Aeromonas sobria infection in farmed mud loach (*Misgurnus mizolepis*) in Korea, a bacteriological survey

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Summary

A disease outbreak occurred in June 2012 among mud loach cultured on pond farms in Jangseong-gun, Jeollanam-do, Korea. Mortality rates reached up to 1.2% in the farm per day. Typical clinical signs were bleeding ulcer at the middle portion of head and haemorrhagic erosion of the operculum. Based on biochemical characteristics, the causative bacterium isolated from diseased fish was identified as *Aeromonas sobria*. The isolate expressed two haemolytic genes, aerolysin (*sob*) and haemolysin (*asa1*) genes. Histopathologically, liver showed hepatocellular vacuolar degeneration and congestion in sinusoids. The spleen exhibited necrotized splenocytes and haemorrhagic pulps. In the kidney, glomerular destruction, renal tubular necrosis and haemorrhage were observed. Experimental infection (infectious dose of 10^6, 10^7, and 10^8 cfu fish^{-1}) of healthy mud loach with the isolate resulted in the development of clinical signs similar to those seen in the farm. By injection with an infectious dose of 10^6 cfu fish^{-1}, the mortality rate was 10.3% within 7 days post infection. A mortality rate of 60.9% was reached within 2 days when an infectious dose of 10^7 cfu fish^{-1} was used. Otherwise, all fish died within 1 day when injected with 10^8 cfu fish^{-1}. The results demonstrated that *A. sobria* is involved in the morbidity and mortality of the farmed mud loach.

Key words: *Aeromonas sobria*, Haemolysin, *Misgurnus mizolepis*, Mud loach

Introduction

Aeromonas species are ubiquitous gram-negative, motile, rod-shaped bacteria which can be commonly isolated from fresh water ponds and are also normal inhabitant of the gastrointestinal tract of fish (Fraire, 1978; Inglis *et al*., 1993; Sersy *et al*., 1996; Yu *et al*., 2010; Modarres Mousavi Behbahani *et al*., 2014). Infected fish show skin ulcers, tail rot, fin erosion, hemodiapedesis and hemorrhagic septicemia. The organisms often invade skin wounds, commonly with the water molds, or ectoparasites (Noga, 1986; Noga, 2010). In particular, *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, *A. salmonicida*, *A. jandaei*, *A. bestiarum* and *A. veronii* form the most predominant clinical isolates that are typically associated with diseased fishes (Toranzo *et al*., 1989; Esteve *et al*., 1993; Kozińska *et al*. 2002; Wahli *et al*., 2005; Austin and Austin, 2007; Noga, 2010; Yu *et al*., 2010; Yi *et al*., 2012). Several researchers suggested that infections caused by *Aeromonas* spp. are closely associated with overcrowding under intensive cultures, water temperature change, abrasive handling, hypoxia, organic pollution and rough weather condition (Inglis *et al*., 1993; Eisa *et al*., 1994; Cipriano, 2001; Noga, 2010; Yu *et al*., 2010; Majtán *et al*., 2012). *Aeromonas sobria* has been recovered from the blood, faeces and wounds in humans (Daily *et al*., 1981; Filler *et al*., 2000; Igbinosa *et al*., 2012). The organisms have also been isolated from wild-spawning gizzard shad, *Dorosoma cepedianum*, farmed perch, *Perca fluviatilis* and mud loach, *Misgurnus mizolepis* (Toranzo *et al*., 1989; Wahli *et al*., 2005; Yu and Park, 2008). Moribund gizzard shad did not display any clinical signs of disease whereas perch and mud loach showed skin lesions and fin rot. The loaches, *Misgurnus* spp. are widely distributed throughout the streams, ditches, reservoirs and rice paddy fields in Korea, China and Japan (Kim, 1993; Kim *et al*., 1995, Ahn *et al*., 2010). Bacterial pathogens such as *Flavobacterium columnarae* (*Flexibacter columnaris*), *Staphylococcus epidermis*, *A. hydrophila*, *A. sobria* have been described in cultured loaches (Chowdhury and Wakabayashi, 1991; Park *et al*., 2002; Yu and Park, 2008; Jun *et al*., 2010). Loach is usually cultured in ponds which suffer from difficulties in controlling water quality and stocking densities. Thus, such conditions can easily stress the fish making them more susceptible to various bacterial infections.

The aim of the present study was to identify the causative agent of mud loach losses in a pond farm and estimate the pathogenic capacity of this agent in terms of Koch's postulates. In addition, pathological lesions in the affected fish and biochemical and genetic properties of the isolated bacterium were described.

Materials and Methods

Fish and microbiology

A disease outbreak occurred in June 2012 in a mud loach farm located in Jangseong-gun, Jeollanam-do, Korea. When the disease outbreak occurred, water temperature in the farm ranged from 23°C to 25°C. Fish (mean length 11.5 ± 1.5 cm; mean weight 8.6 ± 1.1 g) were reared at a density of 15 kg. Mortality rate per day was estimated to be 1.2% of the total fish in the farm. Salt bathing and oxytetracycline administration were adopted for 7 days to prevent the mortality but the treatments were not effective. Skin and gill samples from 20 anesthetized fish with 2-propoxyethanol (40 mg L^{-1}) 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, spleen and brain were aseptically streaked on tryptic soy agar (TSA) (Becton-Dickinson, Franklin Lakes, NJ, US). After incubation at 25°C for 48 h, dominant colonies on TSA agar plates were re-streaked onto TSA to obtain pure isolates. The isolate was identified using API 20E and 50CHE system (BioMérieux, Durham, NC, US) following 48 h incubation at 25°C. Interpretation of the API results was carried out in accordance with the manufacturer's manual. The isolate was streaked onto sheep blood agar plates and then incubated at 10°C and 30°C for hemolytic activity. Antibiotic susceptibility was determined by Kirby-Bauer disk diffusion method (Bauer *et al*., 1966) on Müeller-Hinton agar (Becton-Dickinson, Franklin Lakes, NJ, US). Chemotherapeutic discs (Becton-Dickinson, Franklin Lakes, NJ, US) and their concentrations (µg $disc^{-1}$) used in the test were: ampicillin (10), doxycycline (30), erythromycin (15), gentamycin (10), kanamycin (30), nalidixic acid (30), and tetracycline (30). After 48 h of incubation at 25°C, diameters of the inhibition zone were measured. Two ATCC collections, *Aeromonas sobria* (ATCC43979) and *A. hydrophila* (ATCC700183), and one isolate *A. sobria* (DJ-1) from kidney in dead pond loach (*Misgurnus anguillicaudatus*) were used as controls.

Histopathology

Nine moribund mud loaches were sacrificed for histopathology. The fish were anaesthetized with 2-phenoxyethanol and gills, operculum, liver, spleen, kidney and head lesions were removed and fixed in 10% neutral buffered formalin for 24 h. The tissues were dehydrated in an ethanol series and embedded in paraffin. Sections of 5 µm thickness were stained with Mayer's haematoxylin and eosin (H&E).

Survival ability of the isolate in the fish serum

Survival ability of the isolate in serum was measured with some modifications from Leung *et al*. (1994) and Won and Park (2008). Briefly, blood was sampled from the caudal vein in nine healthy mud loach using a 1 ml disposable syringe and centrifuged with 5,000 rpm for 5 min. The supernatant was used as the serum sample. The isolate suspension was mixed with the same volume of fish serum in 96 well plates (flat bottom). After incubation for 0, 1, 3, 6 and 12 h at 25°C, the absorbance at 600 nm was measured. *Aeromonas sobria* (ATCC43979), *A. hydrophila* (ATCC700183), *A. veronii* (ATCC35623) and DJ-1 were used as controls.

PCR detection of hemolytic genes

Genomic DNA of the isolate was extracted using a commercial genomic DNA extraction kit (Bioneer, Daejeon, Korea). As specific primers of *Aeromonas sobria* aerolysin genes (288 bp), sobf (5′-GCG ACC AAC TAC ACC GAC CTG-3′) and sobb (5′-GGA CTT GAT GAG GGC AAC CCG-3′) designed by Filler *et al*. (2000) were used. The PCR conditions were as follows: initial denaturation 94°C for 5 min, 20 cycles of denaturation at 94°C/1 min, annealing at 57°C/1 min, extension at 72°C/1 min followed by final extension at 72°C/5 min. For amplification of the hemolysine gene (*asa1*) fragment (294 bp), forward primer 5′-TAA AGG GAA ATA ATG ACG GCG-3′ and the reverse primer 5′-GGC TGT AGG TAT CGG TTT TCG-3′ was used (Das *et al*., 2013). Polymerase chain reaction products were analyzed by gel electrophoresis in 1.5% (w/v) agarose gel and viewed, after ethidium bromide staining, under UV light. ATCC43979, ATCC700183 and DJ-1 served as controls.

Haemolysin assay

Haemolysin assay was carried out by the method of Wahli *et al*. (2005). Mud loach blood was washed with sterile phosphate-buffered saline (PBS), pH = 7.4 until the supernatant became clear. Four hundred microliters of packed erythrocytes were then diluted in 50 ml of PBS to obtain a 0.8% suspension of erythrocytes. The isolate (1×10^9 cfu ml^{-1}) was incubated at 25°C for 24 h in tryptic soy broth with gentle agitation. The culture supernatant was collected by centrifugation and filtered using 0.2 µm pore-size membranes. Two-fold serial dilutions of the filtrate were made with PBS. One hundred µL of diluted filtrate were combined with 100 µL of a 0.8% suspension of the mud loach erythrocytes in 96 well plates (flat bottom). The plates were incubated at 25°C for 3 h (without shaking) and visually examined for haemolysis. For positive controls, PBS containing 0.1% sodium dodecyl sulphate was used. Haemolytic activity was expressed as the highest dilution of filtrate producing complete haemolysis. The test was performed in triplicate.

Experimental challenge

Mud loach (mean length 12.3 ± 1.2, mean weight 9.4 ± 0.7 g) that had been grown with ground water were obtained from a commercial mud loach farm. Fish had no history of unusual mortalities or abnormalities before transport to the lab. To adapt to lab conditions, fish were maintained in a 1 ton tank supplied with aerated dechlorinated tap water (dissolved oxygen 6.3~6.5 mg L^{-1}; nitrite 0.3~0.4; pH 6.5~6.9) for 30 days. The water

temperature was maintained at 25°C. During the acclimation, these fish were fed once a day with commercial mud loach feed (Woosung Aquafeed, Korea). Fish were starved for 24 h prior to the commencement of acclimation in order to standardize the dietary status of the fish. The bacterial isolate were grown for 48 h on TSA at 25°C and cell suspensions were then prepared in PBS (pH 7.4). In order to determine the effective bacterial dose for experimental infection, groups of 20 mud loach each were injected intraperitoneally (i.p.) with 100 µL of PBS containing 1×10^6, 1×10^7 and 1×10^8 colony forming units (cfu) of the isolate. A control fish group was injected with 100 µL of PBS. Water temperature, pH and dissolved oxygen during the infection test were 24~25°C, 6.7~6.9 and 6.1~6.6 mg L^{-1}. The course of mortality was recorded daily for 7 days after infection. Fish were not fed throughout the experimental period. Macroscopic alterations in the fish were recorded and dead fish were analyzed for presence of infecting pathogen.

Results

Naturally infected mud loach: clinical signs and bacterial isolation

Affected fish mainly showed petechiae at the lateral portion of body, hyperemia at the base of fins, bleeding ulcer at the middle portion of head and haemorrhagic erosion of operculum (Fig. 1). Grey ulcers were also observed in some individuals. Mucus samples from the skin and gill did not reveal the presence of any parasites. On the fourth day after the fish arrival on the farm, clinical signs began to appear. Internal necropsy showed pale coloration of liver and enlarged haemorrhagic kidney and spleen in the moribund fish. Gram stained imprints of the kidney and spleen exhibited numerous small gram-negative bacteria except for brain. Bacterial isolation was not successful in the brain but the isolates were obtained from the kidney and spleen of all diseased fish. The presence of these bacteria was confirmed by the appearance of round punctuate and pale yellow colonies on TSA after 48 h incubation at 25°C. The isolate was gram-negative, small-sized straight rods and motile. In the API test systems, the isolate produced β-galactosidase, arginine dihydrolase and lysine decarboxylase, but not H_2S and ornithine decarboxylase (Table 1). It acidified glucose, mannitol and sucrose, but not inositol, sorbitol, rhamnose, arabinose and salicin. No ability in the isolate to degrade urea and esculin was observed. The isolate was identified as *Aeromonas sobria* (99.3%). The isolate caused β-haemolysis on sheep blood agar plates when incubated at 30°C but not at 10°C. Among all antibiotics, the isolate was susceptible to erythromycin, gentamicin, kanamycin and nalidixic acid.

Table 1: Comparison of biochemical characteristics among the present study (LBH) and the reference strains, *A. sobria* (DJ-1, ATCC 43979) and *A. hydrophila* (ATCC 700183)

Characteristics	Present isolate	*A. sobria*		*A. hydrophila*
	LBH	DJ-1	ATCC 43979	ATCC 700183
Gram stain	-	-	-	-
Catalase	+	+	+	+
Oxidase	+	+	+	+
Motility	+	+	+	+
Fermentation of glucose	+	+	+	+
0/129 (150 µg)	-	-	-	-
β-Galactosidse	+	+	+	+
Arginine dehydrolase	+	+	+	+
Lysine decarboxylase	+	+	+	+
Ornithine decarboxylase	-	-	-	-
Citrate utilization	+	+	+	+
H_2S production	-	-	-	-
Urease production	-	-	-	-
Tryptophan deaminase	+	-	+	+
Indole production	+	+	+	+
Acetone production	+	+	+	+
Gelatinase	+	+	+	+
Glycerol	+	+	+	+
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	+
D-Ribose	+	+	+	+
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
D-Adonitol	-	-	-	-
Methyl-β-xyloside	-	-	-	-
D-Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	+	+
L-Sorbose	-	-	-	-

L-Rhamnose	-	-	-	+
Dulcitol	-	-	-	-
Inositol	-	-	-	-
D-Mannitol	+	+	+	+
D-Sorbitol	-	-	-	-
α-Methyl-D-mannoside	-	-	-	-
α-Methyl-D-glucoside	-	-	+	+
N-Acetylglucosamine	-	-	-	+
Arbutin	-	-	-	+
Esculin ferric acid	-	-	-	+
Salicin	-	-	-	+
D-cellobiose	+	-	+	-
D-Maltose	+	+	+	+
D-Lactose	-	-	-	-
D-Melibose	-	-	-	-
D-Saccharose	+	+	+	+
D-Trehalose	+	+	+	+
Inulin	-	-	-	-
D-Melezitose	-	-	-	-
D-Raffinose	-	-	-	-
Amidon	+	+	+	+
Glycogen	+	+	+	+
Xylitol	-	-	-	-
Gentiobiose	+	-	-	-
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Potassium gluconate	+	+	+	+
Potassium 2-ketogluconate	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-
Sheep blood agar at 10°C	-	-	-	+
Sheep blood agar at 30°C	+	+	+	+
Susceptibility to				
Ampicillin (10 µg)	R	R	S	R
Doxycycline (30 µg)	R	S	S	S
Erythromycin (15 µg)	S	S	S	I
Gentamycin (10 µg)	S	S	S	S
Kanamycin (30 µg)	S	S	S	S
Nalidixic acid (30 µg)	S	S	S	S
Tetracycline (30 µg)	R	S	S	S

+ Positive, - Negative, R: Resistance, I: Intermediate, and S: Sensitive

Fig. 1: Diseased mud loach displays bleeding ulcerations at the operculum (A) and middle of the head (B) and haemorrhages at the lateral portion of body and basal portion of fins (C)

Haemolytic genes and haemolysin assay

The isolated strains, *A. sobria* (ATCC43979) and DJ-1 generated 288 bp and 294 bp fragments for aerolysin gene and haemolysin gene, respectively, whereas *A. hydrophila* (ATCC700183) did not (Fig. 2). In the haemolysin assay, the isolate did not show any haemolytic activity to mud loach erythrocytes.

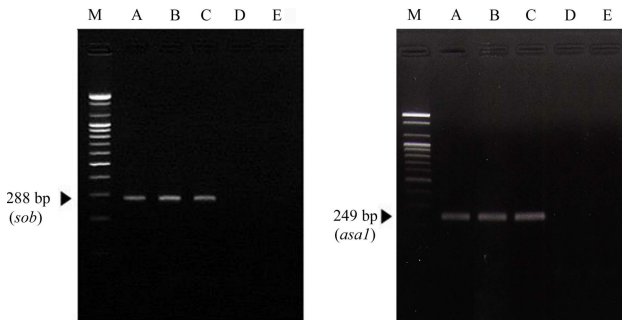

Fig. 2: Amplification of 288 bp and 249 bp by PCR for detection of *Aeromonas sobria* aerolysine (*sob*) and haemolysine (*asa1*) genes, respectively. Lane M: Maker, A: LBH (isolate), B: DJ-1 (*Aeromonas sobria*), C: ATCC43979 (*A. sobria*), D: ATCC700183 (*A. hydrophila*), and E: negative control (without template DNA)

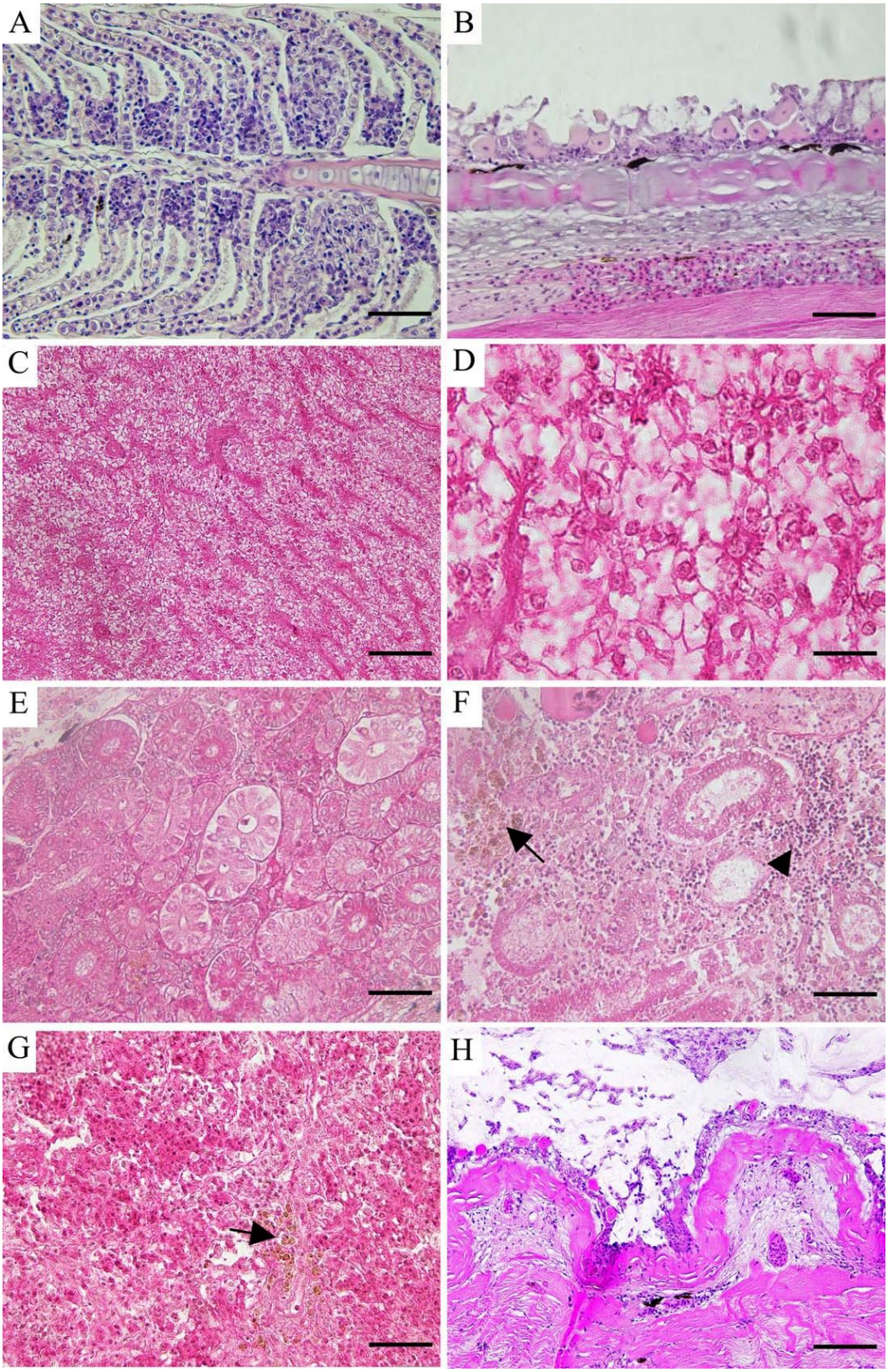

Fig. 3: Histopathology of diseased mud loach, *Misgurnus mizolepis*. (A) Gill showing epithelial lifting and hyperplasia of epithelial cells at the basal portions. (B) Operculum showing damaged epithelial and club cells, and haemorrhage in the underlying dermal loose connective tissues. (C) Liver displaying hepatocellular vacuolar degeneration and congestion in sinusoids. (D) Hepatic cells presented karyorrhexis, karyolysis, karyopyknosis and hypochromatosis of the nuclear membrane. (E) Kidney showing renal parenchymal haemorrhages, tubularr necrosis and peritubular haemorrhages. (F) Interstitial lymphocyte infiltration (arrow head) and haemosiderin granules (arrow) exhibiting in the parenchyma of the kidney. (G) Spleen showing haemorrhage, deposition of haemosiderin (arrow) and destruction of sheathed tissue. (H) Raised lesion on the head displaying epidermal exfoliation and haemorrhages and edema in the underlying dermal loose connective tissue, (scale bars, 100 µm)

Table 2: Experimental infections of the isolate (LBH) from mud loach, *Misgurnus mizolepis*

Control/infectious dose (cfu/fish)	Cumulative mortality (%)	Post-infection days
Group I (10^6)	10.3	7
Group II (10^7)	60.9	2
Group III (10^8)	100	1
Group IV(control)	0	0

Histopathology

Histopathological lesions were observed in gill, operculum, liver, spleen and kidney of affected fish. The lesions observed in the gill consisted of epithelial lifting which is characterized by a desquamation of the outer layer of the lamellar epithelium (Fig. 3A). Hyperplasia was present with an increased number of epithelial cells at the basal portions. The interlamellar epithelium showed some nuclei exhibiting marginal hyper-chromatosis. The operculum exhibited damaged epithelial and club cells and hemorrhage was revealed in the underlying loose dermal connective tissues (Fig. 3B). In the liver, there were hepatocellular vacuolar degeneration and congestion in sinusoids (Fig. 3C). Necrotic cells showed karyorrhexis, karyolysis, karyopyknosis and hypochromatosis of the nuclear membrane (Fig. 3D). Extensive haemorrhage was observed in the parenchyma and peritubular region (Fig. 3E). Necrotic renal tubular epithelial cells showed karyorrhexis, karyopyknosis and hypochromatosis of the nuclear membrane. The interstitial lymphocyte in-filtration and haemosiderin granules were also evident (Fig. 3F). Pulps in the spleen were congested, accompanied by haemorrhages and the destruction of sheathed tissue (Fig. 3G). Deposition of haemosiderin was also observed. The swollen lesion on the head showed epidermal exfoliation and haemorrhages and edema in the underlying loose dermal connective tissue (Fig. 3H).

Survival ability of the isolate in the fish serum

The survival ability in fish serum was stronger in the isolate and DJ-1 after incubation for 12 h than in ATCC43979, ATCC700183 and ATCC35623 (Fig. 4).

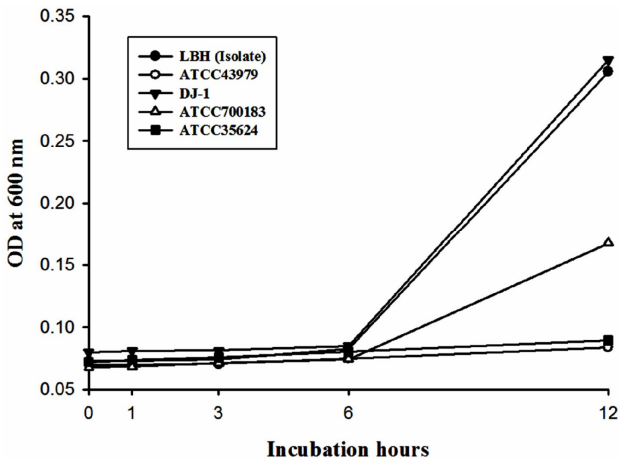

Fig. 4: Survival of isolate (LBH), *Aeromonas sobria* strains (ATCC43979, DJ-1), *A. hydrophila* (ATCC700183), *A. veronii* (ATCC35624) in sera of the mud loach, *Misgurnus mizolepis*

Experimental challenge

All dead fish from intraperitoneal injection showed typical external and internal clinical signs comparable to those found in diseased fish in the farm. A causative bacterial isolate could be reisolated from liver, spleen and kidney of all challenged fish. In injection with an infectious dose of 10^6 cfu $fish^{-1}$ (group I), the mortality rate was 10.3% within 7 days post infection (Table 2). A mortality rate of 60.9% was reached within 2 days when an infectious dose of 10^7 cfu $fish^{-1}$ (group II) was used. Otherwise, all fish died within 1 day when injected with 10^8 cfu $fish^{-1}$ (group III). Control group did not show any abnormal clinical signs or mortality during the experiment. The LD_{50} value was estimated to be 2.1×10^7 cfu $fish^{-1}$.

Discussion

Motile *Aeromonas* spp. are well recognized as significant causes of disease and mortality in fish (Miyazaki and Jo, 1985; Toranzo *et al*., 1989; Inglis *et al*., 1993; Lee *et al*., 1993; Cipriano, 2001; Miyazaki *et al*., 2001; Wahli *et al*., 2005; Austin and Austin, 2007; Yu *et al*., 2010).

The biochemical, morphological and physiological characteristics of the bacterial isolate from diseased mud loach was almost identical to the reference strain *A. sobria* (ATCC 43979) and DJ-1 which utilize glucose, mannitol and sucrose and haemolyse on blood plate agar at 30°C, but not salicin, esculin and arabinose. These findings are thus consistent with the features of *A. sobria* (Inglis *et al*., 1993; Austin and Austin, 2007).

The two haemolytic toxins (aerolysin and haemo-lysin) have been described in *A. sobria* (Filler *et al*., 2000; Das *et al*., 2013). Filler *et al*. (2000) reported that *A. sobria* strains positive for cytotoxin production possessed aerolysine genes (*sob*). Das *et al*. (2013) suggested that haemolysin genes (*asa1*) seem to be predominant virulence factor of motile *A. sobria* associated with *Aeromonas* septicaemia. In this study, we found that the isolated bacteria expressed both *sob* and *asa1* genes, and exhibited haemolytic activity on blood agar plates. It appears that those genes in the isolate are responsible for the virulence factors for cell lysis (haemolysis), causing the fish septicemia. *Aeromonas sobria* contains a cell-bound β-toxin because it has β-haemolytic activity (Sersy *et al*., 1996). Our isolate showed β-haemolytic activity on blood agar plate, so the isolate might have contained a cell-bound haemolysin (β-toxin). Arimi *et al*. (1990) postulated the existence of a cell-bound haemolytic factor that is released intra-cellularly. Haemolytic activity was not detected in the

bacterial supernatant of the isolate tested by the microplate assays. It is presumed that the isolate possess a cell-associated haemolysin.

Serum resistance has been suggested to be a good indicator for virulence in bacteria (Leung *et al*., 1994; Won and Park, 2008). In the present study, the survivability in fish serum was tested and found to be related to the pathogenicity of the isolate. Similarly, *Flavobacterium psychrophilum* in trout serum and *Vibrio harveyi* in flounder serum showed higher survivability (Wiklund and Dalsgaard, 2002; Won and Park, 2008). From this result, we suggest that the ability of *A. sobria* to survive and grow in the mud loach serum plays a major role in septicemia by this organism.

Gross symptoms in fish infected with an *Aeromonas* spp. include dermal ulceration, fin rot, ocular ulceration and haemorrhagic septicemia (Cipriano, 2001; Miyazaki *et al*., 2001; Wahli *et al*., 2005; Yu *et al*., 2010). Diseased mud loaches examined in the present study displayed focal haemorrhage and ulceration on skin (erythro-dermatitis), and operculum erosion. Yu and Park (2008) reported that external signs of *A. sobria* infected mud loach were limited to haemorrhage of skin and fins. In this study, however, some affected fish exhibited haemorrhagic ulceration on the middle of head. It appears that *A. sobria* produces different symptoms in mud loach.

Internal examination revealed liver congestion and enlargement of spleen and kidney. The common gross lesions observed in the diseased fish were in accordance with the findings of earlier studies (Miyazaki and Jo, 1985; Austin and Austin, 1987; Inglis *et al*., 1993; Lee *et al*., 1993; Miyazaki *et al*., 2001; Wahli *et al*., 2005; Yu *et al*., 2010; Noor El Deen *et al*., 2014).

In histological examination, the most prominent pathological changes were noted in the liver, spleen and kidney. In various fish species infected with *Aeromonas* spp., degeneration of the hepatic cells and haemorrhage and destruction of haematopoietic tissues are observed (Miyazaki and Jo, 1985; Lee and Kim, 1993; Miyazaki *et al*., 2001; Yu *et al*., 2010; Noor El Deen *et al*., 2014). *A. hydrophila* infection is also known to cause haemorrhage and destruction of sheathed tissues in spleen, and renal tubular necrosis and haemorrhage in the kidney (Miyazaki and Jo, 1985; Miyazaki *et al*., 2001; Noor El Deen *et al*., 2014). Haemosiderosis is attributed to β-haemolysin secreted by the bacteria that cause haemolysis inside the fish body followed by deposition of haemosiderin (Miyazki and Kaige, 1985; Noor El Deen *et al*., 2014). In this study, we observed haemosiderin granules in the spleen and kidney. This finding suggests that the haemosiderosis is induced by haemolysis because the isolate has haemolytic activity. Clinical signs of the diseased mud loaches are similar to *A. hydrophila* infection in various fishes but we could not isolate the *A. hydrophila* from the lesions. Hence, the lesions of the liver, spleen and kidney were not considered to be the primary pathological changes caused by *A. hydrophila* invasion.

Injection of the isolated strain into healthy fish resulted in high mortality with accompanying symptoms, comparable to those found in diseased fish on the farm. The API test verified that the reisolated bacterium from experimentally infected fish was the same one as used for infection, thereby satisfying Koch's postulates. Yu and Park (2008) reported that *A. sobria* shows weak pathogenicity to mud loach. Likewise, in this study, the concentration of 1×10^6 cfu fish^{-1} did not cause profound mortality, although the isolate displayed haemolytic activity on blood agar plate. From these results, we postulate that the isolate by itself has weak pathogenicity, and its pathogenic properties may be potentiated by environmental factors in fish farms. Several researchers suggested that the infections caused by *Aeromonas* spp. are closely related with overcrowding, rough handling, undesirable weather condition, poor nutritional status and non-bacterial pathogenic infections (Ventura and Grizzle, 1987; Eisa *et al*., 1994; Aoki, 1999; Cipriano, 2001; Wahli *et al*., 2005; Yu and Park, 2008; Yu *et al*., 2010; Majtán *et al*., 2012). Additionally, fish which are under poor environmental conditions such as high nitrite levels, low dissolved oxygen, transfer of fish, or high levels of carbon dioxide are more susceptible to infections caused by *Aeromonas* spp. (Majtán *et al*., 2012). In Korea, it is difficult to control water quality or stocking densities because mud loaches are usually cultured in pond culture system. However, it is not possible to conclude that poor environmental conditions caused the present *A. sobria* infection in the mud loaches because we did not evaluate the relationship between outbreaks of disease in the farm and the environmental parameters such as overcrowding and water conditions. Iversen *et al*. (2005) reported that transportation often involves traumatic events (capture, transport, loading, unloading and stocking) which may induce severe stress factors to fish. The disease outbreak occurred on the fourth day after mud loach arrival at the farm. Thus, we speculate that transportation stress rendered them more susceptible to *A. sobria* infection.

The isolate was sensitive to erythromycin, gentamicin, kanamycin and nalidixic acid whereas it was resistant to ampicillin, doxycycline and tetracycline. According to the results of our study, aminoglycosides (gentamycin and kanamycin) or macrolide (erythro-mycin) could be recommended to treat fish infected with *A. sobria*. However, due to the risks for development of resistance for antibiotics, we recommend that farmers establish good farm management in order to prevent *A. sobria* infection. In this study, we conclude that *A. sobria* is involved in the morbidity and mortality of the cultured mud loach and that this bacterium has the potential to act as a pathogen of mud loach.

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