 120 min (Fig. 7). Host cells infected with DI-7 and FL-15 showed nearly complete lysis. DI-7 contained aerolysin and nuclease genes (Fig. 5; lane 1), while the reference strain contained only the aerolysin gene (Fig. 3; lane 5). The nuclease gene is known to be associate with the development of pathogenicity (Nam *et al.*, 2002). Martins *et al.* (2002) indicated that the prevalence of pathogenicity was higher in clinical isolates than in environmental isolates. *Aeromonas* strains isolated from fish intestine and fish tail lesions are capable of infecting a human intestinal epithelium cell line. Additionally, the presence of these bacterial strains may be fatal in immunocompromised patients.

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