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Mass Mortality of Doctor Fish (*Garra rufa obtusa*) Caused by *Citrobacter freundii* Infection

Gun-Wook Baeck, Ji-Hyung Kim*, Casiano Choresca Jr.*, Dennis K. Gomez*, Sang-Phil Shin*, Jee-Eun Han* and Se-Chang Park*[1]

Department of Marine Biology and Aquaculture, Gyeongsang National University, Tongyeong 650-160, Korea
**Laboratory of Aquatic Animal Medicine and BK21 Program for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*

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Abstract : In this paper, we described a case of mass mortality of doctor fish from a private fish hatchery farm in Korea with a history of abnormal swimming behavior, diffuse bleeding on the skin and fins and sudden death caused by fish pathogenic bacteria, *Citrobacter freundii*. Twelve moribund fish fingerling samples were submitted to College of Veterinary Medicine, Seoul National University in October 2008 for diagnostic examination. Diagnostic results showed that the morphological and biochemical properties of the bacteria isolated from the moribund fish were *C. freundii*. The remaining diseased fish from the hatchery farm were given treatment based on our recommendation and successfully recovered.

Key words **:** mass mortality, doctor fish, *Citrobacter freundii,* treatment.

Introduction

One of the most unusual alternative treatments of human skin disorder is the so-called 'Doctor fish of Kangal' in the Central Anatolia region of Turkey. This alternative treatment was first mentioned in 1989 (17), however the details of the treatment were published recently by O¨zcelik et al. (10). Since there have been many unscientific and misleading names for this kind of skin disorder therapy, Grassberger and Hoch (5) suggest the term 'ichthyotherapy', in accordance with other biotherapy concepts such as maggot therapy (use of sterile fly larvae), hirudotherapy (use of leeches) and apitherapy (use of bee venom). Recently, this ichthyotherapy was adopted and commonly commercialized worldwide including Japan, Croatia, China, Netherlands, Malaysia, Singapore and Korea.

Previous research on the doctor fish reported two different types of fish, which are members of the Cyprinidae family and are adapted to live in hot waters (13). The *Cyprinion macrostomus macrostomus* (striker) has a terminal mouth, is 15 to 20 cm in length, and is covered with relatively large scales that have 6 to 8 irregularly arranged lateral spots of various sizes. The second fish, commonly called as doctor fish, *Garra (G.) rufa obtusa* (licker) has a crescent-shaped ventral mouth and a maximum length of 19 cm. Its body is also covered with large scales, and an immature fish lose their lateral spots during maturation (13). The distribution areas of *G. rufa obtusa* were reported in Iran, Iraq, Jordan and inland waters of Turkey including the Orontes, Euphrates and Tigris river basins and some coastal rivers in southern Turkey and northern Syria (4). Recently, this fish was legally protected from commercial exploitation in Turkey due to concerns of over-harvesting for exportation. At the same time, *G. rufa obtusa* were cultured successfully in fish farm and can possibly provide enough fish for ichthyotherapeutic use in Korea.

In spite of these medical preferences of ichthyotherapy from *G. rufa obtusa*, there was only little knowledge of diseases reported in aquaculture. This paper describes a case of mass mortality of doctor fish *G. rufa obtusa* caused by fish pathogenic bacteria, *Citrobacter (C.) freundii*.

Materials and Methods

Fish source

About 20,000 doctor fish were hatched and grown in a private fish hatchery farm in Korea. According to the fish farm owner, fish were stocked in the 5 ton aquarium tank equipped with aeration system and the water temperature ranged from 32-34°C. During the rearing period in October 2008, some fingerlings showed abnormal swimming behavior, diffuse bleeding on the skin and fins and sudden death. On the first day of mortality, fish were treated with SULFAFISH (Sulfidemethoxine 500 g/kg) (BAYER, Korea) (0.0625 g/l dosage with prolonged immersion treatment), and on the following day, fish were treated with malachite green (0.06 cc/l dosage with prolonged immersion treatment). However, mass

[1]Corresponding author.
E-mail : parksec@snu.ac.kr

mortality was not stopped during the two day period of treatment and more than 9,000 of fingerlings were continuously died showing same symptoms as previously described. On the fourth day of mortality, 12 fingerling fish samples were submitted to College of Veterinary Medicine, Seoul National University for diagnostic examination.

Fish examination

When twelve moribund fingerling samples (4.7 cm average length and 1.3 g average weight) that showed abnormal swimming behavior and lethargy were examined externally, skin, gills, and fins revealed an increase in the quantity of mucous mass on the surface. Diffuse bleeding was observed on the skin, fins and the ventral part of the abdomen. The anal orifice was bloody. The gills exhibited petechial hemorrhages, with severe epithelial hyperplasia with resultant fusion of lamellae to one another (Fig 1). While in the internal examination, a small quantity of reddish fluid was observed in the abdominal cavity. Bleeding was recorded on the internal organs, swim bladder, gonads, intestine, muscles, kidneys, and liver. The spleen was enlarged and the intestine was filled with a bloody fluid (Fig 2).

Detection of the causative agent

In order to rule out disease causing factors of fish, different water parameters was analyzed such as DO, temperature, pH and ammonia. Fungal and parasitic infection was checked using the skin-scratch, fecal, and internal content examinations. Moreover, viral diseases, such as koi herpes virus, iridovirus, betanodavirus, rhabdovirus and birnavirus, were also tested by using PCR assay. For bacterial isolation, sterile swab from the liver, kidney and diffused bleeding of skin specimen were streaked onto tryptic soy agar (TSA) plates, respectively, and the inoculated plate was incubated at 25°C for 24 hrs. Suspected common colonies were re-streaked on TSA to obtain pure cultures, which were then identified on the basis of microscopic analysis and with the aid of the Vitek System® II test (bioMérieux®, France) with biochemical analysis.

Antibiotic susceptibility test by disc diffusion method

Antibiotic susceptibility of bacterial isolate was determined by the disc diffusion method (3). Antibiotic discs (BBL, USA) which were used in this study were shown in Table 1. The sensitivity and resistance of isolated bacteria

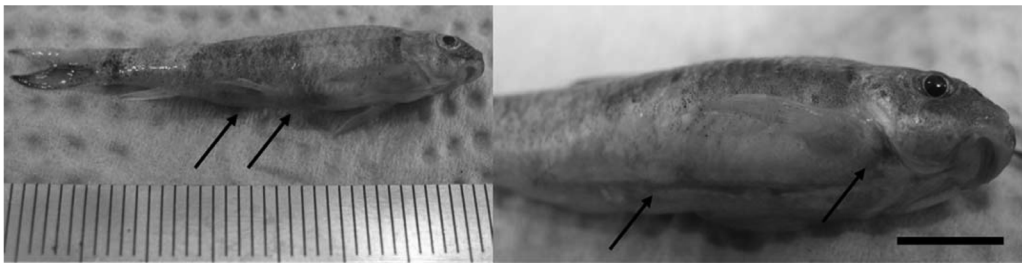

Fig 1. Erosion of the skin, dropping off of scales, bleeding in jugularis region, and diffuse bleeding on the skin (Bar = 10 mm).

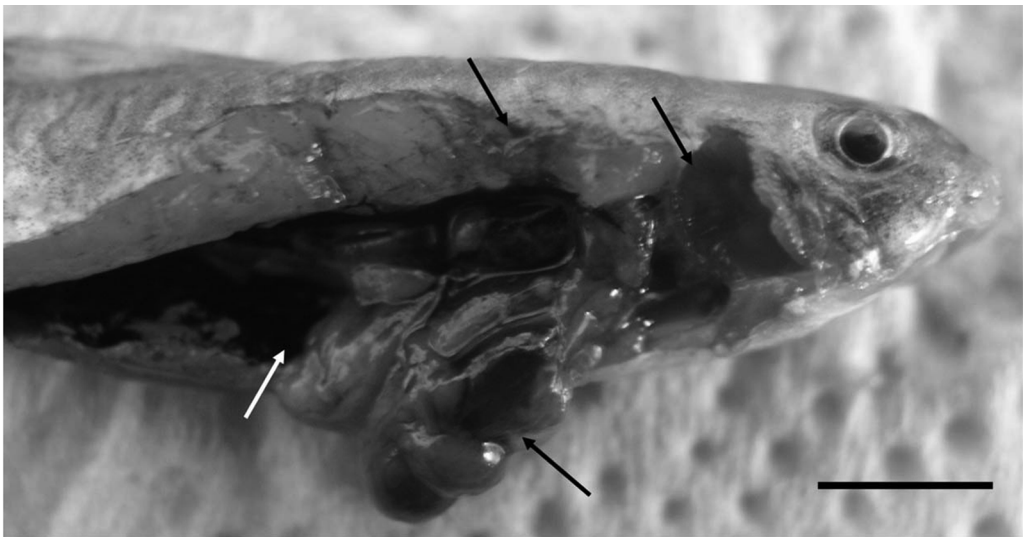

Fig 2. Petechial hemorrhages on the gills associated with necrosis of the tips of the gills. Bleeding on internal organs (Liver, kidneys, gonads and muscles) with a small quantity of reddish fluid in the abdominal cavity (Bar = 10 mm).

Table 1. Antimicrobial susceptibility test of *C. freundii* isolated from diseased doctor fish

Antibiotics (μg)	*C. freundii*	Antibiotics (μg)	*C. freundii*
Amikacin (30)	S[a]	Ofloxacin (5)	S
Ampicillin (10)	S	Polymyxin B (300 IU)	R
Carbenicillin (100)	R[a]	Tetracycline (30)	R
Cefixime (5)	S	Tobramycin (10)	S
Cefoperazone (75)	I[a]	Trimethoprim (5)	R
Gentamicin (10)	S	Enrofloxacin (5)	S
Ciprofloxacin (5)	R	Amoxicilin/clavulanic acid (30)	R
Colistin (10)	S	Oxytetracycline (30)	R
Kanamycin (30)	S	Cefepime (30)	S
Nalidixic acid (30)	R	Cefotaxime (30)	S
Neomycin (30)	I	Chloramphenicol (30)	S
Nitrofurantoin (300)	I	Trimethoprim/Sulfamethoxazole (1.25)/(23.75)	R
Norfloxacin (10)	S		

[a]The category 'S' means sensitive to antibiotic; 'R' means resistant; 'I' means intermediate. And each category was decided by zone diameter interpretive standards (8).

and zone diameter interpretive standards (8) was determined according to the manufacturer's instruction.

Results

Detection of the causative agent

All the water parameters examined showed normal numerical value. Fungal, parasitic and viral diseases were all negative in tested methods. Therefore, bacterial disease was ruled out as the causative agent of mass mortality that occurred in fish hatchery farm. Streaking on TSA gave an apparently pure transparent common bacterial growth from the liver, kidneys, and diffused bleeding of skin of all moribund fish. The result of the microscopic examination revealed that the isolates were identified as gram-negative, motile, oxidase-negative, catalase-positive, anaerobic coli-form of bacteria. Colonies measuring 1-2 mm in diameter with round, smooth, and convex were formed on TSA. It was not pigmented and did not induce hemolysis on blood agar. The isolated bacteria were incubated at 20 and 34°C for temperature sensitivity test, and the growth rate of bacteria was decreased at 20°C comparing to the other. Moreover, as a result of Vitek System® II, *C. freundii* was isolated and showed 99% probability (Data not shown). Also, the bacterial isolate was compared and subjected to taxonomical analysis according to Bergey's Manual for Determinative Bacteriology (6) (Data not shown).

Antibiotic resistance profiles of bacterial isolate

The results of antibiotic resistance profiles of *C. freundii* isolate were shown in Table 1. Bacterial isolate was resistant to carbenicillin, colistin, nalidixic acid, polymyxin B, tetracycline, trimethoprim, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, oxytetracyclin, and was intermediate to cefoperazone, neomycin, nitrofurantoin. However, it was sensitive to amikacin, ampicillin, cefixime, ciprofloxacin, gentamicin, kanamycin, norfloxacin, ofloxacin, tobramycin, enrofloxacin, cefepime, cefotaxime and chloramphenicol.

Fish treatment

Based on the above results of the examination, we have recommended the owner of the fish hatchery farm to lower down the water temperature of the aquarium tank from 32-34°C to 21-22°C with proper aeration. Also, the fish were treated with oxytetracyclin (OTC) (20 mg/l/day dosage with bath treatment) without feeding, according to our recommendations. Due to the severe mortality of doctor fish, OTC was used for treatment without bacterial antibiotic resistance test. Finally, the disease was gradually decreased within 24 hrs after treatment and fish recovered from mortality.

Discussion

C. freundii is a member of the family *Enterobacteriaceae*, gram-negative, oxidase-negative, catalase-positive, motile, anaerobic coli-form bacteria and is often the cause of significant opportunistic infections (2,14). These bacterial strains are widely distributed in nature and are found in soil, water, foodstuffs, and a wide variety of animals, including household pets, birds, and cattle (14,15) as well as human (9). Moreover, the first publication describing pathology and isolation of this species of bacteria from aquarium fish was reported by Sato *et al*. (12). *C. freundii* was subsequently isolated from diseased rainbow trout, which were mix-infected by IPN virus (11), from carp in India which established erosion and hemorrhaging on the skin, focal nodules in the kidneys, and other lesions typical of hemorrhagic septicemia (7), and from carp and rainbow trout fry which were artificially infected by *C. freundii* (16). According to the results from

our fish examination, the symptoms of the doctor fish were identical with other previous reports.

Although *C. freundii* is commonly isolated in eutrophic waters (1), despite the unclearness of bacterial origin, it can be speculated that the urgent mass mortality of doctor fish was caused by acute stress causing factors such as overstocking, poor quality of the water and an increase in the number of coliform bacteria in aquarium water due to high water temperature. Also, the doctor fish, especially *G. rufa obtusa,* is an omnivore, feeds on phytoplankton and zooplankton and is known to be slow-growing, aggressive and predatory fish species (13). In order to use this species for ichthyotherapy, fish rarely feed to maximize aggressive and predatory aptitude (13). The scarcity of natural food sources in the aquarium means that human skin is an attractive and easily obtainable food source for these fish (10). Thus, when there are diseased individuals in the aquarium, schools of fish nibble on the diseased or dead fish, which is a more easily obtained food source (13). By these reasons, it can also be speculated that the spread of bacterial infection was faster than other species of fish.

Due to the severe mass mortality, we recommended two direct treatment methods: dropping down the aquarium water temperature from 32-34°C to 21-22°C without feeding and treatment with OTC. Finally, the mortality was gradually decreased within 24 hrs after treatment and fish were recovered from diseases. According to this successful treatment, even though doctor fish isolated *C. freundii* already showed OTC resistance, the dropping down of water temperature without feeding was efficient for this time and it was proved by the temperature sensitivity test of doctor fish isolate. In addition, it can be speculated that the treated OTC played a role to control the other opportunistic fish pathogenic bacteria.

In conclusion, all of our morphological, biochemical analysis and diagnostic results of the doctor fish indicated that the causative agent of mass mortality was *C. freundii*. This is the first report of doctor fish-associated disease outbreak. Due to the doctor fish characteristics such as high temperature culture and omnivorousness, a strict fish disease control system must be used in the doctor fish aquaculture in Korea.

Acknowledgment

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Citrobacter freundii 감염에 의한 Doctor fish (*Garra rufa obtusa*)의 집단 폐사

백근욱 · 김지형* · 카시아노 초레스카* · 데니스 고메즈* · 신상필* · 한지은* · 박세창*[1]

경상대학교 해양생물교육연구센터 해양산업연구소,
*서울대학교 수의과대학 BK수의과학연구인력양성사업단

요 약 : 최근 doctor fish (*Gara rufa obtusa*)를 이용한 Ichthyotherapy는 인간 피부질환의 대체 치료법으로 각광받고 있으며, 근래 doctor fish의 국내 증식 성공으로 인하여 이러한 추세는 증가되고 있다. 그러나 doctor fish의 질병과 생태에 대해서는 아직 연구가 미미한 실정이다. 본 보고는 국내에서 증식되던 doctor fish가 *Citrobacter freundii*에 감염되어 집단 폐사한 예로서, 병어에 대한 외부 및 내부 진단, 분리된 세균의 형태학적, 생화학적 동정을 통하여 *C. freundii*를 확정하였으며, 분리된 *C. freundii*에 대한 항생제 감수성 검사를 수행하였다. 또한 살아남은 병어에 대한 집단 치료를 시도하여 성공적인 치료 효과를 얻을 수 있었다.

주요어 : doctor fish, *Citrobacter freundii,* 집단 폐사, 치료.