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Isolation of *Photobacterium damselae* subsp. *damselae* from zebra shark *Stegostoma fasciatum*

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Abstract : The zebra shark *Stegostoma fasciatum* which had been reared in the commercial aquaria was found dead and submitted for postmortem examination. A pure bacterial culture was isolated from pale and enlarged liver. The analysis of *ure*C and 16S rRNA genes confirmed the isolate as *Photobacterium* (*P.*) *damselae* subsp. *damselae* and this pathogen was sensitive to gentamicin. Although, no mortality in mouse was observed in the experimental infection study, the isolation of this pathogen in aquarium fish is significant because it can act as a reservoir to other aquatic animals and can also be zoonotic potential to human during aquarium management. This paper describes the first isolation of *P. damselae* subsp. *damselae* from zebra shark.

Keywords : aquarium, *Photobacterium damselae* subsp. *damselae,* zebra shark

Photobacterium (*P.*) *damselae* was originally described as new pathogenic *Vibrio* species, first isolated from damselfish *Chromis punctipinnis* [11] and its pathogenicity has been partly attributed to the production of a powerful cytolysin and exotoxins [10]. *P. damselae* includes Gram-negative marine bacteria that comprises the fish virulent isolates belonging to two different subspecies, namely, *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) [9] and *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damselae*) [13]. The two subspecies develop different diseases, but only subsp. *damselae* is pathogenic for humans [11]. Although, the pathogenicity to mammals constitutes one of the main characteristics to differentiate this subspecies from *P. damselae* subsp. *piscicida* [7], they also differ in important biochemical and physiological traits, as well as in host specificity [12].

P. damselae subsp. *damselae* [14] comprises fish-virulent strains isolated from clinical samples and diseased fish [1] that produce skin ulcers and haemorrhagic septicemia in a wide range of fish species (warm and cold water fish) such as damselfish *Chromis puncipinnis*, eels *Anguilla anguilla*, brown shark *Carcharhinus plumbeus*, yellowtail *Seriola quinqueradiata*, seabream *Sparus auratus* and turbot *Scophthalmus maximus* [6]. In all cases, the main external symptoms of infection with this bacterium are skin ulcerative lesions and extensive hemorrhages, especially in mouth, eyes and musculature [12]. Moreover, it may be a primary pathogen for mammals and it can cause not only wound infections but also primary septicemia in healthy human [4].

Zebra shark *Stegostoma fasciatum* occur in shallow coastal waters of the western Pacific and Indian Oceans and they are popular species for aquaria exhibition due to their ease of domestication and attractive appearance [5]. The zebra shark which had been reared and confined in the private commercial aquarium in Seoul, Korea for indoor exhibition was found dead and submitted to the College of Veterinary Medicine, Seoul National University for diagnostic examination. A pure bacterial culture was isolated from pale and enlarged liver. The result of the bacterial identification yielded *P. damselae* and antibiotic susceptibility and hemolytic

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test were conducted. The pathogen was further characterized and confirmed using PCR method for 16S rRNA and *ure*C genes [13] to further differentiate into the *P. damselae* subsp. *damselae*.

The dead zebra shark (body weight 9.45 kg, total length 132 cm) was reported to show abnormal swimming behavior, slow-moving and leaning towards the bottom of the aquarium, lethargy and loss of appetite for two weeks. After external examination was done, it exhibited dark coloration in the skin and extensive hemorrhages in musculature and urethra tubercle. While, internal examination showed the presence of yellowish fluid in the abdominal cavity, severe hemorrhages inside the stomach lining and liver was pale and enlarged.

Sterile swab from the liver specimen of the zebra shark was streaked onto brain heart infusion agar plates (BHIA) (Becton, Dickinson and Company, USA) supplemented with 2% NaCl (w/v) and the inoculated plate was incubated at 25°C for 24 h. A Vitek System2 test (bioMérieux, France) was used for the purpose of characterizing the isolate. The test was inoculated with the pure isolate and read as described by the identification kit and allowed to incubate overnight for automated reading of the reactions. The isolate was compared to taxonomical analysis according to Bergey's Manual for Determinative Bacteriology [3]. Although the isolate has similar Vitek System2 profile (data not shown), it was not possible to obtain an accurate identification using this rapid system.

The isolate was further confirmed using PCR assay. First, for extraction of bacterial DNAs, the colonies were grown in BHIA supplemented with 2% NaCl were picked and re-suspended in 500 µl of sterilized double distilled water. Bacterial DNA was then extracted by boiling bacterial cells for 5 min and centrifuged at 6,000 g for 5 min. Bacterial DNA was collected on the upper aqueous phase of the supernatant and then stored at –20°C until used.

For accurate identification and detection of pathogen, *P. damselae* subsp. *damselae* from pure culture, the PCR method and primers used in this study was adapted with slight modification as previously described [13]. A 20 µl PCR reaction mixtures containing DNA template, a 10 pmol of each primer (Bioneer, USA) and AccuPower PCR Premix (Bioneer, USA). The amplifications were carried out in a thermocyclers (T-personal 48; Biometra, Germany) with the following parameters: an initial denaturation step of 95°C for 4 min; 30 serial cycles of a denaturation step of 95°C for 1 min, 60°C for 1 min, and 72°C for 40 sec; and a final extension step of 72°C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis in 1% Tris-borate-EDTA buffer. Gels were stained with ethidium bromide (0.5 µg/ml), visualized and photographed under UV illumination.

Antibiotic susceptibility test was examined on Muller Hinton Agar (Difco, USA) and a test for hemolysis was conducted in the pure isolate using 5% sheep blood agar (Korea Media, Korea). Moreover, mice (about 12 week old) were injected intraperitoneally with 0.2 µl (1.18×10^8 CFU/ml) of *P. damselae* subsp. *damselae* isolate for infection experiment. Clinical signs and mortality was observed for 2 weeks.

After diagnostic examination, clinical signs observed in the dead zebra shark were similar to those previously described for vibriosis and pasteurellosis in other fish [2]. Also, from the result of Vitek 2, *P. damselae* was isolated and showed 99% probability. All these results indicate that the isolate is closely related to members of *P. damselae*. Although biochemical and physiological characteristics tests are useful in identifying the subspecies of *P. damselae,* it was further confirmed by PCR assay using 16S rRNA gene and *ure*C gene primers. The isolated strain showed both the amplification products of 267 bp and 448 bp, corresponding to an internal fragment of the 16S rRNA gene and *ure*C gene, respectively. As a result, it was confirmed to be *P. damselae* subsp. *damselae* and differentiated from *P. damselae* subsp. *piscicida* [13]. The result of the PCR profile of *P. damselae* subsp. *damselae* isolated from the zebra shark is shown in (Fig. 1).

The susceptibility pattern of the bacterial isolate from 25 antimicrobial drugs is shown in (Table 1) and this pathogen was considered to have strong sensitivity to gentamicin (10 µg). A hemolysis test showed a clear β-hemolysis and result of the identification revealed that the isolated bacterium was Gram-negative rod-cocci in shape and motile.

P. damselae subsp. *damselae* is known to be a pathogen for poikilotherms and mammals. The disease produced by *P. damselae* subsp. *damselae* can be transmitted through water [8]. This pathogen can be a threat to susceptible fish species such as those in crowded and stressful aquaculture conditions, where the spread of the disease could be accelerated by direct contact of fish [8]. There are many reports for the

Table 1. Antibiotics susceptibility test of *Photobacterium* (*P.*) *damselae* subsp. *damselae* isolated from the *zebra shark*

Antimicrobial discs	*P. damselae* subsp. *damselae*	Antimicrobial discs	*P. damselae* subsp. *damselae*
Amikacin (30 μg)	R	Gentamicin (10 μg)	S
Amoxicilin/Clavulanic acid (30 μg)	R	Kanamycin (30 μg)	R
Ampicillin (10 μg)	R	Nalidixic acid (30 μg)	R
Carbenicillin (100 μg)	R	Neomycin (30 μg)	R
Cefepime (30 μg)	R	Nitrofurantoin (300 μg)	R
Cefixime (5 μg)	R	Norfloxacin (10 μg)	I
Cefoperazone (75 μg)	R	Ofloxacin (5 μg)	I
Cefotaxime (30 μg)	R	Oxytetracycline (30 μg)	R
Chloramphenicol (30 μg)	R	Polymycin B (300 IU/IE/UI)	R
Ciprofloxacin (5 μg)	R	Tetracycline (30 μg)	R
Colistin (10 μg)	R	Tobramycin (10 μg)	R
Enrofloxacin (5 μg)	R	Trimethoprim (5 μg)	R

The category 'R' means resistant; 'S' means sensitive to antibiotic; 'I' means intermediate. Cited from reference No. 10.

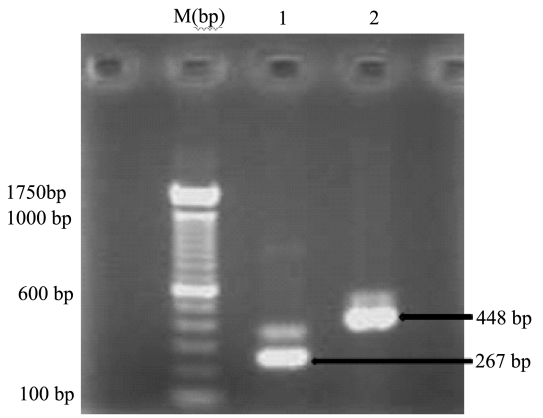

Fig. 1. Polymerase chain reaction (PCR) amplification product profiles of *Photobacterium damselae* subsp. *damselae* isolated from zebra shark *Stegostoma fasciatum*. Lanes M, molecular weight marker using 100 bp ladder; Lane 1, 16S rRNA gene; Lane 2, *ure*C gene. Size of amplication fragments (bp) is shown on the right.

isolation of *P. damselae* subsp. *damselae*, but no paper about the isolation of *P. damselae* subsp. *damselae* in the aquarium fish, until now only in captive and cultured fish.

This isolation *P. damselae* subsp. *damselae* is significant because the aquarium fish like zebra shark, can be a reservoir of this zoonotic pathogen that can be transmitted not only to other aquatic animals in aquarium and may also cause zoonosis to human during aquarium management. Although in the infection experiment, no clinical symptoms and mortality was observed in mouse at post inoculation of this bacterium the present isolate can be considered as non-virulent to mammals or human.

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