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Phylogenetic Identification and Distribution of Enterotoxin Genes in Aeromonas Strains Isolated from Pet Fish

[1]Dae-Cheol Kim, [2]Seung-Won Yi, [2]Ho-Seong Cho, [2]Myung-Jo You and [2]Gee-Wook Shin
[1]Department of Bio-Industrial Machinery Engineering,
College of Agriculture and Life Sciences, [2]Institute of Bio-Safety Research,
College of Veterinary Medicine, Chonbuk National University, Jeonju, Korea

Abstract: In the present study, 47 strains of *Aeromonas* sp. were collected from cultured koi (n = 11) and imported pet fish (n = 36). All strains were identified by phylogenetic analysis using partial sequences of the *gyrB* gene. In addition, PCR assays were used to detect the presence of genes for cytotoxic enterotoxin (act) and cytotonic enterotoxin (alt and ast). In the phylogenetic identification, the strains comprised five species, *A. veronii* (n = 36), *A. hydrophila* (n = 7), *A. jandaei* (n = 1), *A. aquariorum* (n = 1) and *A. allosaccarophila* (n = 2). Of the identified species, *A. jandaei*, *A. aquariorum* and *A. allosaccarophila* were earlier unrecorded in microbiological fields of Korea. In the detection of enterotoxin genes, the *act*, *alt* and *ast* genes were respectively presented in 85.1, 61.7 and 17.0% of strains. The act and *act/alt* gene patterns were prominent among Aeromonas strains, especially *A. veronii.* On the other hand, only *A. hydrophila* strains harbored all three of the *enterotoxin* genes. The results of the present study suggest that pet fish could be a potential risk factor for Aeromonas infection in humans.

Key words: *Aeromonas* sp., phylogenetic identification, gyrB, rpoD, enterotoxin gene, pet fish

INTRODUCTION

Pet fish have an important role in international trade in the aquaculture industry. In Korea, >4 million dollars per year are spent on pet fish imported from parts of South East Asia such as Indonesia, Singapore, Taiwan and China (Korea Customs Service, KCS; www.customs.go.kr). Recently, there have been some reports about frequent isolation of food and water borne pathogens from imported pet fish (Roberts *et al*., 2009; Lowry and Smith, 2007) which could threaten the public health of humans because most of these animals are maintained in indoor aquariums. The most common zoonotic agent was *Aeromonas* sp. (Roberts *et al*., 2009; Lowry and Smith, 2007) which is an autochthonous bacterium of aquatic environments and an opportunistic pathogen that causes infection under stressful host conditions. A human could easily be infected by an Aeromonas-contaminated environment or food and the infected human may exhibit a variety of clinical symptoms, ranging from diarrhea to septicemia (Janda and Abbott, 2010).

The taxonomy of the genus *Aeromonas* is very complex. According to the Bergey's Manual of Systematic Bacteriology (Martin-Carnahan and Joseph, 2005), the genus *Aeromonas* include 16 different species; *A. hydrophila*, *A. veronii*, *A. allosaccarophila*, *A. sobria*, *A. jandaei*, *A. media*, *A. bestiarum*, *A. salmonicida*, *A. eucrenophila*, *A. schubertii*, *A. trota*, *A. encheleia*, *A. popoffii*, *A. caviae* and *Aeromonas* sp. DNA Hybridization Group (HG) 11 and HG13. In addition, novel species such as *A. aquariorum* (Martinez-Murcia *et al*., 2008), *A. sanarellii* (Alperi *et al*., 2010) and *A. rivuli* (Figueras *et al*., 2011a) have been found in fish, human and aquatic environments over the last decade. Therefore, the genus *Aeromonas* consists of at least 27 different species. Due to these taxonomical complexities, the housekeeping genes, *gyrB* and *rpoD* have been suggested to be better genetic markers for species-level identification compared with the conventional method based on the phenotypic characteristics or *16S rRNA* gene analysis (Martinez-Murcia *et al*., 2011; Janda and Abbott, 2010; Beaz-Hidalgo *et al*., 2010; Alperi *et al*., 2010; Soler *et al*., 2004).

Enterotoxins have been reported to play important roles in the pathogenesis of *Aeromonas* sp. The enterotoxins include cytotoxic enterotoxin (Act) and two cytotonic enterotoxins (Alt and Ast) (Sha *et al*., 2002; Albert *et al*., 2000; Xu *et al*., 1998). Act possesses

Corresponding Author: Gee-Wook Shin, Laboratory of Aquatic Animal Medicine, College of Veterinary Medicine, Chonbuk National University, 561-756 Jeonju, South Korea

hemolytic, cytotoxic and enterotoxic activities and is closely related to aerolysin in terms of functions and conserved regions of nucleotide sequences. Enterotoxins induce tissue damage and fluid secretion by enhancing proinflammatory cytokines in macrophages (Galindo *et al*., 2004; Chopra *et al*., 2000; Xu *et al*., 1998). In addition, the *act* gene mutants show an increased LD50 (50% lethal dose) in a mouse model (Xu *et al*., 1998). Alt and Ast produce fluid accumulation in rat animal intestinal loops by increasing cyclic AMP (cAMP) and prostaglandin in Chinese Hamster Ovary (CHO) and intestinal epithelial cells (Chopra *et al*., 1996, 1994). Therefore, the enterotoxin-encoding genes, act, alt and ast could be genetic markers for estimating potential virulent strains among *Aeromonas* sp. isolated from a variety of sources. Pet fish can be carrier animals for pathogenic *Aeromonas* sp. implicated in human disease. To the knowledge, there has been limited information about prevalence on the species level and distribution of enterotoxin genes among Aeromonas strains isolated from pet fish. Therefore, in the present study, researchers sought to investigate the prevalence of *Aeromonas* sp. isolated from pet fish. To that aim, researchers performed phylogenetic identification using *gyrB* or *rpoD* gene sequences from 47 bacterial strains collected from pet fish. In addition, researchers investigated the distribution of the enterotoxin genes, act, alt and ast with important virulence factors in *Aeromonas* sp.

MATERIALS AND METHODS

Bacterial isolation and culture: First, researchers selected 47 strains from presumptive identification using *16S rRNA* gene sequences. There were 36 strains for the imported pet fish and 11 strains for the cultured koi carp. The imported pet fish were cultured in the fresh waters of tropical regions such as Singapore, Indonesia, Taiwan and China. The cultured koi carp were captured as part of a study on koi herpes virus during the summer season. All strains were cultured on Tryptic Soy Agar (TSA) plates at 27°C and single colonies were separately re-cultured in Tryptic Soy Broth (TSB) and stored at -70°C using Cryocare Bacteria Preservers (Key Scientific Products, Inc).

Genomic DNA extraction: The bacteria-attached bead was incubated in TSB overnight at 27°C. Bacterial suspension was subjected to AccuPrep® Genomic extraction (Bioneer Inc., Korea) for purification of bacterial DNA. The purified DNA concentration was determined using an Epoch Spectrophotometer System (Biotek, Wakefield, MA) at 450 nm.

PCR amplification and sequencing: Amplification and sequencing of *gyrB* and *rpoD* genes were performed using primer sets listed in Table 1. The *gyrB* and *rpoD* genes were amplified in Gradient Thermal Block (Bioneer Inc., Korea) with a final volume of 25 uL Accupower® PCR premix (Bioneer, Korea) including 1 uL of 10 mM of each forward and reverse primer (gyrB3F and gyrB14R for gyrB and rpoD70Fs and rpoD70Rs for rpoD), 1 uL bacterial genomic DNA (30~40 ng) and 22 uL of sterile ultrapure water. For amplification of the *gyrB* gene, the reaction was performed at 94°C for 10 min followed by 30 cycles of 94°C for 1 min, 66°C for 1 min and 72°C for 2 min followed by a final extension at 72°C for 10 min. However, amplification of the *rpoD* gene was performed according to the same method as that used for the *gyrB* gene except with an annealing temperature of 64°C. The PCR products for each gene were resolved by 1.5% agarose/TBE gel including RedSafe™ (iNtRON Biotechnology, Korea) and visualized under UV light. The PCR products were purified using an Accupower® Gel Purification kit (Bioneer, Korea). Nucleotide sequences for each gene were analyzed using sequencing primers for the *gyrB* or *rpoD* genes in the Macrogen service center (Korea).

Phylogenetic analysis: Researchers used the CLUSTA_X program (Version 1.8) to independently align the partial

Table 1: List of primers used for phylogenetic identification and detection of enterotoxin gene in Aeromonas strains isolated from pet fish

Target	Primer pair	Sequence (5'-3')	Annealing (°C)	Product (bp)	References
DNA gyrase, β-subunit	gyrB3F	TCCGGCGGTCTGCACGGCGT	68	1,100	Yanez *et al*. (2003)
	gyrB14R	TTGTCCGGGTTGTACTCGTC			
	gyrB7F	GGGGTCTACTGCTTCACCAA			
	gyrB-sch3F	CATGTCTACGAGCAGACCTA			Martinez-Murcia *et al*. (2011)
	gyrB-sch12R	CTCCACGTTCAGGATCTTGCC			
RNA polymerase, σ^{70} factor	rpoD70Fs	ACGACTGACCCGGTACGCATGTA	55	800	Martinez-Murcia *et al*. (2011)
	rpoD70Rs	ATAGAAATAACCAGACGTAAGTT			
AHCYTOEN/aerA	AHCF	GAGAAGGTGACCACCAAGAAGA	65	232	Kingombe *et al*. (1999)
	AHCR	AACTGACATCGGCCTTGAACT			
Ast enterotoxin	astF	TCTCCATGCTTCCCTTCCACT	63	331	Nawaz *et al*. (2010)
	astR	GTGTAGGGATTGAAGAAGAAGCCG			
Alt enterotoxin	altF	TGACCCAGTCCTGGCACGGC	63	442	Nawaz *et al*. (2010)
	altR	GGTGATCGATCACCACCAGC			

nucleotide sequences of gyrB and rpoD of Aeromonas strains isolated from the present study and type strains of Aeromonas enrolled in the *NCBI* gene database. Genetic distances were determined using Kimura's two-parameter model (Kimura, 1980) and phylogenetic trees were constructed by neighbor-joining with the MEGA Program (Kumar *et al*., 2001).

Detection of enterotoxin genes: The detection of virulence genes was performed by single PCR assays using primer sets for act, alt and ast (Table 1). The PCR reaction buffer was the same as described above except for the replacement of each primer set. Thermal cycle conditions consisted of 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, annealing at the Tm of each primer set for 50 sec and 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel with RedSafe and the bands were visualized under UV light.

RESULTS AND DISCUSSION

Phylogenetic identification: The PCR product for the *gyrB* gene was approximately 1100 bp in 47 strains. The sequences of the PCR products were aligned with 888 nucleotides (positions 445~1365 according to *E. coli* numbering) of type strains of *Aeromonas* species to construct the phylogenetic tree (Fig. 1). The phylogenetic tree revealed that all strains except AV024 were grouped with five different species; *A. veronii* (n = 35), *A. hydrophila* (n = 7), *A. jandaei* (n = 1), *A. aquariorum* (n = 1) and *A. allosaccarophila* (n = 2). On the other hand, the gyrB sequence for the AV024 strain resulted in interspecies divergence of 5.6, 4.6 and 3.9% for *A. allosaccarophila*, *A. veronii* and *A. jandaei* strains, respectively in the phylogenetic analysis resulting in the formation of an independent line in the phylogenetic tree. The AV024 strain was re-analyzed by amplification of the *rpoD* gene followed by its partial sequence. The alignment of 649 nucleotides (positions 408~1053 according to *E. coli* numbering) was used to construct a phylogenetic tree with known *Aeromonas* sp. and a divergence of 1.4% was observed between rpoD sequences from AV024 and the *A. veronii* type strain (Fig. 2).

Detection of enterotoxin genes: The *act*, *alt* and *ast* genes were detected in 85.1, 61.7 and 17.0%, respectively of all Aeromonas strains (Table 2). Researchers found that 91.5% of strains tested had one or more enterotoxin genes and the strains were associated with six enterotoxin gene patterns, *act*, *alt*, *act*/*alt*, *act*/*ast* or *act*/*alt*/*ast* (Table 3).

The *act* gene alone was detected in only 36.1% of *A. veronii* strains (27.7% of total strains) while 40.4% of all strains harbored the *act*/*alt* gene pattern which included 17 strains (47.2%) of *A. veronii* and one strain each of *A. aquariorum* and *A. allosaccarophila*. The *alt* gene alone occurred in 6.4% of strains including *A. veronii* and *A. jandaei*. However, the *ast* gene was not singly present in the strains. On the other hand, the *act*/*alt*/*ast* gene pattern was observed in all *A. hydrophila* strains.

The genus *Aeromonas* is taxonomically very diverse groups. For discriminating among *Aeromonas* sp. the housekeeping genes, *gyrB* and *rpoD* have been shown to be useful genetic markers (Martinez-Murcia *et al*., 2011; Janda and Abbott, 2010; Beaz-Hidalgo *et al*., 2010; Alperi *et al*., 2010; Soler *et al*., 2004). However, there are few prevalent studies using housekeeping genes for species-level identification of Aeromonas from the aquaculture industry including from pet fish (Beaz-Hidalgo *et al*., 2010). In earlier phylogenetic identifications using housekeeping genes both sequences of *Aeromonas* sp. had a divergence >3% among species indicating a cut-off value below 3% for species differentiation (Yanez *et al*., 2003; Soler *et al*., 2004; Kupfer *et al*., 2006). Based on the previous results, researchers identified five different species present in all strains except strain AV024. There have been some reports about gyrB mutations in quinolone-resistant bacteria (Kim *et al*., 2010; Spigaglia *et al*., 2009). This might be a reason for separating the AV024 strain from the other Aeromonas type strains in phylogenetic analyses using the *gyrB* gene sequences. This could be supported by repeating the phylogenetic analysis using the *rpoD* gene of the AV024 strain.

There have been many reports about the inaccuracy of phenotypic methods in identifying *Aeromonas* sp. for instance, the inaccuracy rates of conventional phenotypic identification were reported for 75.5% of 90 strains from diseased fish (Beaz-Hidalgo *et al*., 2010) and 71.5% of 82 strains from frozen fish. On the other hand, Lamy *et al*. (2010) reported overall species inaccuracies of 8~32.2% according to commercial phenotypic systems on identification of *Aeromonas* sp. In addition, *A. sobria* has been misnamed as *A. veronii* bv. sobria because of common phenotypes such as esculin, salicin and L-arabinose negativity, shared by both species (Janda and Abbott, 2010). In spite of this fact, the earlier studies have widely used conventional and commercial phenotypic systems for species-level identification of Aeromonas and have reported frequent isolations of *A. caviae*, *A. sobria* and *A. hydrophila* from cultured and imported pet fish (Cizek *et al*., 2010; Dixon and Issvoran,

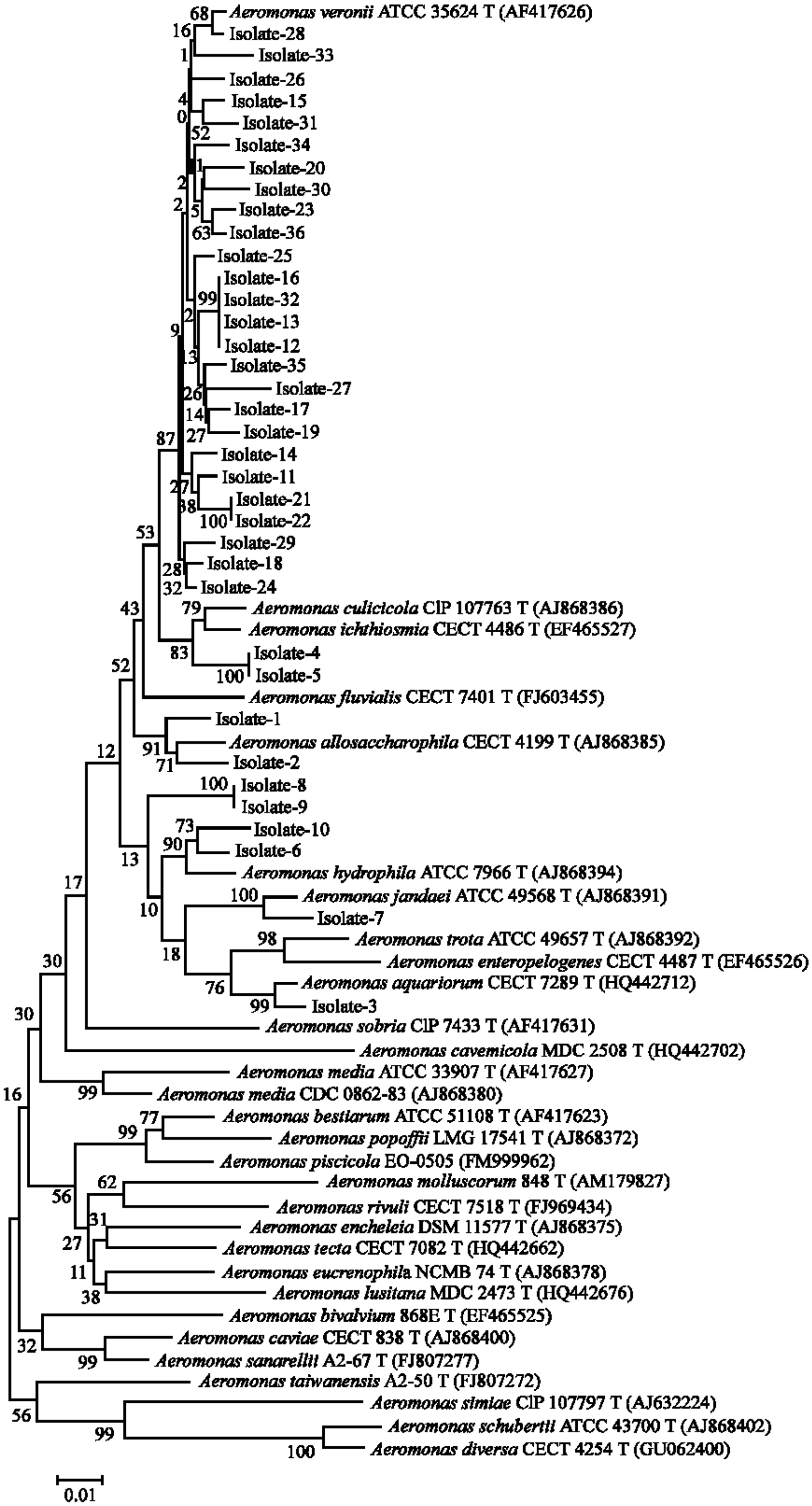

Fig. 1: Unrooted neighbor-joining phylogenetic tree based on the *gyrB* gene sequences showing the relationship within the genus *Aeromonas* of 47 strains isolated from pet fish. Numbers at nodes indicate bootstrap values (1000 replicates)

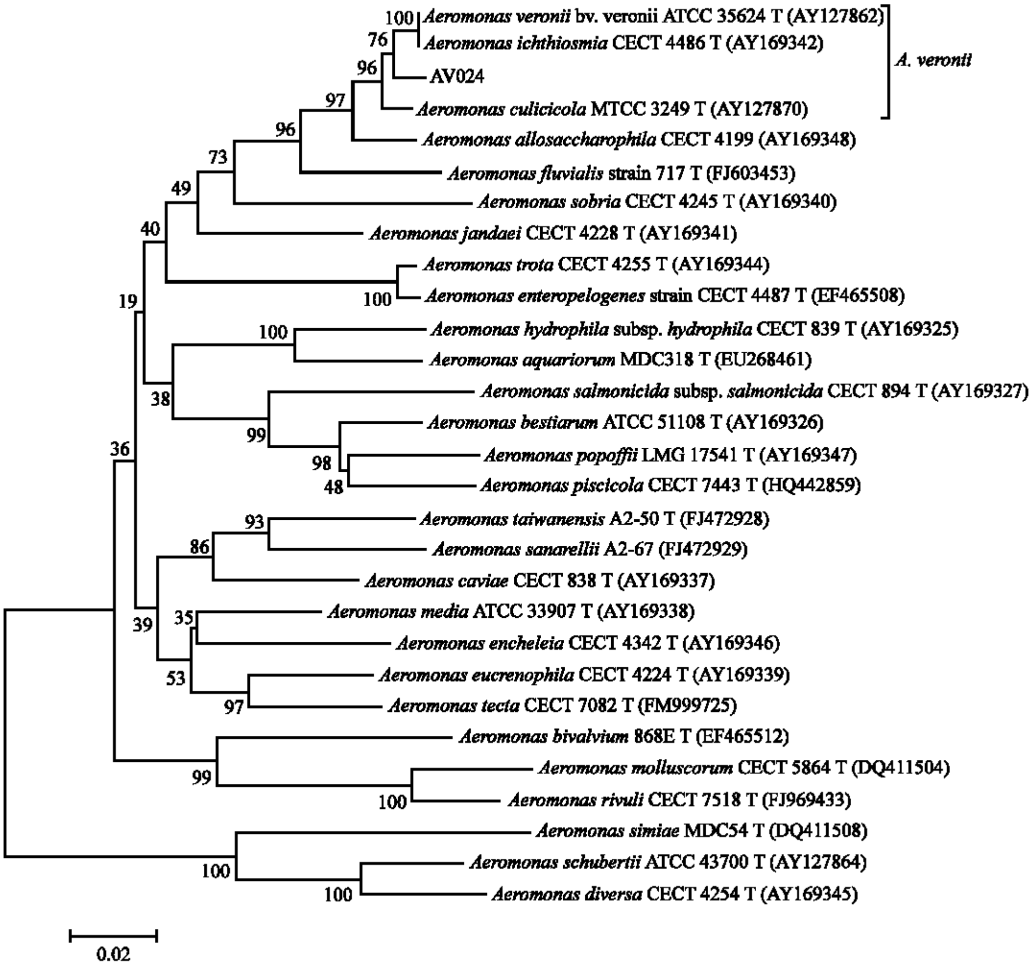

Fig. 2: Unrooted neighbor-joining phylogenetic tree based on the *rpoD* gene sequences showing the relationship within the genus *Aeromonas* of the strain AV024 unidentified in phylogenetic identification using gyrB sequences. Numbers at nodes indicate bootstrap values (1000 replicates)

Table 2: Prevalence and presence of enterotoxin genes in *Aeromonas* sp. isolated from imported pet fish and cultured koi carp

		No. of strains positive for the gene (%)		
Host	Species	act	alt	Ast
Pet fish	*A. veronii* (n = 31)	26 (83.9)	18 (58.1)	1 (3.2)
(n = 36)	*A. allosaccarophila* (n = 2)	1 (50.0)	1 (50.0)	0 (0)
	A. aquariorum (n = 1)	1 (100)	1 (100)	0 (0)
	A. hydrophila (n = 1)	1 (100)	1 (100)	1 (100)
	A. jandaei (n = 1)	0 (0)	1 (100)	0 (0)
	Subtotal (n = 36)	29 (80.6)	22 (61.1)	2 (5.6)
Koi (n = 11)	*A. veronii* (n = 5)	5 (100)	1 (20.0)	0 (0)
	A. hydrophila (n = 6)	6 (100)	6 (100)	6 (100)
	Subtotal (n = 11)	11 (100)	7 (63.6)	6 (54.6)
	Total (n = 47)	40 (85.1)	29 (61.7)	8 (17.0)

1992; Lowry and Smith, 2007; Verner-Jeffreys *et al*., 2009). In disagreement with earlier studies, researchers found that *A. veronii* was the most dominant species from imported pet fish and its identification rate was equivalent to that of *A. hydrophila* among strains from koi carp. This disagreement could clearly be due to differences in methods used for species-level identification of Aeromonas strains. On the other hand, *A. aquariorum*, *A. jandaei* and *A. allosaccarophila* were separately identified in four strains of imported pet fish but were not presented in the cultured koi. To the knowledge, there has not been a report about the three different species in microbiological fields for human, veterinary and environment in Korea. Since, *A. aquariorum* was first isolated from pet fish in 2008 (Martinez-Murcia *et al*., 2008), it has been widely isolated from clinical and environmental samples (Figueras *et al*., 2011b, 2009; Aravena-Roman *et al*., 2011). In addition, the species was identified in 15.9% of extraintestinal clinical samples in Taiwan (Figueras *et al*., 2009) which is a neighboring country of and major exporter of pet fish to South

Table 3: Distribution of enterotoxin genes in *Aeromonas* sp.

Species	nd	act	alt	act/alt	act/ast	act/alt/ast
A. *veronii* (n = 36)	3 (8.3)	13 (36.1)	2 (5.6)	17 (47.2)	1 (2.8)	0
A. hydrophila (n = 7)	0	0	0	0	0	7 (100)
A. aquariorum (n = 1)	0	0	0	1 (100)	0	0
A. jandaei (n = 1)	0	0	1 (100)	0	0	0
A. allosaccarophila (n = 2)	1 (50.0)	0	0	1 (50)	0	0
Total	4 (8.5)	13 (27.7)	3 (6.4)	19 (40.4)	1 (2.1)	7 (14.9)

Korea. Therefore, pet fish could be a transport animal for transmission of new *Aeromonas* species to importers.

In the present study, the detection of the *act* gene was significantly higher than that in previous studies, showing up in 65% of food strains from Canada (Kingombe *et al.*, 2010), 62% of clinical strains from Southern Taiwan (Wu *et al.*, 2007) and 70% of strains from drinking water in the US (Sen and Rodgers, 2004). Some researchers revealed that the *act* gene was much less prevalent in *A. caviae* than in *A. veronii* and *A. hydrophila* (Albert *et al.*, 2000; Sen and Rodgers, 2004; Wu *et al.*, 2007; Kingombe *et al.*, 2010). The present study could not identify *A. caviae* from isolated strains which may explain the higher prevalence of the *act* gene among the present strains. On the other hand, a greater percentage of alt-positive *A. veronii* strains were observed in the present study compared with earlier studies (Albert *et al.*, 2000; Sen and Rodgers, 2004; Wu *et al.*, 2007; Kingombe *et al.*, 2010). The difference might be related to the geographical variation in detection of the *alt* gene. Actually, the present study showed a higher prevalence of the *alt* gene in *A. veronii* from the imported pet fish compared to that from the cultured koi. Some researchers have revealed a relationship between enterotoxin gene patterns and potential roles in the pathogenesis of diarrhea (Albert *et al.*, 2000; Sha *et al.*, 2002). Sha *et al.* (2002) showed that the act, act/alt and act/alt/ast mutants resulted in 64, 73 and 100% decreases, respectively in fluid secretions in the ileal loops of mouse models compared with wild-type *A. hydrophila*. On the other hand, Albert *et al.* (2000) reported that the *alt/ast* genes and *alt* gene were associated with watery stools and loose stools, respectively. The present study showed that 91.5% of Aeromonas strains harbored one or more enterotoxin genes. In addition, *act*, *act/alt* and *act/alt/ast* gene patterns were present in 83% of Aeromonas strains. Given the results from the earlier studies (Xu *et al.*, 1998; Albert *et al.*, 2000; Sha *et al.*, 2002) most Aeromonas strains isolated from pet fish are thought to be pathogens for humans as well as fish.

CONCLUSION

In conclusion, imported pet fish carry a variety of *Aeromonas* sp. such as *A. veronii*, *A. hydrophila*, *A. aquariorum*, *A. jandaei* and *A. allosaccarophila*. Of the identified species, *A. aquariorum*, *A. jandaei* and *A. allosaccarophila* are earlier unrecorded in Korea. In addition, most *Aeromonas* sp. harbor more than one gene encoding an enterotoxin that plays a key role in pathogenesis of Aeromonas in humans as well as fish. Therefore, the results of the present study suggest that pet fish are an important reservoir for pathogens that threaten the animal and public health of the importing country.

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