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Bacteriological Study about the Death of Cultured Doctor Fish, *Garra rufa* in the Aquarium

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Since April 2012, doctor fish in the breeding tank and in the quarantine tank in Hanwha Aquaplanet Yeosu Aquarium have been dying, accompanied by diffuse bleeding around the mouth, in the chin, and at the bottom of the abdomen. In this study, the cause of death would be examined through the bacteriological study of doctor fish and the rearing water quality in the aquarium. The water quality and the bacterial counts of the rearing water in the exhibit tank and in the quarantine tank were analyzed once a week, starting from August to November 2014. Water quality was measured based on the following data: temperature was in the range of 24.5~26.8℃, pH at 6.77~7.94, DO at 6.15~8.61 ppm, ammonia at 0~0.93 ppm, nitrite at 0.009~0.075 ppm, and nitrate at 1.1~40.9 ppm. Studies revealed that the differences in these water quality factors were not related to the death of doctor fish. Bacterial counts in the rearing waters of *Garra rufa* slightly increased to 10^3~10^4 CFU/ml, just before the death of the doctor fish. Twelve strains of bacteria were isolated from the dead fish and rearing waters. The isolates were identified as *Aeromonas veronii*, *Citrobacter freundii*, *Pseudorhodoferax aquiterrae*, *Shewanella putrefaciens*, and *Vibrio anguillarum* on the basis of 16S rRNA gene sequences. The most dominant species was *C. freundii*, which showed medium sensitivity to florfenicol and norfloxacin, and was resistant to amoxacillin, doxycycline, oxytetracycline, tetracycline, and trimethoprim. Ten isolates were confirmed to be pathogenic to the doctor fish. Doctor fish infected with *C. freundii* and *S. putrefaciens* showed high mortality in the experimental groups. These results indicate that the variation in bacterial numbers in the rearing water was related to the death of doctor fish. *C. freundii* and *S. putrefaciens* were directly implicated in causing the death of doctor fish in the aquarium.

Keywords: Doctor fish, *Garra rufa*, *Citrobacter freundii*, *Shewanella putrefaciens*

Introduction

Garra fishes are distributed in Southwest Asia, Africa, and Southeast Asia (Durna et al., 2010). *Garra rufa* belongs to the Cyprinidae family of fishes (Gözükara and Çavaş, 2004), and is known to live in Ceyhan river, Müftü stream, Tigris in Turkey, Euphrates basins, northeast part of the Mediterranean basin in Anatolia, and western Iran (Durna et al., 2010; Patimar et al., 2010).

Ichthyotherapy using *G. rufa*, which is commonly known as doctor fish (Gözükara and Çavaş, 2004), was first practiced as a treatment method at Kangal spa located in Anatolia, central Turkey. It is known to be effective especially for patients with psoriasis or eczema (Majtán et al., 2012).

While *G. rufa* is frequently used commercially to treat skin disease, little is known about its diseases and ecology.

Ruane et al. (2013) isolated *Streptococcus agalactiae* and aquatic birnavirus from *G. rufa*. L. Baeck et al. (2009) reported the mass mortality of *G. rufa* obtuse infected with *Citrobacter freundii*, and Majtán et al. (2012) reported the death of *G. rufa* due to *Aeromonas sobria*.

Aquaplanet Yeosu has been exhibiting doctor fishes for experience. It has observed, however, the incessant deaths of the fishes, both reared and exhibited, since April 2012, and raised issues on the maintenance of the fishes.

The death rate tended to subside over time, but frequent deaths of doctor fishes continued. In this study, the cause of death of

doctor fishes reared in the aquarium will be analyzed through bacteriological research.

Materials and Methods

1. Rearing condition and Quality Measurement of the rearing water

The exhibit tank and the quarantine tank contained about 600 ℓ of water and 400 ℓ, respectively. The rearing water was filtered in a recirculation system, while commercial feed was supplied once a day. Fishes were added, and the filtering system was cleaned and replaced when necessary.

The quality of the rearing water was measured once a week in the Aquaplanet Yeosu Marine Organism Research Center by taking 100 ml each from the exhibit tank and the quarantine tank.

Water temperature, pH, and DO were measured using a pH and DO meter (HQ40d, HACH company, USA), while the nutrient salt (ammonia, nitrite, nitrate) was measured using a spectrophotometer (DR3900, HACH company, USA). Method 8155 was followed for ammonia, method 8507 for nitrite, and method 8039 for nitrate.

2. Total bacterial count

Water samples from the exhibit tank and the quarantine tank was then placed in a biological water sampler (2 ℓ, New-Kukje Science Corporation Korea) and underwent serial dilution by 1/10 in the lab. After each dilution, samples were smeared on the Nutrient agar (NA, BD, France) plate with 0.5% NaCl, and cultured 48 hours at 25℃ to determine total bacterial count.

3. Isolation and identification of bacteria from rearing water and dead fish

The rearing water of the doctor fish and the liver, kidney, and intestines separated from fish right before death were smeared on the NA medium with 0.5% NaCl, and cultured 48 hours at 25℃. Isolated bacteria underwent polymerase chain reaction (PCR) using 16S rRNA universal primers (fD1, AGAGTTTGATCCTGGCTCAG; rP2, ACGGCTACCTTGTTACGACTT) (Pre-denaturation, 95℃, 5 min, 1 cycle; Denaturation, 95℃, 30 sec; Annealing, 43~55℃, 30~60 sec; Extension, 72℃, 90 sec, 30 cycle; Final-extension, 72℃, 5 min, 1 cycle). PCR products purified with a PCR purification kit (AccuPrep, Bioneer, Korea) underwent sequencing (Solgent Corp., Korea) to obtain the base sequence. It was then analyzed by using the BLAST search of NCBI (National Center for Biotechnology Information, USA).

4. Antimicrobial sensitivity test

The antimicrobial sensitivity of the isolated bacteria was evaluated through the disc diffusion method. The used antimicrobials were amoxacillin, ampicillin, ciprofloxacin, doxycycline, erythromycin, florfenicol, flumequine, neomycin, norfloxacin, novobiocin, oxolinic acid, oxytetracycline, sulfonamide, tetracycline, and trimethoprim of Oxoid.

5. Artificial infection

The pathogenicity of the isolated bacteria toward doctor fish was tested with 10 selected bacteria. A total of 10 doctor fishes that were 2~5 cm long and about 0.5 g in weight were accommodated in each tank containing fresh water. The isolated bacteria were inserted into the water at the concentration of 10^6 CFU/ml. The bacteria were also mixed with 1 g feed at the concentration of 1.5×10^9 CFU/ml and then dried. It was fed once the fish at 1% of the total fish weight. The internal organs of dead fish were smeared on the NA medium and cultured 48 hours at 25℃ before re-separation of the bacteria.

Results

1. Mortality of doctor fish

The number of dead doctor fish at the Aquaplanet Yeosu in 2014 is shown in Fig. 1 and 2. The exhibit tank had peak values in January and April, and the quarantine tank in March and August.

2. Water quality

Water quality in the exhibit tank and in the quarantine tank was measured when there were deaths, and likewise when there were no reported deaths, as shown in Table 1. When there were deaths in the exhibit tank, the average water temperature was 26℃, pH 7.45, and DO 7.68 ppm. Also, ammonia was 0.01 ppm, nitrite 0.01 ppm, and nitrate 26.2 ppm.

When there were no deaths in the exhibit tank, the average water temperature was 25.9℃, pH 7.42, and DO 6.69 ppm. Also, ammonia was 0.03 ppm, nitrite 0.02 ppm, and nitrate 27 ppm.

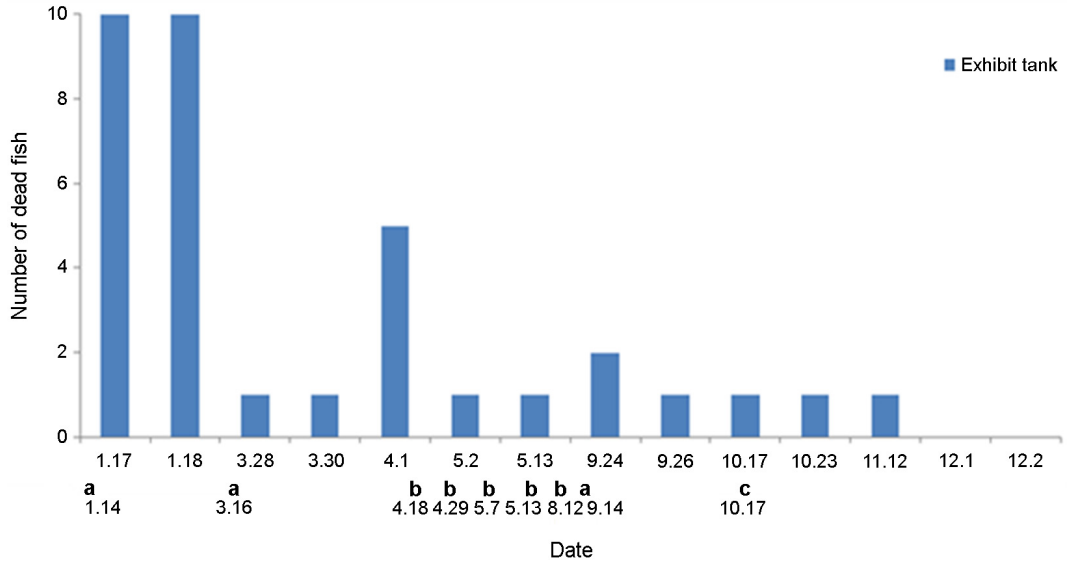

Fig. 1. The number of dead fish based on the date of death in the exhibit tank of doctor fish in 2014. a, new addition of doctor fish in the tank; b, washing of filter media in sump; c, change of sump.

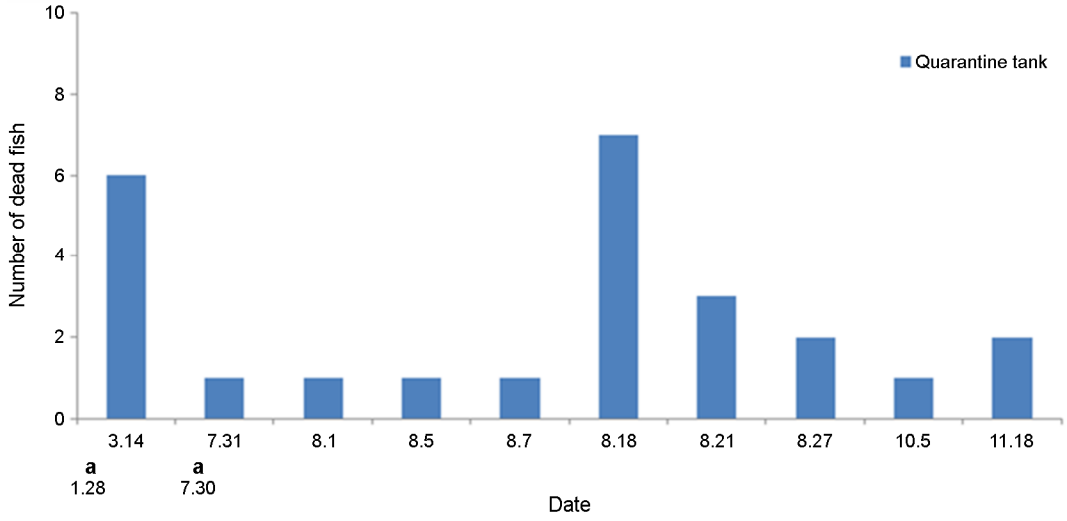

Fig. 2. The number of dead fish based on the date of death in the quarantine tank of doctor fish in 2014. a: new addition of doctor fish in the tank.

The water quality results from the exhibit tank showed that there were no significant differences in the measured value whether there were deaths or there were none. The quarantine tank also showed similar results.

3. Total bacterial count in rearing water

The bacterial count in the rearing water of doctor fish was higher in the exhibit tank than in the quarantine tank (Fig. 3 and 4).

The bacterial count in the exhibit tank was approximately 5,000 CFU/ml on average during the period of August 5 through

Table 1. The range of the measured value of water quality factors in the exhibit tank and in the quarantine tank when deaths of doctor fish occurred or not

		Water temperature (℃)	pH	DO (ppm)	Ammonia (ppm)	Nitrite (ppm)	Nitrate (ppm)
Exhibit tank	A*	25.7~26.6	7.11~7.73	7.3~8.18	0.01~0.02	0.007~0.012	24.1~30.5
	B**	25.3~26.6	7.28~7.59	5.65~7.5	0~0.12	0.004~0.075	14.8~40.9
Quarantine tank	A	24~26.2	6.94~7.42	7.23~7.54	0~0.17	0.008~0.011	2.8~4
	B	24.9~26.8	6.77~7.55	6.43~8.28	0~0.04	0.003~0.04	1.1~4.2

*: When there were deaths, **: When there were no deaths

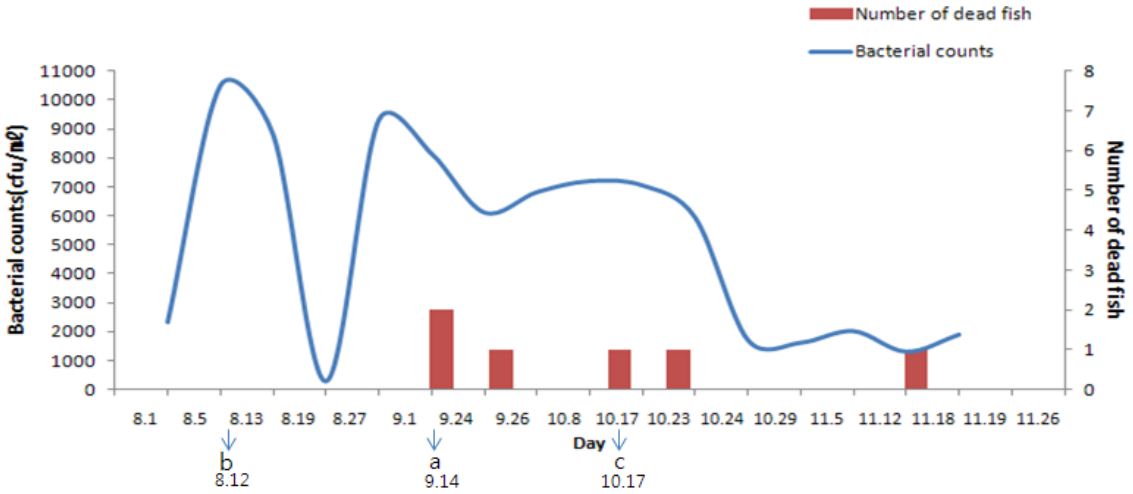

Fig. 3. Bacterial counts in the rearing water of the exhibit tank of doctor fish. a, new addition of doctor fish in the tank; b, washing of filter media in sump; c, change of sump.

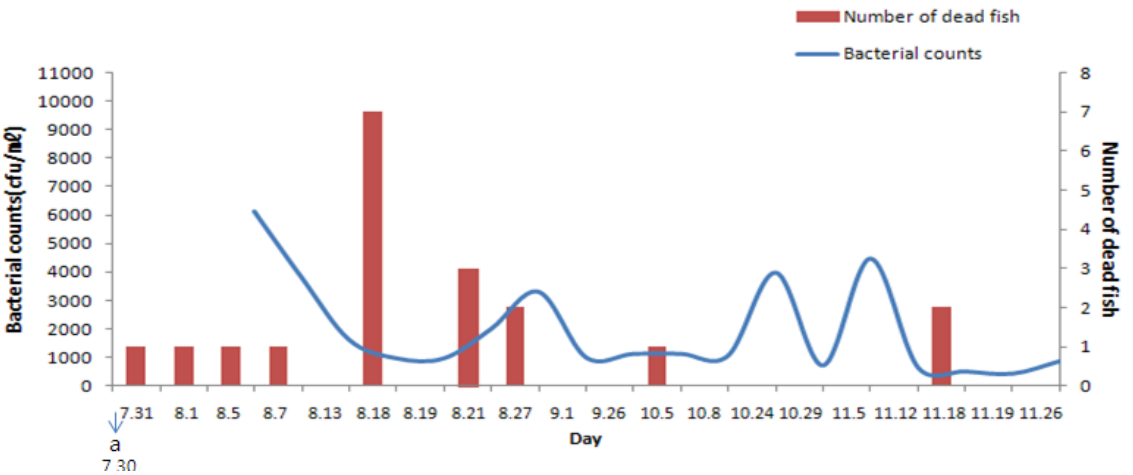

Fig. 4. Bacterial counts in the rearing water of the quarantine tank of doctor fish. a: new addition of doctor fish in the tank.

November 26, with the exception of August 27, which seemed to be an experimental error. The bacterial count in the quarantine tank was about 2,000 CFU/ml.

Table 2. Identification of 12 strains of bacteria isolated from the rearing water of the quarantine tank and doctor fish, *Garra rufa*

Strain	BLAST identification	Similarity
DF1	*Vibrio anguillarum*	100%
DF2	*Aeromonas veronii*	96%
DF3, DF4, DF7, DF9	*Aeromonas veronii*	>=99%
DF5	*Shewanella putrefaciens*	100%
DF6, DF8, DF10, DWF1	*Citrobacter freundii*	>=99%
DWF2	*Pseudorhodoferax aquiterrae*	99%

*DF: Strains isolated from the doctor fish, *Garra rufa*
*DWF: Strains isolated from the rearing water

4. Identification of the isolates

A total of 12 bacteria were isolated and identified from the rearing water and the dying doctor fish (Table 2). Five kinds of bacteria have been identified, including *Vibrio anguillarum*, *Aeromonas veronii*, *Shewanella putrefaciens*, *Citrobacter freundii*, and *Pseudorhodoferax aquiterrae*. Among them, *C. freundii* and *A. veronii* appeared frequently.

5. Antimicrobial sensitivity of isolated bacteri

The sensitivity test of the 12 isolated bacteria against 15 antimicrobial agents (Table 3) showed that the most isolated bacteria were sensitive against florfenicol, and resistance against ampicillin, amoxacillin, and sulfonamide. In particular, the bacteria identified as *C. freundii* were resistance against amoxacillin, doxycycline, oxytetracycline, tetracycline, florfenicol, and trimethoprim.

Table 3. Antimicrobial sensitivity of 12 bacterial strains isolated from the rearing water of the quarantine tank and the dead fish, *Garra rufa*

Antimicrobial agent	*Aeromonas veronii*					*Citrobacter freundii*				*Vibrio anguillarum*	*Shewanella putrefaciens*	*Pseudorhodoferax aquiterrae*
	DF2	DF3	DF4	DF7	DF9	DF6	DF8	DF10	DFW1	DF1	DF5	DFW2
Ampicillin	–	–	–	–	–	–	11*	–	10	–	–	–
Amoxacillin	–	–	8	10	–	–	–	–	–	–	–	–
Ciprofloxacin	10	16	15	31	–	14	14	13	32	–	–	17
Norfloxacin	10	15	12	24	–	14	16	12	34	–	–	13
Doxycycline	14	10	14	10	–	–	–	–	–	18	21	35
Oxytetracycline	10	10	9	–	–	–	–	–	–	14	9	29
Tetracycline	9	8	8	10	–	–	–	–	–	25	9	22
Erythromycin	15	20	17	17	16	–	–	–	12	14	29	13
Neomycin	13	12	14	15	12	13	13	13	–	11	14	13
Florfenicol	26	18	35	11	–	–	–	–	–	37	26	25
Flumequine	11	11	11	27	–	13	13	11	23	11	1	17
Novobiocin	12	10	9	19	10	–	–	–	11	32	9	18
Oxolinic acid	–	–	–	16	–	9	9	9	20	–	–	–
Sulfonamide	–	–	34	–	–	–	–	–	–	–	–	–
Trimethoprim	13	19	31	22	–	–	–	–	–	15	–	–

*Clear zone, mm in diameter

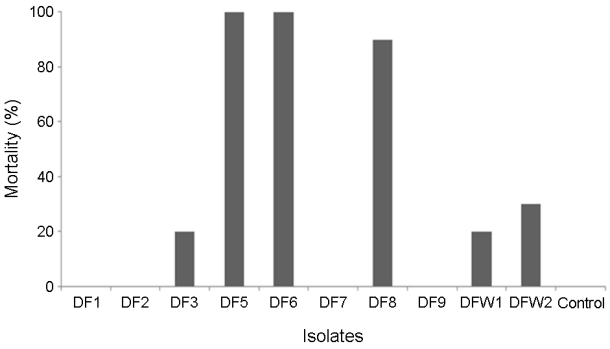

Fig. 5. Mortality of doctor fish after infection with various bacterial isolates (DF1, *V. anguillarum*; DF2, DF3, DF4, DF7, DF9, *A. veronii*; DF5, *S. putrefaciens*; DF6, DF8, DF10, DWF1, *C. freundii*; DWF2, *P. aquiterrae*).

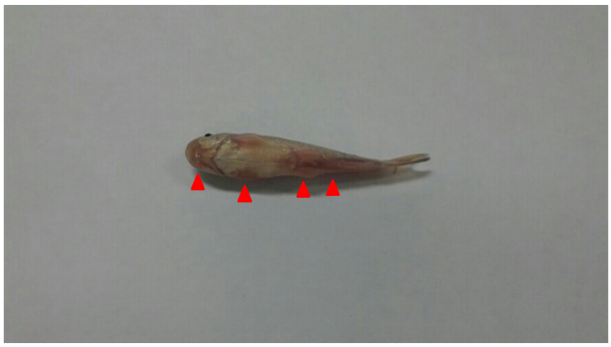

Fig. 6. Diffuse bleeding (▲) on the skin and fins of dead doctor fish.

6. Pathopenicity

The results of artificially infecting toward doctor fish with 10 isolated bacteria (Fig. 5) showed that the group infected with *C. freundii* (DF6, DF8) and *S. putrefaciens* (DF5) had higher mortality. Most dead fish had diffuse bleeding on the skin, fins, and abdomen as in the original dead fish of aquarium. Bleeding was also visible at the anus (Fig. 6). As internal symptoms, some fish exhibited ascites, but there were no special clinical symptoms. And the bacteria inoculated were reisolated from all the dead fish.

Discussion

The analysis on the deaths of doctor fish in the exhibit tank and in the quarantine tank at the Aquaplanet Yeosu in 2014 revealed that there were no seasonal changes, and that there was a tendency of higher mortality when there were physical changes, such as addition of fishes or filtering system cleaning. Water quality was measured on 6 different factors during the experiment. The results were normal, and it was believed that water quality does not affect the death of doctor fish.

The filtering system was replaced on the 17th of October to increase filtering power. After such replacement, there was no sign of sudden deaths of doctor fish, the number of bacteria decreased, and the tendency continued for a certain period (Fig. 3). After the increase in the number of bacteria then showed a tendency to increased number and frequency of the dying doctor fish. After the number of bacteria decreased, the mortality rate of fish tended to drop. Thus, it was estimated that the increase of bacteria was related to the increase of the mortality rate of doctor fish.

C. freundii is a gram-negative bacillus belonging to Enterobacteriaceae (Badger et al., 1999), and is an opportunistic pathogen (Öoztüurk and Altınok, 2014). It exists extensively in nature, and is generally found in eutrophicated water (Allen et al., 1983).

Svetlana et al. (2003) points to murky water, oxygen decrease, and colon bacillus increase as the conditions in which *C. freundii* causes disease in trout. Also, Baeck et al. (2009) reported that *C. freundii* was a pathogen frequently isolated in eutrophicated water, and that the stress from the coliform bacteria, which increased in the tanks due to water quality deterioration, and temperature increase could be a cause of the sudden mass mortality of doctor fish.

A pathogenicity test of the isolated bacteria also showed that the tank infected with *C. freundii* and *S. putrefaciens* had the same symptoms as naturally diseased fish and high mortality. Thus, it was found that these two bacteria are related with the death of doctor fish in the aquarium.

Sato et al. (1982) reported that 25 out of 29 sunfish, *Mola mola*, which were reared in the Matsushima Aquarium in Japan, died after numerous fatty granuloma in the kidneys were detected, and there was bleeding rash on the surface of the bodies of the fish. They first isolated *C. freundii* from the dead fish's granuloma, kidney, and spleen.

Svetlana et al. (2003) intraperitoneally injected *C. freundii* into *Cyprinids* and reported that the mortality rate reached 50%, and that there were petechia and diffuse bleeding on the skin of the dead fish, and bleeding and discoloration in the internal organs. These symptoms were similar to those of the infected fishes in this study.

In this study, the isolated *C. freundii* was resistant to tetracycline.

Baeck et al. (2009) also reported that *C. freundii* was resistant to oxytetracycline. Nawaz et al. (2008) reported that the *Citrobacter* spp. isolated from catfish were resistant to tetracycline as well.

S. putrefaciens is a pathogen known to cause illnesses to human beings (Khashe and Janda, 1998). *S. putrefaciens* is also an opportunistic pathogen against fish, and stress is pointed out as the cause of disease (Kozinska and Pekala, 2004). Fish infection was first reported in 1985 at a fish farm for Red Sea rabbit fish, *Siganus rivulatus*. It recorded high mortality with symptoms such as discoloration of the body surface and mouth, bleeding necrosis, fin damage, and exophthalmos (Saeed et al., 1987).

While there are many cases in which omnivorous doctor fish gets starved to increase its predatory behaviour for effective keratin treatment, it was reported that hungry doctor fish could eat the infected or dead entities, and the bacterial disease could spread more rapidly compared to other fishes (Baeck et al., 2009).

In this study, it was found that the deaths of doctor fish in the aquarium were related to the physical changes, such as addition of new fishes or filtering system cleaning, and the temporary increase in the number of bacteria. It was believed that *C. freundii* and *S. putrefaciens*, which were isolated from the dead fish, acted as a direct cause of deaths. Since these bacteria could flourish with the deterioration of water quality, it is suggested that adequate water quality control could fully prevent diseases.

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