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Full Length Research Paper

Occurrence of tetracycline-resistant *Aeromonas hydrophila* infection in Korean cyprinid loach (*Misgurnus anguillicaudatus*)

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In this paper, we described a mass mortality of cyprinid loaches, *Misgurnus anguillicaudatus* from a private fish farm in Korea. Diffuse bleeding was observed on the skin around the anal orifice. Bacterial pathogens from kidneys samples of moribund fish were cultured, identified and confirmed to be *Aeromonas hydrophila* using morphological, biochemical test and genetical analysis. The isolated *A. hydrophila* strains were resistant to commercial antibiotics and the presence of the tetracycline resistance gene (*tet* E) was detected by genetical analysis. Pathogenecity test was performed using healthy 4-week-old cyprinid loaches by intraperitoneal (IP) injection and the LD_{50} concentration of the pathogen was determined to be 6.0×10^7 CFU $fish^{-1}$. In this paper, we confirmed the infection of farm-raised cyprinid loaches with tetracycline-resistant *A. hydrophila*.

Key words: Cyprinid loach (*Misgurnus anguillicaudatus*), *Aeromonas hydrophila*, tetracycline resistance gene, pathogenecity test.

INTRODUCTION

The genus *Aeromonas* is a member of the family *Aeromonadaceae* that are primarily aquatic organisms found in water. Some *Aeromonas* spp. are pathogenic for humans as well as fish (Tsukamoto et al., 1993). The organisms in this family produce a clear zone of β-hemolysis on blood agar (Khardori and Faintein, 1988). *Aeromonas hydrophila* is a member of motile aeromonads and it can cause disease in fish, resulting in high mortality (Ishimura et al., 1988; Alvarado and Boehm, 1989; Angka, 1990; McGarey et al., 1991; Esteve et al., 1993). There has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments (Rhodes et al., 2000; Schmidt et al., 2001a, b). Five classes of genetically distinguishable tetracycline resistance determinants designated A through E, have been described among aerobic enteric gram-negative bacteria (Nawaz et al., 2006). Several studies have shown *tet*E to be the predominant determinant among the different classes of tetracycline-resistant genes (DePaola and Roberts, 1995; Miranda et al., 2003; Schmidt et al., 2001a).

The loach (*Misgurnus* spp.) is a member of the Cobitidae family (Lacepede, 1803) and inhabits freshwater systems by nature (Kim et al., 1994b). Two species of loaches (*Misgurnus* spp.), the mud loach (*M. mizolepis*) and the cyprinid loach (*Misgurnus anguillicaudatus*), are cultured mostly for food and sometimes for Buddhism ceremonies in Korea (Kim et al., 1994a). The annual demand for loaches in Korea and Japan was over 100,000 tonne (t) in 2004 due to its high nutritional value and use in folk medicine (Jiangsu Meteorological Bureau, 2004). Aquaculture of loach in 2008 was over 432 t in Korea (Korea National Statistical office, 2008). Jeollabuk-do province in Korea is famous for the aquaculture of loaches, with over 384 t in 2008, which was 89% of the total loach aquaculture in the entire country (Jeollabuk-do Province Office, 2008).

There have been few reports about *A. hydrophila* in Korea since the previous publication about isolation of *A.*

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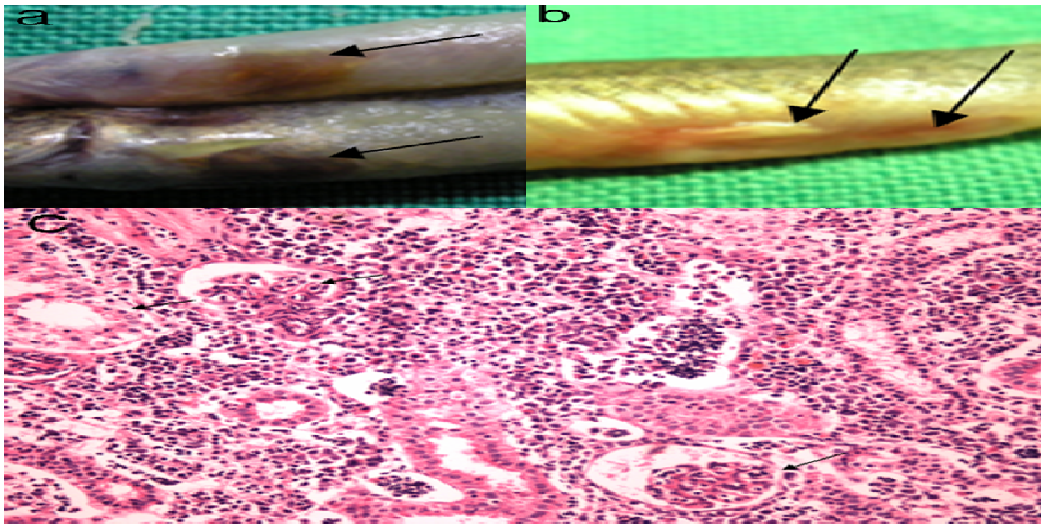

Figure 1. Diseased cyprinid loach (*M. anguillicaudatus*) showing diffuse bleeding and hemorrhages at the ventral part (a) and the protrusion of intestine and hemorrhages on the skin around the anal orifice (b). Section through the kidney of a cyprinid loach with *A. hydrophila* septicemia. There is considerable tubular degeneration with eosinophilic detritus within the lumina. A degenerate glomerulus is in this figure. The hemopoietic tissue of the renal interstitium is considerably reduced and remaining cells are necrotic. H + E ×400 (c).

hydrophila from rainbow trouts in Korea (Lee et al., 2000). Although, the majority of the loach population is cultured and *A. hydrophila* is one of the main causes of its mass mortality in Korea, there was only little knowledge of this bacterium from cyprinid loach. This paper describes a case of mass mortality of cyprinid loaches caused by the pathogenic bacteria, *A. hydrophila*. We report for the first time the isolation and identification of tetracycline-resistant *A. hydrophila* from the farm-raised cyprinid loach, *M. anguillicaudatus.*

MATERIALS AND METHODS

Fish sample

Fish were cultured in a private fish farm equipped with an aeration system and water temperature ranging from 25 to 27°C in Jeollabuk-do province, Korea. In April 2009, some fingerlings (20 - 30 days old) showed abnormal swimming behavior, diffuse bleeding on the skin around the anal orifice and gradual mortality. The mortality rate was over 1% per day and fish were treated with oxytetracycline (OTC) (20 mg l^{-1} day^{-1} dosage with bath treatment). However, mass mortality continued during the antibiotic treatment and approximately 50% of the fingerlings died, showing same symptoms as previously described. When 90 moribund fingerling samples (7.8 cm average length and 3.4 g average weight) that exhibited abnormal swimming behavior and lethargy were examined externally, the skin and gills revealed an increase in the quantity of mucous on the surface. Diffuse bleeding and hemorrhages were observed on the skin around the swollen anal orifice of moribund fish (Figure 1a and b). Nine fish samples were randomly selected for further analysis. Renal tubular epithelial cells exhibited vacuolar degeneration accompanied by nuclear degeneration and necrosis followed by tubular destruction (Figure 1c). There were no remarkable findings on any organs in the internal examination.

Morphological and biochemical analysis

Gram staining and motility test were performed. For bacterial isolation, sterile swabs from the kidneys of the 9 fish samples were streaked onto tryptic soy agar (TSA) and the inoculated plates were incubated at 25°C for 24 h. Suspected common colonies were re-streaked on TSA to obtain pure cultures, which were then simply identified on the basis of microscopic analysis and with the aid of the Vitek System®2 (bioMérieux®, France) and API 20E test (BioMerieux, France) for biochemical analysis.

Extraction of bacterial DNA and PCR amplification for *A. hydrophila*

The isolated strains were re-suspended in 500 µl of sterilized double-distilled water (DDW). The bacterial DNA was extracted by boiling cells for 5 min and centrifuging the solution at 6000 g for 5 min. Bacterial DNA was collected on the upper aqueous phase of the supernatant and then stored at -20°C until needed. Multiplex PCR for simultaneous detection of *A. hydrophila* extracellular hemolysin gene *ahh1* (130 bp) and *A. hydrophila* aerolysin gene *aerA* (309 bp) was performed as previously described (Wang et al., 2003). Amplification was carried out in a T-personal 48 thermocycler (Biometra, Gottingen, Germany) with previously published PCR conditions (Wang et al., 2003). Negative (DDW) and positive controls (*A. hydrophila*, ATCC 7966) were included in the PCR. The PCR products were analyzed by 1.5% agarose gel electrophoresis in 1% Tris-borate-EDTA buffer. Gels were stained with ethidium bromide (0.5 µg ml^{-1}), visualized, and photographed under ultraviolet (UV) illumination.

Nine samples of the 16S rRNA gene of the bacterial species were amplified by PCR using universal primers 27F and 1492R at the Macrogen Genomic Division, Korea. The isolation and purification of genomic DNA from the sample was done using the

Table 1. List of oligonucleotide primers, target genes, amplicon sizes and sources of gene sequences used for PCR in this study.

Primer pair	Sequence (5'to 3')	Target gene	Amplicon size	Source
AHH1F	GCCGAGCGCCCAGAAGGTGAGTT	*ahh1*[a]	130 bp	(Wang et al., 2003)
AHH1R	GAGCGGCTGGATGCGGTTGT			
AH-aerAF	CAAGAACAAGTTCAAGTGGCCA	*A. hydrophila aerA*	309 bp	(Wang et al., 2003)
AH-aerAR	ACGAAGGTGTGGTTCCAGT			
27F	AGAGTTTGATCMTGGCTCAG	16S rRNA		(universal primers)
1492R	TACGGYTACCTTGTTACGACTT			
518F	CCAGCAGCCGCGGTAATACG	16S rRNA		(Lane et al., 1991)
800R	TACCAGGGTATCTAATCC			
*tet*AF	GCTACATCCTGCTTGCCTTC	*tet*A	211 bp	(Nawaz et al., 2006)
*tet*AR	GCATAGATCGCCGTGAAGAG			
ClassB *tet*AF	TCATTGCCGATACCACCTCAG	*tet*B	391 bp	(Nawaz et al., 2006)
ClassB *tet*AR	CCAACCATCATGCTATTCCATCC			
ClassC *tet*AF	CTGCTCGCTTCGCTACTTG	*tet*C	897 bp	(Nawaz et al., 2006)
ClassC *tet*AR	GCCTACAATCCATGCCAACC			
ClassD *tet*AF	TGTGCTGTGGATGTTGTATCTC	*tet*D	844 bp	(Nawaz et al., 2006)
ClassD *tet*AR	CAGTGCCGTGCCAATCAG			
ClassE *tet*AF	ATGAACCGCACTGTGATGATG	*tet*E	744 bp	(Nawaz et al., 2006)
ClassE *tet*AR	ACCGACCATTACGCCATCC			

[a] From strain ATCC 7966.

D Neasy® Tissue Kit (QIAGEN, Hilden, Germany). Sequencing of the purified PCR products was performed using the ABI PRISM Big Dye TM Terminator Cycle Sequencing Kit (Applied BioSystems, California, USA) and sequencing primers (518F and 800R) at the Macrogen Genomic Division, Korea (Lane et al., 1991). Electrophoresis of sequencing reactions was completed using the automated ABI PRISM 3730XL DNA Sequencing System (Applied BioSystems, California, USA). The rRNA sequence genes of the bacterial strains obtained in this study were aligned with other bacteria of the same species (EU770274.1, FJ515776.1, FJ515777.1 and FJ794069.1) available from GenBank database using the multiple alignment algorithms in the MegAlign package (Windows Version 3.12e; DNASTAR Software Package, Wisconsin, USA) and percentage sequence similarities were determined.

Multiplex PCR was performed to amplify the tetracycline resistance genes (*tet*A, 211 bp; *tet*B, 391 bp; *tet*C, 897 bp; *tet*D, 844 bp; *tet*E, 744 bp) using one representative strain (SNUFPC-Aeh01) as previously described (Nawaz et al., 2006). Sequencing of the purified PCR product was performed to confirm the presence of the tetracycline resistance genes at the Macrogen Genomic Division, Korea. The sequence of the tetracycline resistance genes obtained in this study was aligned with those of *Aeromonas salmonicida* plasmid tetracycline resistance genes (CP000645.1 and DQ366299.1) available from GenBank database and percentage sequence similarities were determined. All the primers used in this study were shown in Table 1.

Antibiotic susceptibility test by disc diffusion method

Antibiotic susceptibility of bacterial isolates was determined by the disc diffusion method (Bauer et al., 1966). Antibiotic discs (BBL, USA) used in this study were shown in Table 2. The sensitivity and resistance of isolated bacteria and zone diameter interpretive standards were determined according to the CLSI (Clinical and Laboratory Standards Institute) criteria for animal isolates (CLSI, 2006).

Experimental infection

The bacteria isolated from moribund fish were stored at -80℃ in sterile 20% glycerol until use. To determine the LD_{50} concentration of the pathogen, the stored bacteria from the moribund fish were used for the infection experiment after growing for 24 h at 25℃ in TSA. The bacterial concentration was determined from the optical density at 600 nm, confirmed the following day by plate count, and serially diluted ten-fold with saline. 240 healthy 4-week-old cyprinid loaches were divided into six groups in 4 L fiber plastic tanks at 23 - 24℃. The fish were challenged with 0.1 ml of the bacterial suspension by intraperitoneal (IP) injection. The final doses of infections in the experimental groups ranged from 10^4 to 10^8 CFU $fish^{-1}$. The control group was injected with 0.1 ml of sterile saline. After injection, these fish were kept for 2 weeks. Dead fish were sampled everyday for isolation of bacteria from the kidneys using TSA and incubated at 25℃ for 24 h. After cultivation, the isolates were checked by PCR.

RESULTS

Detection of the causative agent

Several morphological and biochemical identical strains were isolated from 9 randomly selected loaches. Streaking on TSA gave an apparently pure transparent common bacterial growth from the kidneys of all moribund fish. The result of the microscopic examination revealed that the isolates were identified as gram-negative, motile, oxidase-positive, catalase-positive, single or paired rod-form of bacteria. Colonies measuring 3 - 5 mm in diameter formed on TSA. They were white with a pale brown pigment, and induced β-hemolysis on blood agar. The isolated bacteria were incubated at 20

Table 2. Antimicrobial susceptibility test of *A. hydrophila* isolated from cyprinid loach.

Antibiotics(μg)	Sensitivity
Amikacin (30)	S
Amoxicillin/clavulanic acid (30)	R
Ampicillin (10)	R
Cefepime (30)	S
Cefotaxime (30)	S
Chloramphenicol (30)	R
Ciprofloxacin (5)	S
Gentamicin (10)	S
Tetracycline (30)	R
Trimethoprim/Sulfamethoxazole (1.25)/(23.75)	R

The category 'S' means sensitive to antibiotic; 'R' means resistant. And each category was decided by zone diameter interpretive standards (CLSI, 2006).

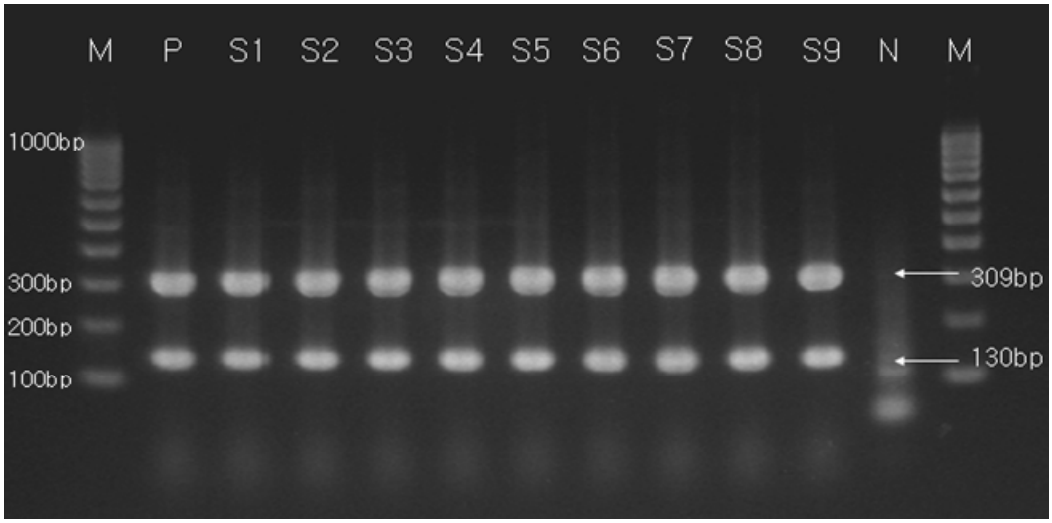

Figure 2. Representative amplification products obtained using the multiplex PCR assay for detection of *A. hydrophila* in cyprinid loach. Lanes M, 100 bp DNA ladder; Lane P, positive control (*A. hydrophila* ATCC 7966, 130 bp of the *ahh1* gene for *A. hydrophila* extracellular hemolysin gene and 309 bp of the *aerA* gene for *A. hydrophila* aerolysin gene); Lane S1 – S9, positive for *A. hydrophila* (130 and 309 bp); Lane N, negative control.

and 36.5℃ to test for temperature sensitivity, and the growth rate of bacteria was nearly the same at both temperatures. From the result of Vitek System®2, *A. hydrophila* was isolated and showed 98% probability. According to the result of API 20E test, the isolates were identical to the reference of Bergey's Manual of Determinative Bacteriology. Characterization (based on their morphological and biochemical reactions using the API 20E test and Vitek System®2) showed that these isolates were phenotypically identified as *A. hydrophila*. The specific PCR products corresponding to the 130 bp fragment of the *ahh1* gene and the 309 bp fragment of the *aerA* gene were detected from pure cultures (Figure 2). Moreover, as a result of 16S rRNA gene sequencing, *A. hydrophila* consisting of approximately 936 nucleotides was isolated and showed 100% sequence similarity with other strains of *A. hydrophila* available in the GenBank.

Antibiotic resistance profiles of bacterial isolate

Antibiotic resistance profiles of *A. hydrophila* strains were identical and shown in Table 2. The bacterial isolate was resistant to amoxicillin/clavulanic acid, ampicillin, chloramphenicol, tetracycline and trimethoprim/ sulfamethoxazole. However, it was sensitive to amikacin,

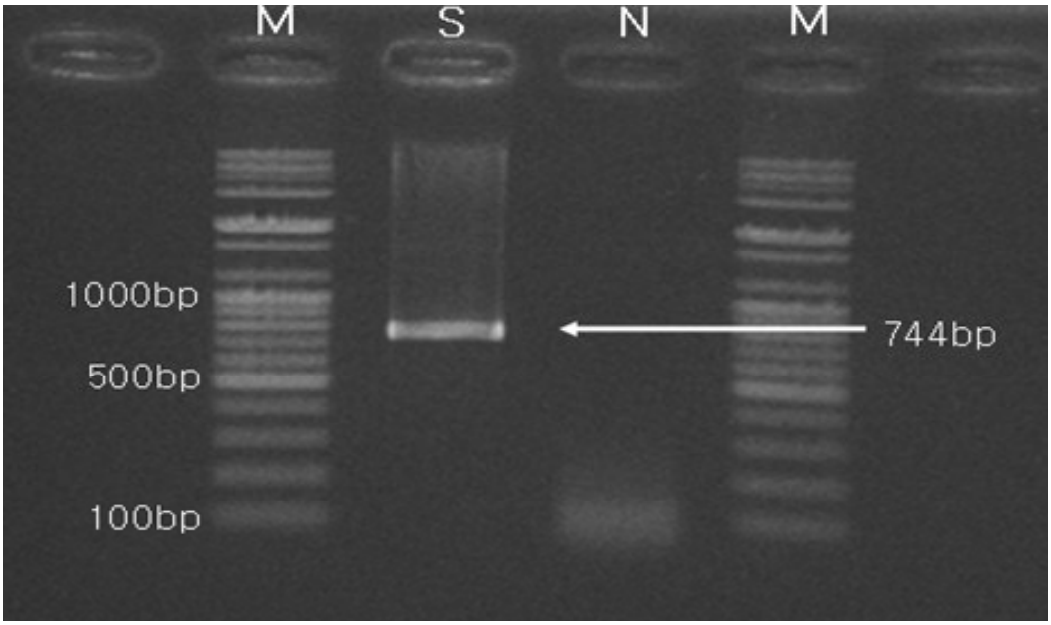

Figure 3. A multiplex PCR assay was performed to amplify the tetracycline resistance genes (*tet*A to E) from the isolates. Lane M, 100 bp DNA ladder; Lane S, the presence of the *tet*E gene (744 bp); Lane N, negative control.

cefepime, cefotaxime, ciprofloxacin and gentamicin. The assay detected the presence of the *tet*E gene in *A. hydrophila* by amplifying the 744 bp PCR product from the genomic DNA of these isolates (Figure 3). *tet*E gene sequencing showed 100% similarity with other reported *tet*E genes of *A. salmonicida* subsp. *salmonicida* A449 plasmid 4 (CP000645.1) and *A. salmonicida* plasmid pYA90644 (DQ366299.1) available in the GenBank.

Experimental infection

Mortality started at the 3rd day post-injection and cumulative mortalities of the groups injected with 6.0×10^8, 6.0×10^7, 6.0×10^6, 6.0×10^5 and 6.0×10^4 CFU fish^{-1} were 80, 80, 20, 10 and 10%, respectively. No mortality was observed in the control group (Figure 4). Characteristic feature of *A. hydrophila* pathogenicity shows that the mortality of the group injected with 6.0×10^7 CFU fish^{-1} already reached 50% at the 5th day post-infection.

DISCUSSION

Although there is a previous report about *A. hydrophila* isolates from rainbow trouts in Korea (Lee et al., 2000), there is little accurate analysis about other Korean isolates of the same species. Confirmation with isolation in pure culture and PCR assay from the affected cyprinid loaches in this study clearly indicated that *A. hydrophila* was the causative agent of the mass mortality of cyprinid loaches in the private fish hatchery farm. A number of virulence factors derived from *A. hydrophila* have been proposed in an effort to explain the pathogenesis of infections (Wang et al., 2003). Toxins with hemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas* spp. (Namdari and Bottone, 1990; Chopra and Houston, 1999). Although a number of toxins are produced by different species, single isolates often carry the genes encoding multiple toxins (Wang et al., 2003). Mutagenesis studies indicated that the hemolytic activity of *A. hydrophila* is related to both the hemolysin and the aerolysin genes (Wang et al., 2003). The *A. hydrophila* hemolysin and aerolysin genes that were detected from the samples showed that *A. hydrophila* was pathogenic enough to cause mortality.

A. hydrophila is generally considered a pathogen of low virulence, rarely reported as causing infections in humans. However, the β-hemolytic activity of *A. hydrophila* has been used as an indicator of enterotoxicity and may be responsible for outbreaks of diarrhea (Rahim et al., 1984). It has been also recognized as causing infection in wounds and categorized as an emerging human pathogen (Katz and Smith, 1980; Kozlova et al., 2008). Several reports indicate that a previously healthy man can be infected from a puncture wound sustained in a fresh water lake (Katz and Smith, 1980). Because loaches are edible fish, it is also possible to be infected during cooking. Many strains of *Aeromonas* are not sensitive to the antibiotics commonly used for wound infections (Skiendzielewski and O'Keefe, 1990), and *A. hydrophila* infections can be so dangerous that proper emergency treatment could be necessary. Our PCR data indicated that *A. hydrophila* isolated from cyprinid loach

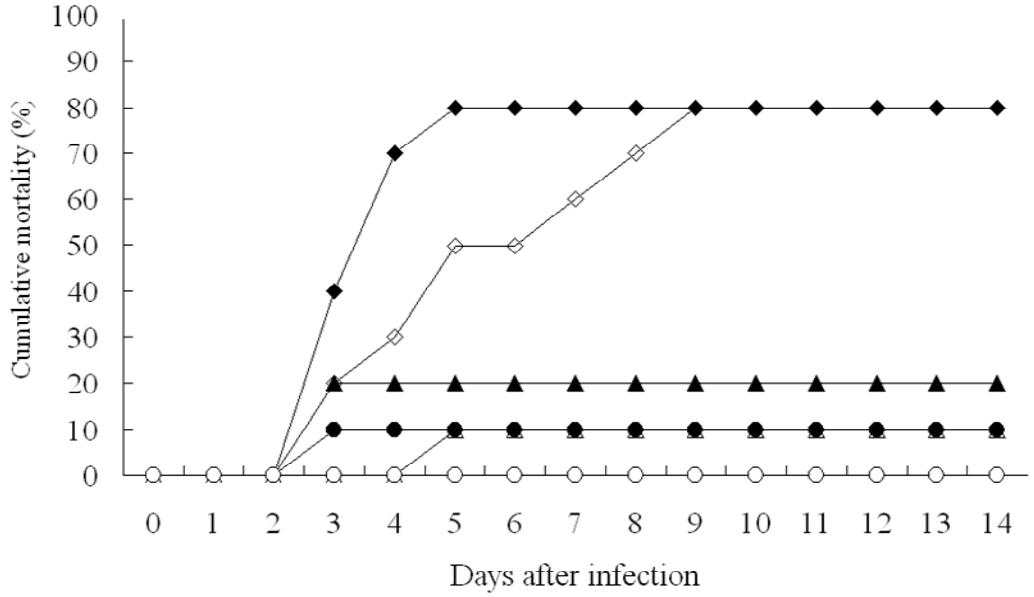

Figure 4. Experimental infection of cyprinid loaches and experimental infection by intraperitoneal injecting. Five test groups of ten cyprinid loaches were intraperitoneally injectected (dose volume 0.1 ml) with 6.0×10^8 CFU fish^{-1} (∅), 6.0×10^7 CFU fish^{-1} (∩), 6.0×10^6 CFU fish^{-1} (▲), 6.0×10^5 CFU fish^{-1} (≥) and 6.0×10^4 CFU fish^{-1} (●) of *A. hydrophila*. Control (○) group of ten cyprinid loaches was intraperitoneally injected with saline.

contained *tet*E and the sequencing data indicated that it had the identical plasmid *tet*E gene as *A. salmonicida*. It had been determined that 86% of *A. hydrophila* isolated from catfish contained *tet* genes (DePaola et al., 1988). Antimicrobial resistance determinants selected in aquaculture ecosystems may be transmitted to human pathogenic bacteria (Smith et al., 1994). Antimicrobial resistant bacteria present in an aquaculture setting may be transferred to fish, other animals and humans who come in contact with this ecosystem via wound infections, in fish-farm workers or processing-plant workers with open wounds by exposure to contaminated water or fish during the handling of fish for food preparation or consumption of aquaculture fish (Petersen and Dalsgaard, 2003).

The LD_{50} of *A. hydrophila* was 2.94×10^7 cfu ml^{-1} in common carp (*Cyprinus carpio*) (Selvaraj et al., 2009). In this study, the LD_{50} of *A. hydrophila* was 6.0×10^7 cfu ml^{-1}, which corresponded to the previous report. Although, the LD_{50} of *A. hydrophila* is high and *A. hydrophila* is commonly isolated in an aquatic environment, it can be concluded that the urgent mass mortality of cyprinid loaches was caused by acute stress causing factors such as overstocking and poor quality of the water. Those findings are so alarming that fish farmers should realize that *A. hydrophila* can contribute high mortality and economic loss to loach aquaculture.

Internationally, ox tetracycline has been most frequently used antibiotic in aquaculture (Jacobs and Chenia, 2007). In this study, we proved that mass mortality of cyprinid loaches was caused by *A. hydrophila*. Additionally, it was proved that *A. hydrophila* containing the *tet*E gene exists in Korean aquaculture system and has virulence. In conclusion, more standardized guidelines and legislations for antimicrobial use in aquaculture systems are necessary and international multi-center collaborative studies are needed.

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