# Infection of farmed sub-adult semicircle angelfish, *Pomacanthus semicirculatus* in Taiwan caused by *Photobacterium damselae* subsp. *piscicida*

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**Abstract**

The semicircle angelfish (*Pomacanthus semicirculatus*) is an extremely popular coral reef teleost. An outbreak occurred in Taiwan with 98% (53 out of 54) cumulative mortality within 10 days among the cultured sub-adult semicircle angelfish. The most significant gross pathological change was the enlargement of the spleen. Histopathologically, bacterial microcolonies were observed in the vascular blood of the gill and internal organs. The identification of micro-organisms isolated from internal organs was verified by multiplex PCR assay for *Photobacterium damselae* subsp. *piscicida* that gave the expected specific amplicon of 267 bp of the 16S rRNA sequence but no amplification of partial urease gene occurred. Additionally, partial sequence of the 16S rDNA gene of the isolate from the fish was also phylogenetically compared and produced 99.83% sequence identity with *P. damselae* subsp. *piscicida* (GenBank accession number KU245711). This investigation is the first published on *P. damselae* subsp. *piscicida* infection in farmed semicircle angelfish.

## Introduction

Photobacteriosis or fish (pasteurellosis) is a bacterial disease common among fish and is considered as one of the most devastating bacterial diseases in aquaculture with significant economic losses worldwide (Kusuda and Salati, 1993; Magariños et al., 1996). The causative agent of this disease, *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), has a broad host range, high mortality rate and ubiquitous distribution (Barnes et al., 2005). The pathogen is known to infect a wide range of marine fish, such as white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) in USA (Snieszko et al., 1964), yellowtail (*Seriola quinqueradiata*), ayu (*Plecoglosus altivelis*), seabream (*Acanthopagrus schlegeli*), red grouper (*Epinephelus akaara*), oval file fish (*Navodan modestus*) and hybrid striped

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bass (*M. chrysops*) in Japan (Kusuda and Miura, 1972; Romalde, 2002), gilthead seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*) in the Mediterranean area (Magariños et al., 1996), golden pompano (*Trachinotus ovatus*) in China and cobia (*Rachycentron canadum*) in Taiwan (Wang et al., 2013). The clinical signs of infected fish are varying but may be accompanied with granuloma-like lesions in the internal organs, characterised by white tubercles of 0.3-0.5 mm in diameter, an enlarged spleen and/or kidney, and multifocal necrosis in the liver, spleen and kidney and bacteria accumulation freely in phagocytes, capillaries and interstitial spaces (Magariños et al., 1996). In Taiwan, photobacteriosis in cobia caused by *P. damselae* subsp. *piscicida* has been reported by Liu et al. (2003). The main symptoms were granulomas in internal organs. The bacterium isolated from sub-adult cobia (chronic form) was found to be virulent to young cobia causing an acute form of the disease.

In the past three decades, culturing of coral reef fishes has changed drastically with the cultivation of new species (Asche et al., 2009). With the bulk of interest focused on spawning and development (Olivotto et al., 2006; Leu et al., 2009), there is still a paucity of information in the understanding and extent of diseases of these fish in aquaculture. In the present paper, we describe the first isolation and characterisation of a pathogenic *P. damselae* subsp. *piscicida* obtained from diseased semicircle angelfish (*Pomacanthus semicirculatus*) with high mortality during an outbreak of photobacteriosis in Taiwan.

## Materials and methods

### *Collection of fish samples*

The fifty-four captive-breeding semicircle angelfish reported in this study were farmed in 7000L fibreglass tanks in south Taiwan. The disease developed in May, 2017 in a tank with approximately one year old fish. The water quality parameters had been recorded in May including water temperature: 28.10±0.66 °C , salinity: 34.24±0.31 psu, dissolved oxygen (DO): 5.93±0.33 mg/L, pH: 8.31±0.03 and Ammonium-nitrogen (NH3-N): 0.05±0.04 mg/L. The fish had an average body weight of 8.8±2.8g and an average length of 6.8±0.9cm. The accumulated mortality was 98.2% (53/54) within 7 days. Ten fish were sampled for histopathology and bacteriology.

### *Bacteriology*

Sterile inoculating loops were taken from the spleen and liver, and streaked onto tryptic soy agar (TSA; Difco) including 1.5% NaCl or supplemented with 5% goat blood. These isolates were cultured at 25 °C for up to 48 h.

### *Pathology*

The kidney, spleen, liver, gill and other internal organs with lesions were fixed in 10% buffered formalin and processed for paraffin sectioning. The sections were stained with haematoxylin and eosin (H&E).

### *Biochemical characterisation*

Biochemical characteristics of the isolate was then determined at 25 °C using the API 20NE (bioMerieux) according to the manufacturer's protocol. The Gram reaction, catalase and oxidase activity, and motility were determined after 24 h.

### *Multiplex PCR assay*

Genomic DNA was extracted following a modification to the method described by Tsai et al. (2012). The PCR amplification was performed by using the oligonucleotide primers car1 and

car2, ure5 and ure3 according to the manufacturer's protocol and that of Osorio et al (2000). The specific primer set car1-car2 targets 16S rRNA gene of *P. damselae* and yields the 267 bp amplicons for *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*. Another specific primer set car1-car2 targets *ure*C gene of *P. damselae* subsp. *damselae* and yields the 448 bp amplicons for *P. damselae* subsp. *damselae*.

***16S rRNA sequencing analysis***

The gene fragment of 16S rRNA was amplified with PCR using the VB1 and VB6 primer modified from that of Chow and Clarridge (2014). The Tri-I Biotech Company, Taiwan, sequenced the amplicon. Sequences in GenBank databases were compared using BLAST (http://www.ncbi.nlm.nih.gov), to search the Entrez database for homologous sequences. Sequences were aligned using Clustal W. MEGA4. The topologies of unrooted phylogenetic trees, prepared by the neighbour-joining method, were evaluated by bootstrap analyses using 1000 times resampling.

***Experimental infections***

Healthy farmed semicircle angelfish (115.5±39.8 g body weight) were obtained from Pingtung, Taiwan with no history of disease, and held at a density of 6 fish in a tank with continuous aeration containing 80 L of sea water at approximately 25 °C for 7 days until they were acclimatised to laboratory conditions. Fish were fed twice daily with commercial pellets, and waste was removed daily. *P. damselae* subsp. *piscicida* strain NM106030S was grown on tryptic soy broth (TSB; Difco) including 1.5% NaCl at 25 °C for 24 h. A bacterial suspension was prepared in 0.85% saline solution to a final concentration of $10^8$ CFU $mL^{-1}$. The experimental group of six fish were then injected intra- peritoneally (ip) with 0.1 mL of bacterial concentrations of $10^7$ CFU $fish^{-1}$. Control group fish (n = 4) were injected ip with sterile saline. Following injection, each group was maintained separately in an 80 L aquarium under the same conditions as described above.

Finally, the fish were monitored continuously for morbidity and mortality and sampled for histopathological and bacteriological analyses. The experiment was terminated within 10 days following inoculation.

## Results

Clinical signs of spontaneously affected semicircle angelfish initially revealed mortality without external lesions. Gross pathological changes included liver and brain hyperaemia, enlarged spleen (Figure 1) and intestine filled fluid. Smears from fresh spleen, head kidney and liver of diseased fish indicated numerous bipolar rod shaped organisms under a light microscope. Histopathologically, bacterial micro colonies were observed in the vascular blood of internal organs, including liver, spleen, and gill etc. (Figure 2).

The bacteria were grown on TSA including 1.5% NaCl and TSA with 5% goat blood (BA) at 25°C for 48h and they represented only one type of colony on all culture plates. Table 1 shows the biochemical and physiological characteristics of bacterial strain NM106030S purified from diseased semicircle angelfish in Taiwan. The isolate was then examined using the API 20NE for the conventional biochemical tests. The bacterium was gram-negative short rod-shaped, non-motile, oxidase- and catalase-positive. Additionally, the phenotypic characterisation of the bacterium appeared to be positive for arginine

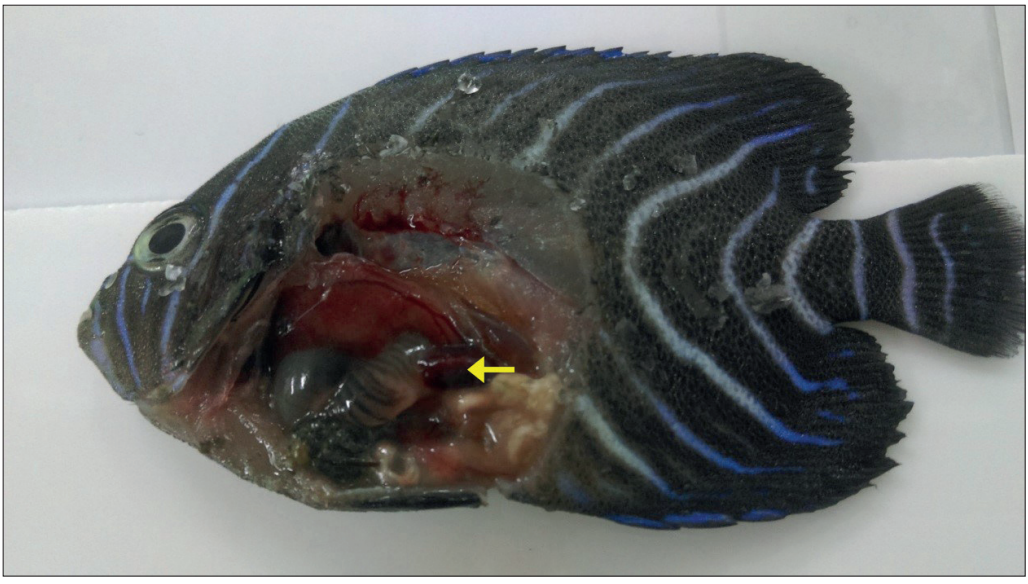

**Figure 1.** Infection of farmed sub-adult semicircle angelfish, *Pomacanthus semicirculatus* by *Photobacterium damselae* subsp. *piscicida*, showing enlarged spleen (arrow).

**Table 1.** Biochemical characteristics of *P. damselae* subsp. *piscicida* from semicircle angelfish using by microbiology and API 20NE.

| **Characteristic** | **NM106030S** |
|---|---|
| Gram | - |
| Shape | bacilliform |
| Oxidase | + |
| Catalase | + |
| motility | - |
| Nitrate | - |
| Tryptophan | - |
| Glucose (acidification) | - |
| Arginine | + |
| Urea | - |
| Aesculin | - |
| Gelatin | - |
| PNPG | - |
| Assimilation of: | |
| Glucose | - |
| Arabinose | - |
| Mannose | - |
| Mannitol | - |
| N-acetylglucosamine | - |
| Maltose | - |
| Gluconate | - |
| Caprate | - |
| Adipate | - |
| Malate | - |
| Citrate | - |
| Phenyl acetate | - |

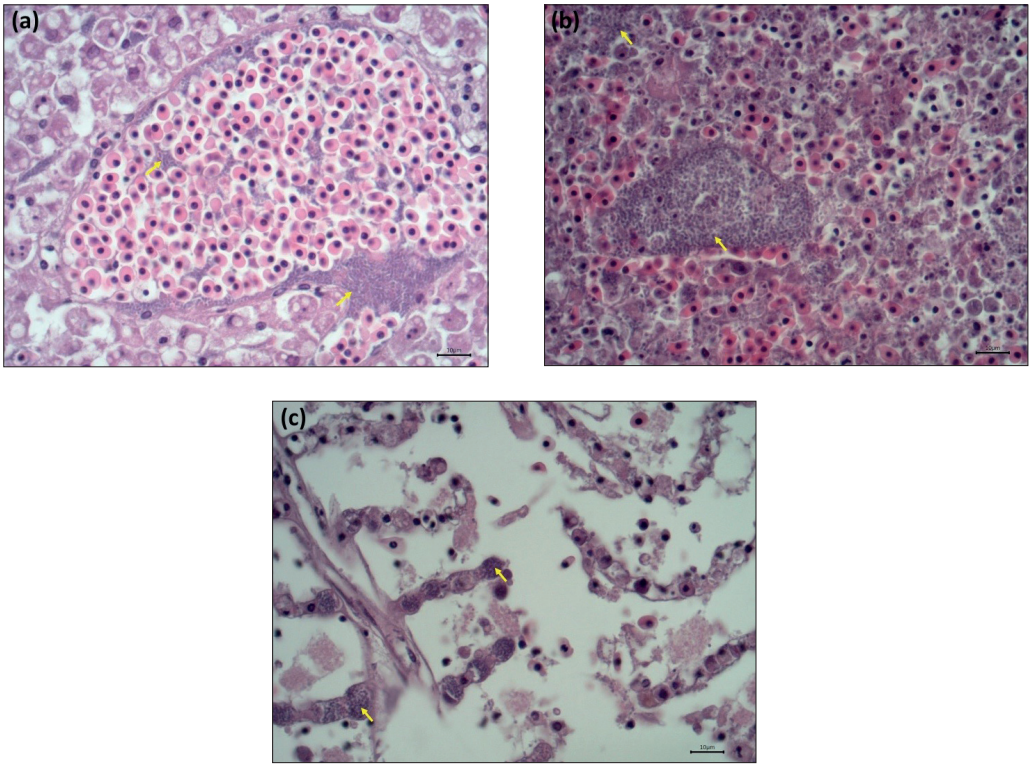


**Figure 2.** Histopathology of sub-adult semicircle angelfish, *Pomacanthus semicirculatus* infected with *Photobacterium damselae* subsp. *piscicida*. Histopathologically, bacterial microcolonies (arrow) were observed in the vascular blood of internal organs, liver (A), spleen (B), and gill (C). (H&E stain, 1000X).

and negative for the other 19 tests by API 20NE. The identification of micro-organisms isolated from semicircle angelfish was verified by multiplex PCR assay for *P. damselae* subsp. *piscicida* that gave the expected specific amplicon size of 267 bp of the 16S rRNA sequence but no amplification of partial *ure*C gene (448bp) (data not shown).

The 16S rRNA of the strain NM106030S (GenBank accession number MH472944) from the semicircle angelfish exhibited identities of 99.83% sequence identity with reference strains *P. damselae* subsp. *piscicida* (KF956381) and *P. damselae* subsp. *damselae* (EF635307). Using the software package CLUSTAL W, we have found that the strain forms a unique clade with two *P. damselae* subspecies as mentioned above, at a distance from other Gram-negative bacteria (Figure 3). This relationship was emphasised by the relatively high nucleotide similarity value and the high bootstrap support value based on the neighbour-joining method.

Six fish were injected intra peritoneally with $10^7$ CFU of bacterial strain NM106030S. Observed fish mortality rate was 66.7% (4/6) within 10 days of the inoculation. *P. damselae* subsp. *piscicida* were re-isolated from moribund and dead fish that were challenged with the isolated strain.

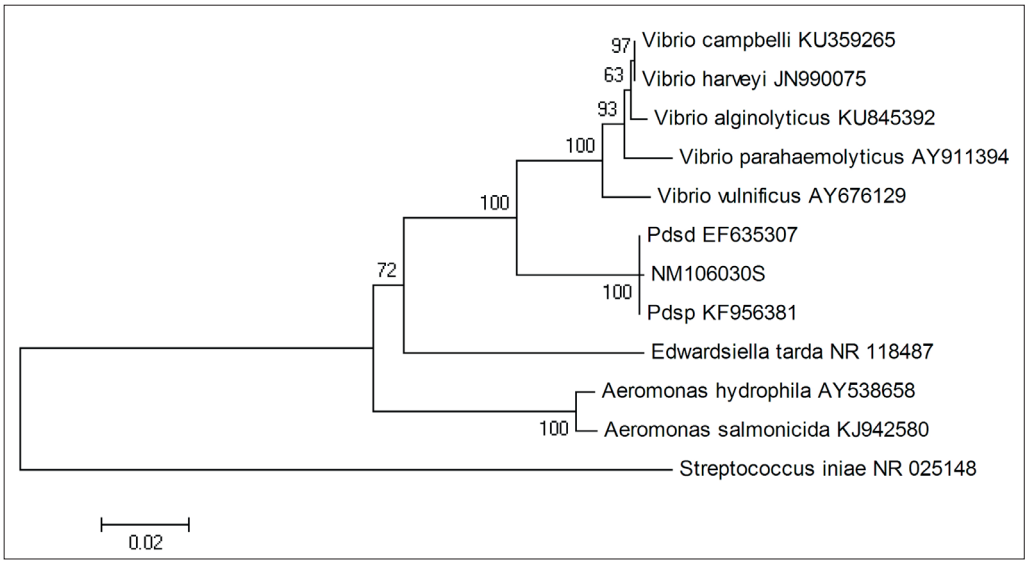


**Figure 3.** Phylogenic tree of the nucleotide sequence of 16S ribosomal RNA in NM106030S strain from semicircle angelfish (*Pomacanthus semicirculatus*) and reference strains using the Clustal W program of the MEGA 4 software package. The tree was generated by using the neighbor-joining method. Pdsp: *Photobacterium damselae* subsp. *piscicida*; Pdsd: *P. damselae* subsp. *damselae*.

## Discussion

Photobacterosis is also known as pseudotubercullosis because it is characterised by the presence, in the chronic form of the disease, of creamy-white granulomatous nodules or whitish tubercules in several internal organs (Romalde, 2002). However the mortality and histopathology were of the acute form in this case. During the study all the moribund/dead fish exhibited darkness in colour with no gross or external lesions. In addition they had enlarged spleens. The gross pathological changes observed in the dead semicircle angelfish were similar to those previously described for photobacterosis in other fish (Toranzo et al., 1991).

Gram- negative, short rod- shaped, non- motile bacterial micro colonies were isolated from the vascular of internal organs such as liver and spleen etc. The same characteristics of isolated bacteria from infected fish were recorded in several previous studies (Hawke et al., 1987, Toranzo et al., 1991, Liu et al., 2003, 2011; Wang et al., 2013). Although the pathogen is not included in the API-20NE code index, the system is valuable for biochemical characteristics examination of the bacterium. The strain produces arginine di-hydrolase and it was negative for the other 19 tests by API 20NE. The present findings are in accordance with Hawke et al., 1987, Margarinos et al., 1992 and Liu et al., 2003 in which the authors have found the same characteristics in the isolated strains in their study.

The confirmatory diagnosis of the isolated strain has been done by multiplex PCR assay for *P. damselae* subsp. *piscicida* in which the strain gave the expected specific amplicon of 267 bp of the 16S rRNA sequence but no amplification of partial *ure*C gene (448bp) occurred.

This difference in the multiplex PCR assay is the most accurate analysis to discriminate two sub-species of *P. damselae* subsp *damselae* and *piscicida* (Oscorio et al., 2000). Furthermore the 16S rRNA of the strain NM106030S from the semicircle angelfish exhibited identities of 99.83% sequence match with reference strains *P. damselae* subsp. *piscicida* (GenBank accession number KF956381) and *P. damselae* subsp. *damselae* (EF635307). This corroborates that the isolated strain from semicircle angelfish was *P. damselae* subsp. *piscicida.*

The accumulated mortality in the cultured semicircle angelfish within 7 days of the natural outbreak of photobacteriosis was 98.2%. The extracellular products (ECP) secreted by *Pasteurella piscicida* are one of the most significant virulence factors which are capable of being lethal to different fish species like rainbow trout, gilthead seabream, turbot and sea bass ( Magarinos et al., 1992) and for cobia (Liu et al., 2003). A previous study reported that the bacteria maintain their infectivity in fish via the intra- peritoneal route, whilst the lesions of organs are found to be associated with the blood circulation system (Fouz et al., 1998). The mortality rate of the intraperitoneally infected fish was 66.7% within 10 days in an artificial infection experiment. The average body weight of the naturally infected fish was 8.8±2.8g whilst the experimentally infected fish had an average body weight of 115.5±39. 8 g conveying that the experimentally infected fish were older than that of those who experienced the natural outbreak. The main reason for this may be the varying degree of susceptibility to photobacteriosis depending on the size and the age of the fish. A study by Noya et al. (1995) on gilthead seabream found that the age of the fish governs the susceptibility to the disease. Higher mortalities were found in juveniles compared to the mortality rate observed in infected adults owing to the adult's greater potential of producing an effective phagocytosis response and killing bacteria by neutrophils and macrophages.

Although the water quality records were normal for marine angelfish other potentially exacerbating activities may be attributable to the mortalities observed such as the feeding diet of Antarctic krill (*Eupfausia superba*) and various frozen raw fish, such as carangid and scombrid species. Furthermore transmission could be implicated through the supplemented sea water without ultra violet steriliser treatment, which may have led to pathogen spread to fish. However, further research is needed to confirm the transmission route in this case. Based on the present findings *P. damselae* subsp. *piscicida* has been identified by specific multiplex PCR as the causative agent of the natural outbreak of the photobacteriosis causing high mortality in ornamental coral reef fish, semicircle angelfish in Taiwan.

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