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First report of *Aeromonas veronii* infection in farmed Israeli carp *Cyprinus carpio* in Korea

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In May 2007, mass mortality of Israeli carp, *Cyprinus carpio* L., was occured on a pond farm located in Jeollabukdo Province, Korea. The mortality rates reached up to 2% of the total fish in the farm per day. Typical clinical signs were abdominal distension, reddish foci on the skin, enteritis, liver congestion and enlarged spleen and kidney. On the basis of biochemical characteristics and 16S rDNA sequence, the causative bacteria isolated from affected carp were identified as Aeromonas veronii. Histologically, degeneration of hepatocytes and congestion in sinusoids were observed in the liver. Spleen showed hemorrhage and the destruction of the sheathed tissues. In kidney, necrotized renal tubules and glomerular destructions were observed. Intestinal tissues revealed necrotized and severe hemorrhage. Mass hemorrhage was observed in muscles. This is the first report that A. veronii caused mortality in cultured Israeli carp in Korea.

Key words : Aeromonas veronii, Israeli carp, Cyprinus carpio, Histopathology

The motile aeromonads are one of the ubiquitous members of the aquatic ecosystem and can be pathogenic to fishes, reptiles and even human (Fraire, 1978; Inglis *et al.*, 1994; Sersy *et al.*, 1996; Park & Oh, 2008). In aquaculture, *Aeromonas* sp. infection in fish is a significant concern as it often involves high mortality and morbidity rates (Goldschmidt-clermont *et al.*, 2008). In particular, *Aeromonas hydrophila*, *A. caviae*, *A. sobira*, *A. salmonicida* and *A. bestiarum* comprise the most predominant clinical isolates that are typically associated with diseased fishes (Austin *et al.*, 1989; Cipriano, 2001; Wahli *et al.*, 2005; 김 등, 2006; Austin & Austin, 2007; Park & Oh, 2008). Although motile aeromonads receive much notoriety as pathogens of fish, it is important to note that these bacteria also constitute part of the normal intestinal microflora of a healthy fish (Trust *et al.*, 1974).

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Aeromonas veronii, a member of the genus aeromonads, is a Gram-negative rod and has been recovered from human wounds, feces and sputum and fish ulcerative lesions (Hickman-Brenner *et al.*, 1987; Abbott *et al.*, 1994; Rahman *et al.*, 2002). Rhaman *et al.* (2002) suggested that *A. veronii* can be a causative agent of epizootic ulcerative syndrome (EUS) in fish.

The aim of the present study was to identify the causative agent of farmed Israeli carp losses on a farm, verify the pathogenic capacity of this agent by fulfillment of Koch's postulates. In addition, histopathology resulting from *A. veronii* infection in farmed Israeli carp and biochemical and genetic properties of *A. veronii* were described.

Materials and methods

1. Fish and Microbiology

A disease outbreak occurred in May 2007 on an Israeli carp farm located in the Jeollabukdo Province, Korea. Water temperature, pH and dissolved oxygen in the farm water ranged from 20°C to 23°C, 6.4～6.6 and 5.8～6.3 mg L^{-1}, respectively during this period. Fish (mean length 17.7±2.8 cm; mean weight 83.6±3.9 g) were reared at a density of 9-11 kg m^{-2} and the mortality rates per day reached up to 2% of the total fish in the farm.

Skin and gill samples from 13 fish were examined for the presence of parasites under a light microscope (CH2, Olympus, Tokyo, Japan). Gross necropsy was performed and imprints of the spleen and kidney were taken and Gram stained. For bacteriological analysis, kidney and spleen were aseptically streaked on tryptic soy agar (TSA) (Becton-Dickinson, Franklin Lakes, NJ, USA). After incubation at 22°C for 24 h, a dominant colony on TSA agar plates was re-streaked onto TSA to obtain pure isolate. The isolate was identified using API 20E and 50CHE system (BioMérieux, Durham, NC, USA) following 24 h incubation at 22°C. Interpretation of the API results was carried out in accordance with the manufacturer's instructions. The isolate was streaked onto sheep blood agar plates and then incubated at 10°C and 30°C for hemolytic activity. *Aeromonas sobria* ATCC 43979 and *A. veronii* ATCC 35623 were used as controls.

Antibiotic susceptibility was assessed by the disc diffusion method (Bauer *et al.* 1966) on Müller-Hinton agar (Becton-Dickinson, Franklin Lakes, NJ, USA). The available chemotherapeutic discs (Becton-Dickinson, Franklin Lakes, NJ, USA) in Korea and their concentrations (μg $disc^{-1}$) used in the test were: norfloxacin (10), ciprofloxacin (5), oxolinic acid (2), kanamycin (30), ampicillin (10), oxytetracycline (30) and erythromycin (15). After 24 h of incubation at 22°C, diameters of the inhibition zone were measured.

2. 16S rDNA sequence analysis

Genomic DNA of the isolate was extracted using a genomic DNA extraction kit (Bioneer, Daedeok-gu, Daejeon, Korea) following the manufacturer's protocol. The DNA templates were amplified by a polymerase chain reaction (PCR) thermocycler (Gene Amp 9700, Perkin Elmer, Chicago, IL, USA) using PCR PreMix (Bioneer, Daedeok-gu, Daejeon, Korea) with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACT T-3′) (Pratten *et al.*, 2003). The PCR condition was as follows:

initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min, and extension at 72°C for 30 sec followed by final extension at 72°C for 10 min.

The reaction product was purified using PCR purification kits (SolGent, Songpa-gu, Seoul, Korea). The analysis of the sequences was performed using a genetic analyzer (ABI 3130 XL, Applied Biosystems, Foster city, CA, USA) at miGenome Research Institute (Kunsan National University, Korea).

3. Histopathology

Eight diseased fish were sacrificed for histology. Dissected tissues of liver, spleen, kidney, intestine and skin were fixed in 10% phosphate-buffered formalin for 24 h, dehydrated in an ethanol series and embedded in paraffin. Sections of 5 μm thickness were stained with Mayer's hematoxylin-eosin (H&E).

4. Experimental infection

For infection experiments, carp (mean length 16.5±0.6 cm; mean weight 81.6±2.9 g) were obtained from a commercial culture farm supplied with ground water. Fish had no history of unusual mortalities or abnormalities before transport to the lab. In order to adapt to laboratory condition, fish were maintained in 100 L aquaria supplied with dechlorinated tap water in a flow through system (dissolved oxygen 6.1～6.4 mg L^{-1}; nitrite 0.4～0.5 mg L^{-1}; pH 6.6～7.0) for 15 days. The tanks were aerated and the water temperature was maintained at 22°C. During the acclimation, these fish were fed twice a day with commercial carp feed (Woosung Aquafeed, Daejeon, Korea). Fish were starved for 24 h prior to the commencement of acclimation in order to standardize the dietary status of the fish.

Six groups (Group I, II, III, IV, V and VI) were used in the infectivity studies. The isolate was grown overnight on TSA medium at 22°C and cell suspensions were prepared in sterile saline (0.85%). Fish were intraperitoneally injected 0.1 mL $fish^{-1}$ with bacterial suspensions of 1×10^3 ～10^7 viable cells mL^{-1}, 10-fold difference between doses. The bacterial concentration was determined from the optical density at 600 nm. A control fish group was injected with 0.1 ml of 0.85% saline. Water temperature was maintained at 22 ± 0.5°C and pH 6.8～7.1. The cumulative mortality was recorded daily for 10 days after infection. Fish were not fed throughout the experimental period. Macroscopic alterations in the fish were recorded and dead fish were analyzed for the presence of infecting pathogen. These tests were performed in triplicate with a parallel group for sampling with 20 fish per group per dose.

Results

1. Clinical sings

Affected fish showed abdominal distention and ulcerative lesions with reddish foci on the skin. Gill samples from moribund fish revealed the presence of *Dactylogyrus* sp. although this parasite did not seem to be the major cause of death. No parasite was observed on the skin.

Internally, the affected fish showed abdominal dropsy, enteritis, slight liver congestion and enlarged spleen and kidney. Gram stained imprints of the spleen and kidney exhibited numerous small Gram-negative

bacteria.

2. Bacterial characterization and 16SrDNA sequence

Pure bacterial isolate obtained after 24 h incubation appeared as round punctate colonies on TSA medium. The isolate was Gram-negative, small-sized straight rods (0.6 μm × 0.9～1.3 μm) and motile. It was also cytochrome-oxidase and catalase positive (Table 1). The isolate caused hemolysis on sheep blood agar plates when incubated only at 30°C but not at 10°C. Using the API system, these bacterial isolate produced arginine dihydrolase, β-galactosidase, indole and lysine decarboxylase, but not H_2S or ornithine decarboxylase. It acidified glucose, mannitol and sucrose, but not salicin, sorbitol, inositol, arabinose or rahmnose. No ability in the isolate to degrade esculin or urea was observed. The isolate was found to belong to the group of *Aeromonas sobria* (99.9%). Antibiotic susceptibility test indicated that the isolate was sensitive to norfloxacin, ciprofloxacin, oxolinic acid and erythromycin but resistant to kanamycin, ampicillin and oxytetracycline (Table 2). In the analysis of the 16S rDNA sequence, the isolates showed 100% homology with *A. veronii* 457C(EU488696), WE08(EU7703006) and IH317(EU770297), and 99.9% with *A. veronii* WS-03(EU770309) and 798C(EU488694).

Table 1. Biochemical characteristics of isolated strain from Israeli carp, *Cyprinus carpio*

Characteristics	**Present isolate (DJ-2)**	***Aeromonas sobria* (ATCC 43979)**	***Aeromonas veronii* (ATCC 35623)**
Gram stain	-	-	-
Catalase	+	+	+
Cytochrome-oxidase	+	+	+
Motility	+	+	+
OF test	+	+	+
0/129 (150μg)	-	-	-
β-galactosidase	+	+	+
Arginine dehydrolase	+	+	-
Lysin decarboxylase	+	+	+
Ornithine decarboxylase	-	-	+
Citrate utilization	+	+	+
H_2S production	-	-	-
Urease production	-	-	-
Trytophan deaminase	-	+	+
Indole production	+	+	+
VP reaction	+	+	+
Glycerol	+	+	+
Erythritol	-	-	-

D-Arabinose	-	-	-
L- Arabinose	-	-	-
Ribose	+	+	+
D-Xylose	-	-	-
L-Xylose	-	-	-
Salicin	-	-	-
Esculin	-	-	-
Adonitol	-	-	-
β-Methyl-xyloside	-	-	-
Galactose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
D-Glucose	+	+	+
L-Sorbose	-	-	-
Dulcitol	-	-	-
α-Methyl-D-mannoside	-	-	-
α-Methyl-D-glucoside	+	+	+
N-Acetylglucosamine	+	+	+
Gelatin hydrolysis	+	+	+
Gas from glucose	+	+	+
Amygdalin	-	-	-
Cellobiose	-	+	-
Maltose	+	+	+
Lactose	-	-	-
Trehalose	+	+	+
Insulin	-	-	-
Melezitose	-	-	-
D-Raffinose	-	-	-
Amidon (starch)	+	+	+
Glycogen	+	+	+
Xylitol	-	-	-
β-gentobiose	-	-	-
D-Turanose	-	-	-
D-Lyxose	-	-	-
D-Tagatose	-	-	-
D-Fucose	-	-	-
D-Arabitol	-	-	-

L-Arabitol	-	-	-
Gluconate	+	+	+
2-Ketogluconate	-	-	-
5-Ketogluconate	-	-	-
Melibose	-	-	-
Sorbitol	-	-	-
Mannitol	+	+	+
Inositol	-	-	-
Rhamnose	-	-	-
Sucrose	+	+	+
McConkey agar	+	+	+
Growth at 37°C	+	+	-
Blood agar at 10°C /30°C	-/+	-/+	-/+

Table 2. Antibiotic susceptibility of the isolated strain from Israeli carp, *Cyprinus carpio,*
(S, sensitive; R, resistant)

Chemotherapeutics	Present isolate
Norfloxacin (10 μg)	S
Ciprofloxacin (5 μg)	S
Oxolinic acid (2 μg)	S
Erythromycin (15 μg)	S
Kanamycin (30 μg)	R
Oxytetracycline (30 μg)	R
Ampicillin (10 μg)	R

S, > 10 mm inhibition diameter; R, < 10 mm inhibition diameter

3. Histopathology

Pathological lesions were found in the liver, spleen, kidney, intestine and skin. In the liver, there were hepatocellular vacuolar degeneration and congestion in sinusoids but bacterial invasions into hepatocytes were not observed (Fig. 1A). In the pancreases, hemorrhage was observed and pancreatic cells underwent vacuolar-degeneration and atrophy (Fig. 1B). Pulps in the spleen were congested, accompanied by hemorrhage and the destruction of the sheathed tissue (Fig. 1C). Numerous bacterial invasions were found in the hemorrhagic splenic pulps. Kidney exhibited multiple hemorrhagic foci in the hematopoietic tissue with bacterial invasions, necrotized renal tubules and glomerular destructions (Fig. 1D). There was hemorrhage in sub-mucosal layer and sloughing of the epithelial lining in the intestine

(Fig. 1E). In the skin, hemorrhage with bacterial invasions into the subcutaneous muscle was observed and some muscle fibers were necrotized (Fig. 1F).

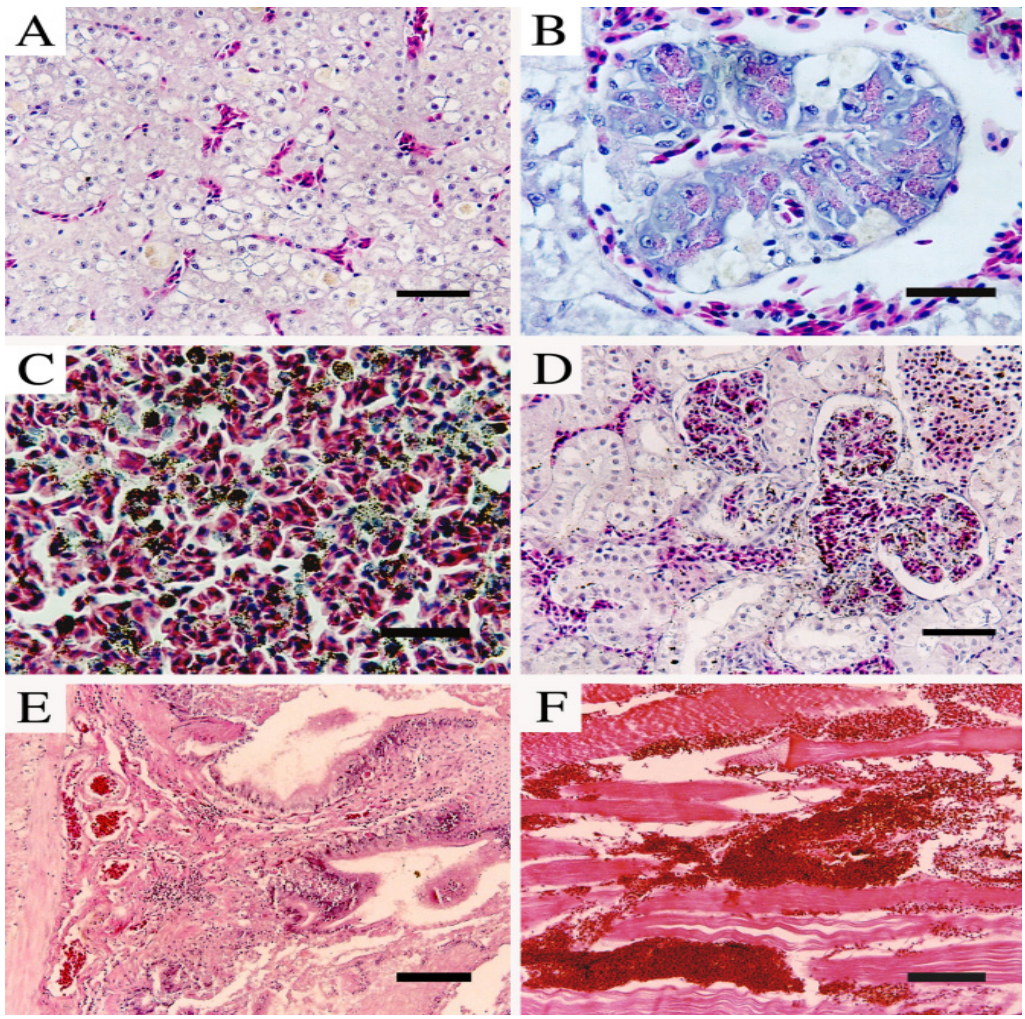

Fig. 1. Histopathology of diseased Israeli carp, *Cyprinus carpio*. (A) Liver showing congestion in sinusoids and degenerated hepatic cells. Scale bar = 50 μm. (B) Pancreatic cells displaying vacuolar degeneration and hemorrhage. Scale bar = 30 μm. (C) Spleen exhibiting congestion, necrosis and bacterial invasion. Scale bar = 30 μm. (D) Kidney displaying vacuolar degeneration of tubular epithelial cells, reduction of hematopoietic tissues and glomerular hemorrhage. Scale bar = 50 μm. (E) Intestine showing hemorrhage in sub-mucosa layer, and sloughing of epithelium mucosa. Scale bar = 100 μm. (F) Hemorrhage occurring within damaged muscles. Scale bar = 100 μm.

4. Infection experiment

All dead fish showed typical external and internal clinical signs comparable to those found in diseased fish on the farm. A causative bacterial isolate could be reisolated from spleen and kidney of experimentally infected fish. Injection with bacterial dose of 10^3 or 10^4 CFU $fish^{-1}$ induced neither visible sings nor any mortality (Table 3). When an infectious dose of 10^5 CFU $fish^{-1}$ was injected the mortality was 16.7% within 10 days. In the injection group with 10^6 CFU $fish^{-1}$, the mortality reached up to 65.0% within 9 days, whereas all fish died within 7 days when injected with 10^7 CFU $fish^{-1}$. The control group did not show any abnormal clinical signs or mortality during the experiment.

Table 3 Cumulative mortality of Israeli carp, *Cyprinus carpio,* infected with the isolated strain

Infectious dose (CFU fish-1)	Cumulative mortality (%)	Post-infection days
Group I (0.85% saline)	0	10
Group II (10^3)	0	10
Group III (10^4)	0	10
Group IV (10^5)	16.7	10
Group V (10^6)	65.0	9
Group VI (10^7)	100	7

Discussion

It was attempted to identify the cause of massive mortality occurred in the farmed Israeli carp. The causative bacterium was identified as *A. sobria* using biochemical experiment whereas in 16Sr DNA sequencing analysis, it was identified as *A. veronii*. Although commercial systems such as API system still refer to *A. veronii* as *A. sobria, A. sobria* is actually genetically *A. veronii* (Buller, 2003). Therefore, we could identify the isolate from diseased fish as *A. veronii*.

Both *A. hydrophila* and *A. veronii* are associated with hemorrhagic septicemia in fresh water fishes (Paniagua *et al.*, 1990; Lee *et al.*, 1993; Inglis *et al.*, 1994; Cipriano, 2001; 김 등, 2006). Gross symptoms in cyprinids infected with *A. hydrophila* include dermal ulceration, fin rot, ocular ulceration, hemorrhagic septicemia and scale protrusion (전, 1985; Cipriano, 2001; Park & Oh, 2008). Diseased carp examined in this study exhibited dropsy symptoms and cutaneous lesions (erythro-dermatitis). Internal examination revealed clear ascites, liver congestion, enlargement of spleen and kidney and enteritis, which were very similar to the signs found in fishes suffering from motile aeromonad infections (Ausin & Austin, 1987; Inglis *et al.*, 1994; Kim *et al.*, 2006; Park & Oh, 2008).

In histopathological examinations, the most prominent pathological changes were noted in the spleen and kidney. Necrotic lesions occurred in theses organs were accompanied by edema and hemorrhage. Bacterial invasion was commonly observed in lesions of the organs. *A. hydrophila* infection is known to cause

hemorrhage and destruction of sheathed tissues in spleen, and renal tubular necrosis and hemorrhage in the kidney (Miyazaki & Jo, 1985; Miyazaki *et al.*, 2001; 김 등, 2006; 박 & 오, 2008). Thus, although superficial sings of the disease in the present carp are similar to *A. hydrophila* infection, we could not isolate the *A. hydrophila* from the lesion. The lesions of the spleen and kidney were thus not considered to be the primary pathological changes caused at least by *A. hydrophila* invasion.

Injection of the bacterial isolates into healthy fish resulted in mortality and developed symptoms comparable to those found in diseased fish on the farm. The API system and 16S rDNA sequencing verified that the reisolated bacteria from experimentally infected fish were the same one as used for infection. The reisolation of the isolates from experimentally infected fish presenting sings of disease fulfilled Koch's postulates. Interestingly, in the infection tests, the concentration of 10^6 or 10^7 CFU ml^{-1} caused acute mortality of the fish. However, the inoculation with lower levels of 10^3, 10^4 or 10^5 CFU ml^{-1} neither led to bacterial growth within the fish nor caused high mortality, although the isolates displayed hemolytic activity on sheep blood agar plates. These results indicate that the isolates by themselves have weak pathogenicity, and its pathogenic properties may be potentiated by environmental factors in fish farms (e.g. temperature, pH, higher organic compounds) as proposed by Park and Yu (2008).

In the present study, *Dactylogyrus* sp. was occasionally observed in some affected fish on the farm (data not shown). However, both gross and histopathological signs were different from those observable parasitic diseases. Therefore, the parasite did not seem to be the main factor that caused mass mortality in the carp farm.

In cyprinids, a very common feature of motile *Aeromonas* sp. infections is the high prevalence of disease outbreaks in spring, when many parameters such as water temperature, metabolic rate and water organic concentration are on the ascending limb (전, 1985; Inglis *et al.*, 1994; 김 등, 2006; 박 & 오, 2008; Park & Yu, 2008). The motile aeromonads' presence, by itself, is not indicative of disease and, consequently, stress is often considered to be the contributing factor in disease occurrences caused by these bacteria (Trust *et al.*, 1974; Cipriano, 2001). Several researchers suggested that the aeromonad diseases are closely associated with high stocking densities under intensive cultures, water temperature change, rough handling, low dissolved oxygen and rough weather condition. (Roberts, 1993; Eisa *et al.*, 1994; Ventura and Grizzle, 1987; Aoki, 1999; Cipriano, 2001; Wahli *et al.*, 2005; Park and Yu, 2008). In Korea, because carp are usually cultured in pond culture system, it is difficult to regulate water changes or stocking densities with any ease. In this study, we did not analyze the relationship between outbreaks of disease in the farm and the environmental parameters such as water temperature and overcrowding. However, those factors could have stressed the fish and rendered them more sensitive to *A. veronii* infection.

A. veronii isolated from carp in this study was sensitive to available antibiotics for aquaculture such as norfloxacin, ciprofloxacin, oxolinic acid and erythromycin. These antibiotics can be considered efficacious in controlling the *A. veronii* infections. However, all of

theses antibiotics are widely and often improperly used in Korean fish farms owing to good efficacy against various bacterial diseases. There is thus a likelihood of resistance development in the future in *A. veronii* to antibiotics analyzed in the present study.

A. veronii has not been previously reported to be pathogen for Israeli carp in Korean fish farm. Therefore, this is the first report on the occurrence of *A. veronii* infection in cultured Israeli carp in Korea.

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