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Two-tailed fancy goldfish challenged with *Bacteroides* spp. isolated from shubunkin goldfish

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ABSTRACT

This study aimed to isolate, morpho-biochemically characterize and identify the most common bacterium in diseased shubunkin goldfish, and challenge the two-tailed fancy goldfish using the identified bacterium. With the aid of Bergey's Manual of Determinative Bacteriology, the bacterium was identified as *Bacteroides* spp. The bacterium was characterized as follows: Gram-negative, coccobacillus, facultative anaerobe, hemolytic, glucose and sucrose fermentative but not lactose and xylose, negative to catalase, Methyl red, Voges-Proskauer and hydrogen sulphide production tests, and positive to Simmon's citrate utilization, liquefaction of gelatin, nitrate reduction, urea hydrolysis and indole production. Two-tailed goldfish challenged with *Bacteroides* spp. showed clinical signs such as gasping for air, irritability, sloughing of scale, lesion and watery internal organs.

Keywords: Shubunkin goldfish, two-tailed goldfish, biochemical tests, *Bacteroides*

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INTRODUCTION

The ornamental fish industry is an aquaculture based business which is very popular to hobbyist around the world. This industry supports the rural people in developing countries. There are more than 4,500 species of ornamental freshwater fish and 1,450 species of marine ornamental fish according to published report of the European Pet Food Industry Federation. More than 50% of ornamental fish originate from Thailand, Japan, China, Malaysia and Sri Lanka[1].

Aquarium fish commonly live in sub-optimal conditions that involve limited volumes of water in aquarium systems with a restricted capacity to maintain adequate water quantity. Unlike wild fish species, aquarium fish cannot escape a potentially harmful environment. Thus, keeping of fish in aquaria has negative influence on the fish well-being[2].

The worldwide development of the ornamental fish trade favors the wide spread of their pathogens which are often transported with fish. This present scenario also favors an increase risk of human infection[3]. Bacterial disease is the most common infectious problem of ornamental fishes. Bacterial organisms may be the primary cause of disease, or they may be secondary invaders, taking advantage of a breach in the fish's integument or compromise of its immune system. The majority of bacterial infections are mainly caused by Gram-negative microorganisms such as *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium*, *Mycobacterium* and *Pseudomonas*. Gram-positive bacterium like *Streptococcus* has been recorded to cause disease in ornamental fishes[4].

The goldfish (*Carassius auratus*) is a freshwater fish in the family Cyprinidae of order Cypriniformes. It is one of the most commonly kept aquarium fish. This fish is native to East Asia. It was first selectively bred in Ancient China more than a thousand years ago, and several distinct breeds have since been developed. Goldfish breeds vary greatly in size, body shape, fin configuration and coloration. Gold fish is known as susceptible to *Aeromonas* infections; in severe cases, it can prove fatal to the fish[5].

This study aimed to isolate, morpho-biochemically characterize and identify the most common bacterium in diseased shubunkin goldfish, and challenge the two-tailed fancy goldfish using the identified bacterium.

MATERIALS AND METHODS

Collection of Shubunkin Goldfish

Shubunkin goldfish were bought from Cartimar Pet Center in Taft Avenue, Pasay City, Philippines. Cartimar Pasay houses a

number of stores that sell exotic, domesticated and highly priced animals. Around 25 stores sell aquarium fishes. It was observed in every store that the unhealthy fish of the same kind were intensively kept (around 50-60 pieces of assorted sizes) in separate aquaria (1.5 x 0.5 ft) provided with aerator. Even though the quality of water in aquaria was well maintained, the sick fish do not undergo any disease treatment nor provided with any special diet, therefore, their health conditions were even worsen. The unhealthy fish usually command lower price (25-75% lower). The five shubunkin samples bought from the said pet shop have manifested one or combination of the following clinical signs of bacterial diseases: swimming at the surface (gasping for air), inactivity (usually stays at the bottom), sunken eyes, deteriorated gills and skin, lesion, sloughing of scales and fin rot (Figure 1).

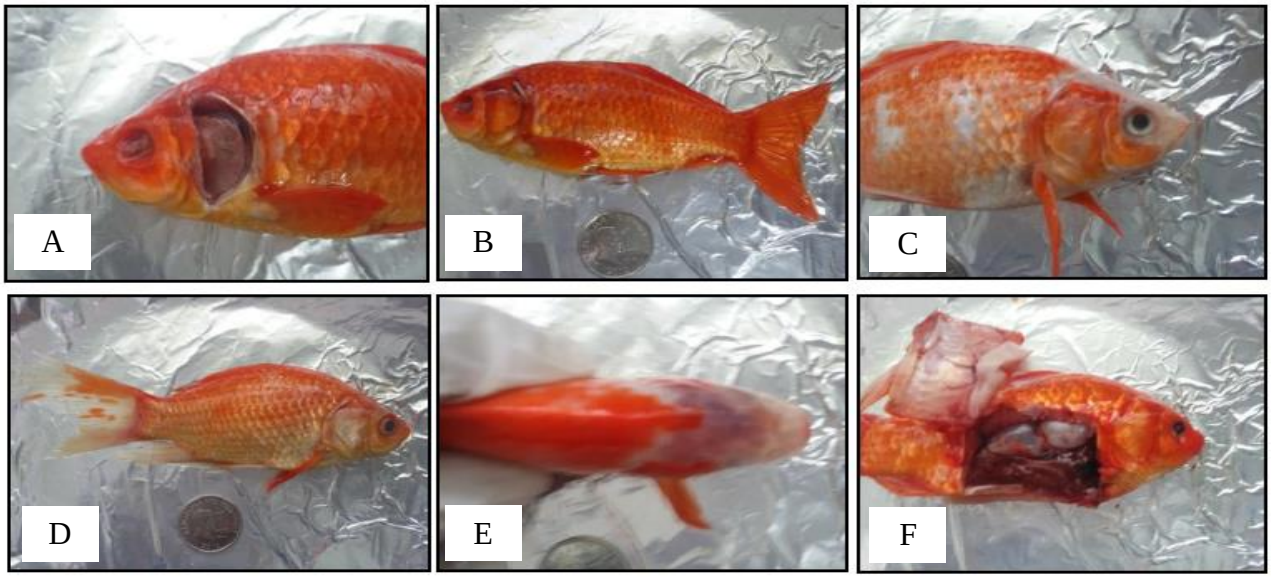

Figure 1. Shubunkin goldfish showing clinical signs of infection (A. deteriorated gills; B. missing eye; C. lesion on head; D. caudal fin rot; E. lesion with hemorrhage on head; and F. swelling of liver)

Bacterial Isolation

Shubunkin goldfish were dissected to reveal its internal organs. Infected tissues from skin, gills, kidney and spleen were macerated using mortar and pestle and afterwards diluted to Phosphate Buffer Solution (1 mL tissue: 5 mL PBS). The mixture was treated with antibiotics (chloramphenicol, 100 mg/L). A sterilized inoculating loop was dipped in the tissue mixture and quadrant streaking was employed in plates containing Blood Agar in order to get isolated colonies. The plates were incubated for 24 hours at 37 °C. After 24 hours, the most common colony in Blood Agar was grown in Nutrient broth.

Morpho-biochemical Tests for the Identification of the Isolate

Gram-staining, microscopy and a number of biochemical tests were done in order to identify the grown bacterium in Nutrient broth (Table 1; Figure 2). Bergey's Manual of Determinative Bacteriology was used as an aid in the identification of the unknown bacterium.

Table 1. Various tests used in the identification of the unknown isolate.

Tests	Purpose	Possible Results
Gram-staining	Identification of gram positive or gram negative bacterium	Gram positive: blue/purple Gram negative: red
Microscopy	Cell shape	Coccus, bacillus, coccobacillus, etc.
Oxygen requirement (fluid thiogylcollate agar)	Oxygen requirement	Obligate aerobe, strict anaerobe, aerotolerant anaerobe, facultative anaerobe or microaerophile
Catalase test	Ability to produce catalase	Positive: release of oxygen bubbles
Oxidation test	Oxidation of sugars	Positive: change of color from yellow to pink
Glucose fermentation (Triple Sugar Iron)	Carbohydrate fermentation; H_2S production	K/A, A/A, K/K, K/NC, etc
Methyl Red (MR) test	Mixed acid fermentation	Positive: production of red color
Voges-Proskauer (VP) test	Butaneidol or acetoin production	Positive: formation of pink to red complex in the interface of the medium
Simmon's citrate test	Citric acid utilization	Positive: change of color from green to Prussian blue
Liquefaction of nutrient gelatin	Gelatin hydrolysis or liquefaction of gelatin	Positive: liquid at low temperature
Nitrate reduction	Presence of enzyme nitrate reductase	Positive: formation of red color
Urea hydrolysis	Presence of enzyme urease	Positive: formation of red/violet color

Indole production	Presence of enzyme trytophanase; indole production	Positive: formation of red colored rings on the interface
H_2S production (SIM medium)	Production of hydrogen sulphide; Motility	Positive: browning on the surface and along the line of puncture Positive: diffused growth along the line of inoculums

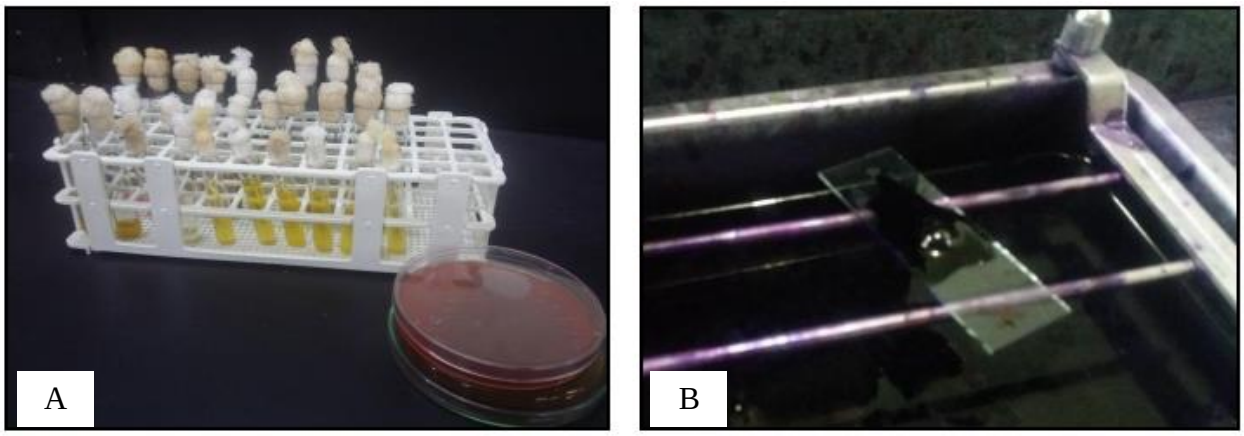

Figure 2. A. Prepared media for the conduct of biochemical tests; B. Gram-staining

Preparation of Bacterial Suspension

From the pure bacterial culture, five colonies were transferred to 5 mL PBS. The broth was incubated at 37 ˚C until it exceeds the turbidity of 0.5 Mc Farland standards. The bacterial suspension was used in the challenge test.

Set-up Preparation and Challenge Test

Healthy two-tailed fancy goldfish were bought from a pet shop in Los Baños, Laguna, Philippines. The experimental fish were conditioned for two days and treated with the antibiotics cloramphenicol (100 mg/L) via 30 minutes immersion. The individual weight of the fish was determined using digital weighing scale. The fish were stocked in aerated circular plastic containers with water capacity of 2.5 L. The study was composed of two controls and two treatments (Table 2). The fish in Treatment 1 and Treatment 2 received 0.2 mL of the bacterial suspension via intraperitoneal injection. Physical and behavioral signs of bacterial infection were observed for a period of 1 week.

Table 2. Description of the controls and treatments used in the study.

	No. of Fish Stocked	Individual Weight (g)	Mode of Introduction
Control 1	2	4.60	Intraperitoneal injection (0.2 mL PBS)
Control 2	2	6.50	Intraperitoneal injection (0.2 mL PBS)
Treatment 1	2	4.60	Intraperitoneal injection (0.2 mL bacterial suspension)
Treatment 2	2	6.50	Intraperitoneal injection (0.2 mL bacterial suspension)

Set-up Maintenance

The fish were fed four times a day using goldfish starter. Uneaten feeds and feces were siphoned daily. Water replacement (25-50%) was done every after two days.

Necropsy

Dead and live fish were necropsied in order to evaluate the possible effects of the bacterium on the challenged fish as compared with the control.

Recovery of Bacterium

The injected bacterium was recovered from dead and remaining live fish in controls and treatments. External and internal smears were streaked in McConkey Agar and the plates were incubated overnight at 37 °C. Isolated colony that has comparable morphological characteristics with that of the injected bacterium was grown in Nutrient Broth. The recovered bacterium was subjected to gram-staining, microscopy and a number of biochemical tests for identification. The results on recovered bacterium were compared with the morpho-biochemical characteristics of the injected bacterium.

RESULTS AND DISCUSSION

Identification of the Isolate

Using Appendix III of Bergey's Manual of Determinative Bacteriology, the bacterium isolated from diseased shubunkin goldfish belongs to genus *Bacteroides*. The result of the morpho-biochemical test of *Bacterioides* spp. as compared to closely related bacteria is provided in Table 3. The easiest way to identify *Bacteroides* against *Bordetella* and *Brucella* was based on the tests catalase, gelatin hydrolysis and Simmon's citrate. These there genera were very distinct from other bacteria because of their tiny cocco-bacillary shape.

Table 3. Morpho-biochemical profiles of *Bacteroides* and closely related coocobacilli bacteria.

Tests	*Bacteroides*	*Bordetella*	*Brucella*
Gram-staining	Negative	Negative	Negative
Microscopy	Coccobacillus	Coccobacillus	Coccobacillus
Motility	Non-motile	Non-motile	Non-motile
Haemolysis	Positive	Positive	Positive
Oxygen requirement (fluid thiogylcollate agar)	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Catalase test	Negative	Positive	Positive
Oxidation test	Glucose = positive Sucrose = positive Lactose = negative Xylose = negative	Glucose = negative	Glucose = negative
Glucose fermentation (Triple Sugar Iron)	Glucose fermentation only (K/A)	---	---
Methyl Red (MR) test	Negative	---	---
Voges-Proskauer (VP) test	Negative	---	---
Simmon's citrate test	Positive	Negative	Negative
Liquefaction of nutrient gelatin	Positive	Negative	Negative
Nitrate reduction	Positive	Positive	Negative
Urea hydrolysis	Positive	Positive	Positive
Indole production	Positive	Positive	Positive
H_2S production (SIM medium)	Negative	Negative	Negative

At present, 22 species of *Bacteroides* are recorded both in humans and animals. This bacterium was isolated in gut of siluriform fishes (*Hypostomus auroguttatus* and *Pimelodus maculates*), wild and cultured Asian seabass (*Lates calcarifer*), pond-reared grass carp (*Ctenopharyngodon idellus*), common carp (*Cyprinus carpio*) and aquarium-reared goldfish (*Carassius auratus*)[6]. *Bacteroides* species are normally gut commensals. However, these organisms can also be responsible for infections with significant morbidity and mortality. *Bacteriodes* infection might occur if the bacterium is introduced into the bloodstream or surrounding tissue[7]. *Bacteroides* in fishes is usually associated with *Aeromonas hydrophila* and causes intestinal abscess resulting to liquefaction and lesion in parts of the body where the bacterium is introduced. The uptake of the bacterium from the environment was influenced by the feeding habit of the fish. In aquarium conditions, *Bacteroides* is introduced in water via fecal discharge. High load of bacterium in water coupled with poor water quality and high stocking density could trigger morbidity and mortality. According to Gilmore and Ferretti (2003), organisms such as *Bacteroides* with such a large genome bank at their disposal may simply need to turn on certain genes to change from friendly commensal to dangerous threat[8]. For instance, the virulence of *B. fragilis* can be subdivided into three categories: (a) adherence to tissues, (b) protection from the host immune response (such as oxygen toxicity and phagocytosis), or (c) destruction of tissues. *Bacteroides* strains may possess all of these characteristics. The fimbriae and agglutinins of *B. fragilis* function as adhesins, allowing them to be established in the host tissue. The polysaccharide capsule, LPS and a variety of enzymes protect it from the host immune response[9]. The capsule is responsible for abscess formation, and histolytic enzymes found in *B. fragilis* can mediate tissue destruction[10].

Two-tailed Goldfish Challenged with *Bacteroides* spp.

Two-tailed goldfish challenged with *Bacteroides* spp. showed similar clinical signs such as gasping for air, irritability and sloughing of scales. These manifestations were observed Day 2 after the challenge test. Both the larger size goldfish (Treatment 2) died in Day 4 (Table 4). Aside from the removed scales in some parts of their bodies, lesion was also detected in the top of their heads (nape) (Figure 3). Necropsy also revealed the presence of watery internal organs (Figure 3) which is considered the most prominent pathological effect of some species of *Bacteroides*. One of the smaller goldfish (Treatment 1) was able to survive until Day 7 even though the clinical signs have continued. Internal organs observation of alive and dead goldfish in Treatment 1 showed no distinct difference as compared with the internal organs of controls.

Table 4. Daily observation of fish in controls and treatments.

	Control 1	Control 2	Treatment 1	Treatment 2
Day 1	Normal	Normal	Normal	Normal
Day 2	Normal	Normal	Stay at the bottom or gasping air in the surface, scratching itself in the edge of the hose	Stay at the bottom or gasping air in the surface, scratching itself in the edge of the hose
Day 3	Normal	Normal	Stay at the bottom or gasping air in the surface, scratching itself in the edge of the hose, removal of scales	Stay at the bottom or gasping air in the surface, scratching itself in the edge of the hose, removal of scales
Day 4	Normal	Normal	Stay at the bottom or gasping air in the surface, scratching itself in the edge of the hose, removal of scales	Both fish died in the afternoon

Day 5	Normal	Normal	No observation	---
Day 6	Normal	Normal	No observation	---
Day 7	Normal	Normal	Stay at the bottom or gasping air in the surface, scratching itself in the edge of the hose, removal of scales One fish died in the afternoon	---

Note: Normal = aggressive feeding, continuous movement, no physical signs of bacterial infection

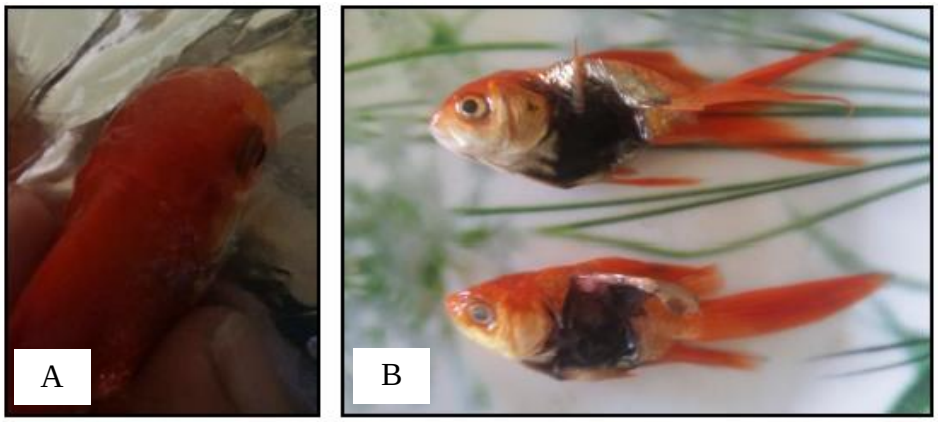

Figure 3. Dead fish in Treatments 1 and 2 showing signs of bacterial infection (A. lesion on the head; B. liquefied internal organs)

Recovery of the Injected *Bacteriodes* spp.

In order to determine whether *Bacteroides* spp. is the possible cause of death in two goldfish in Treatment 2 and one goldfish in Treatment 1, the injected bacterium was recovered, grown in nutrient broth and morpho-biochemically characterized. The same procedure was done in goldfish in Control 1, Control 2 and the other live goldfish in Treatment 1.

No bacteria were recovered in one fish in Control 1 and both fish in Control 2. The morpho-biochemical characteristics of injected *Bacteroides* spp. were 100% compatible with the morpho-biochemical results of recovered bacteria in goldfish in Treatment 3 and Treatment 4. Hence, the bacterium *Bacteroides* spp. could be associated on the death of one fish in Treatment 1 and two fish in Treatment 2. The isolated bacterium in one fish in Control 1 was not 100% compatible with *Bacteroides* spp. based on results of some biochemical tests like lactose oxidation, citrate utilization and gelatin hydrolysis (Table 5).

Table 5. Comparison of morpho-biochemical characteristics of *Bacteroides* spp. with the recovered bacteria in the control and treatment.

Tests	***Bacteroides* spp.**	**Control 1 Fish 1**	**Treatment 1 Fish 1**	**Treatment 1 Fish 2***	**Treatment 2 Fish 1***	**Treatment 2 Fish 2***
Gram-staining	Negative	Negative	Negative	Negative	Negative	Negative
Microscopy	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Haemolysis	Positive	---	---	---	---	Positive
Oxygen requirement (fluid thiogylcollate agar)	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Catalase test	Negative	Negative	Negative	Negative	Negative	Negative
Oxidation test	Glucose = positive Sucrose = positive Lactose = negative Xylose = negative	Glucose = positive Sucrose = positive Lactose = positive Xylose = negative	Glucose = positive Sucrose = positive Lactose = negative Xylose = negative	Glucose = positive Sucrose = positive Lactose = negative Xylose = negative	Glucose = positive Sucrose = positive Lactose = negative Xylose = negative	Glucose = positive Sucrose = positive Lactose = negative Xylose = negative
Glucose fermentation (Triple Sugar Iron)	Glucose fermentation only (K/A)	Glucose fermentation only (K/A)	Glucose fermentation only (K/A)	Glucose fermentation only (K/A)	Glucose fermentation only (K/A)	Glucose fermentation only (K/A)
Methyl Red (MR)	Negative	Positive	Negative	Negative	Negative	Negative

test						
Voges-Proskauer (VP) test	Negative	Negative	Negative	Negative	Negative	Negative
Simmon's citrate test	Positive	Negative	Positive	Positive	Positive	Positive
Liquefaction of nutrient gelatin	Positive	Negative	Positive	Positive	Positive	Positive
Nitrate reduction	Positive	Positive	Positive	Positive	Positive	Positive
Urea hydrolysis	Positive	Positive	Positive	Positive	Positive	Positive
Indole production	Positive	---	---	---	---	Positive
H_2S production (SIM medium)	Negative	Negative	Negative	Negative	Negative	Negative

Note: *Dead fish

CONCLUSION

Using Appendix III of Bergey's Manual of Determinative Bacteriology, the bacterium isolated from diseased shubunkin goldfish belongs to genus *Bacteroides*. Two-tailed goldfish challenged with *Bacteroides* spp. showed similar clinical signs such as gasping for air, irritability, sloughing of scales and lesion. Necropsy revealed the presence of watery internal organs which is considered the most prominent pathological effect of some species of *Bacteroides*. Infection caused by this bacterium could be prevented through maintaining good quality of water and moderate stocking density.

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