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Notes

Recovery of *Pseudomonas anguilliseptica* from Diseased Striped Beakperch (*Oplegnathus fasciatus*) in Korea

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In recent years, several southern coastal fish farms in Korea have experienced 2-30% mortality in striped beakperch suffering from bacterial infections during the spring. In this study, we identified a bacterium isolated from diseased striped beakperch as *Pseudomonas anguilliseptica* via a biochemical test and 16S rDNA sequence analysis. To evaluate the susceptibility of striped beakperch to *P. anguilliseptica*, 4.39×10^7 or 4.39×10^5 CFU/fish of bacteria were injected intraperitoneally at 18 ± 1°C into fish weighing 5.5 g. Cumulative mortalities reached 100% and 45% in the 4.39×10^7 and 4.39×10^5 CFU/fish infected groups, respectively. Experimentally infected fish showed cell-associated inflammation as well as bacteria in the kidneys and spleen. This study is the first report of striped beakperch mortality caused by *P. anguilliseptica*, which has pathogenicity in striped beakperch.

Key words: *Pseudomonas anguilliseptica*, Striped beakperch, 16S rDNA, Pathogenicity

Introduction

Pseudomonas anguilliseptica is an emergent and opportunistic fish pathogen that has gained clinical significance by causing mortality outbreaks in various fish species. It is the etiological agent of "red spot disease," also called "Sekiten-byo," in pond-cultured Japanese eels (*Anguilla japonica*) (Temminck and Schlegel) in Japan (Wakabayashi and Egusa, 1972). *P. anguilliseptica* has been also isolated from several marine fish including black sea bream [*Acanthopagrus schlegeli* (Bleeker)], ayu [*Plecoglossus altivelis* (Schlegel)], Atlantic salmon [*Salmo salar* (L.)], sea trout [*Salmo trutta* (L.)], gilthead sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), striped jack [*Pseudocaranx dentex* (Bloch and Schneider)], and cod [*Gadus morhua* (L.)] (Nakajima et al., 1983; Nakai et al., 1985; Berthe et al., 1995; Kusuda et al., 1995; Ferguson et al., 2004).

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Total production of cultured marine fish in Korea has increased during the past decade. Striped beakperch, *Oplegnathus fasciatus,* is one of the most important aquaculture species in Korea. To increase striped beakperch resources, artificial breeding methods have been developed, and large quantities of cultured fry have been released into Korean coastal waters. Striped beakperch is known to be particularly susceptible to megalocytivirus, which causes systemic infections and forms enlarged cells, necrosis of splenocytes, and hematopoietic cells during the summer season when the water temperature exceeds 22°C (Jung and Oh, 2000; Chao et al., 2004; Oh et al., 2006).

However, in recent years, mass mortalities of both juvenile and adult striped beakperch have occurred in early summer, when water temperatures are 16–19°C. At farms, naturally infected striped beakperch have shown slow and abnormal one-sided swimming on the water surface, as well as hemorrhagic lesions on the body surface and intestine.

This paper describes the clinical signs of the disease,

histological manifestations, characterization of the pathogen, and experimental infections of striped beakperch.

Materials and Methods

Dead or moribund striped beakperch (6 months old; 56-67 mm length) were collected in June 2006 from marine fish farms located at Dolsan-eup in Yeosu on the southern coast of Korea. Samples were processed for bacterial isolation and histological studies of the brains, kidneys, and spleens of the dead or moribund striped beakperch. The bacterium was incubated at 15°C for 1 week on brain-heart infusion (BHI) agar (Difco, Detroit, MI, USA).

Biochemical tests were performed at 15°C for 3 days using an API-20E kit (bioMerieux, Inc., Hazelwood, MO, USA) according to the manufacturer's directions. Catalase activity was determined by bubble production from 3% (v/v) H_2O_2, and oxidase activity was tested using 1% (w/v) tetramethyl-*p*-phenylenediamine.

To investigate the 16S rDNA of the bacterium, a 1454-bp-long PCR product was sequenced. The position was between 8 and 1512 (*Escherichia coli* numbering) (Weisburg et al., 1991). The fD1 primer was 5'-AGAGTTTGATCCTGGCTCAG-3', and the rP2 primer was 5'-ACGGCTACCTTGTTACGAC TT-3'. PCR conditions consisted of 30 cycles at 95°C (2 min), 57°C (30 sec), and 72°C (4 min). For the final cycle, the elongation time was extended to 5 min. Sequencing was performed using a DNA sequencing kit (Applied Biosystems, Tokyo, Japan) and the ABI PRISM 310 DNA sequencer (Perkin Elmer, Waltham, MA, USA). Pairwise evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980). A phylogenetic tree was constructed from the distance matrices by the neighbor-joining method (Saitou & Nei, 1987), and its topology was evaluated using GENETYX-WIN (Ver. 5.0) bootstrap analysis with 1000 replicates.

For the experimental infection trial, we infected fish with the bacteria isolated in this study via intraperitoneal injection (IP), and the effects of infection were investigated. Striped beakperch (*O. fasciatus*) weighing approximately 5.5 g were acclimatized in 500 L of aerated seawater at 18 ± 1°C for at least 2 weeks prior to use. The fish were fed twice a day with commercial striped beakperch pellets. Control and experimental groups of 20 fish each were held in 20 L tanks and maintained at 17°C. Experimental groups were intraperitoneally inoculated with *P. anguilliseptica* (4.39×10^5 or 4.39×10^7 CFU/100 μL/fish), whereas the control group was injected with 100 μL of PBS. After injection, 5% of the water was changed daily, and the fish were monitored for 14 days.

Results and Discussion

The isolated bacterium was aerobic, Gram-negative, and a filamentous rod. The isolate was identified as *Pseudomonas* sp. from biochemical and enzymatic profiles. Those bacteria grew at 10-25°C; however, they produced slow-growing colonies on BHI agar after incubation for 3-4 days at 25°C. The bacteria grew better on BHI with 2% NaCl than on BHI without salt. They were positive for catalase, cytochrome oxydase, nitrate reduction, and motility, but did not utilize organic carbon sources such as

Table 1. Biochemical characteristics of strains isolated from striped beakperch

Character	This study	Wiklund and Bylund (1990)	Kusuda et al (1995)	Type strain NCMB 1949
Gram stain	-	-	-	-
Cell morphology	rod	rod	rod	rod
Motility	+	+	+	+
Growth				
Temperature (°C)	10-25	5-30	10-30	5-30
NaCl%	0-3	0-4	0-3	0-4
Catalase	+	+	+	+
Cytochrome oxydase	+	+	+	+
Voges proskauer reaction	-	-	ND	-
Nitrate reduction	+	-	-	-
H_2S production	-	-	-	-
Gelatin hydrolysis	-	+ (3/16)	-	+
ONPG	-	-	-	-
Indole production	-	-	-	-

ND; not done.

glucose, mannitol, or citrate (Table 1). Preliminary

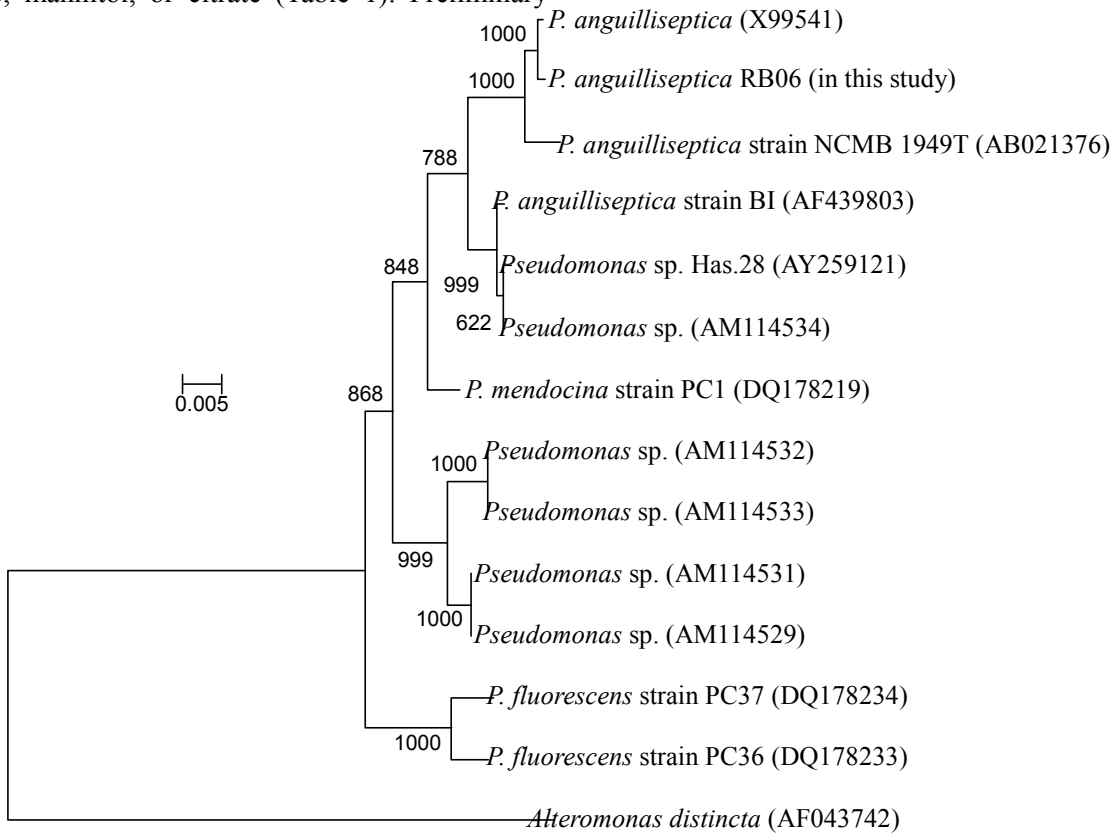

Fig. 1. Molecular phylogenetic tree for the genetic relationships among 14 isolates as based on the nucleotide sequence of 16S ribosomal DNA. Bootstrap values at 1000 times construction are shown at the major nodes in the tree. The scale bar is a genetic distance marker (number of replacement nucleotides per site). The number in parentheses indicates the GenBank accession number.

sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that strain RB06 was closely related to the genus Pseudomonas and showed the highest 16S rRNA gene sequence similarity with *P. anguilliseptica* (Accession number: X99541, 100 %) (Fig. 1).

Dead, moribund, and surviving fish were collected after 14 days for re-isolation of the bacteria and for histological examination. The results of the pathogenicity study are presented in Fig. 2. In the group treated with 4.39×10^7 CFU/fish (Fig. 2♦), the first mortality occurred 2 days after infection, and cumulative mortality reached 100% by the day 4 after injection. In the experimental group infected with 4.39×10^5 CFU/fish, the first mortality occurred on day 5 post inoculation (Fig. 2■), with cumulative mortality reaching 45% on day 6 post injection and remaining constant to the end of the experimental period. Mortality was not observed in the control group (Fig. 2▲). Domenech et al. (1977) reported that the average mortality of sea bass infected by *P. anguilliseptica* was approximately 10-15% and

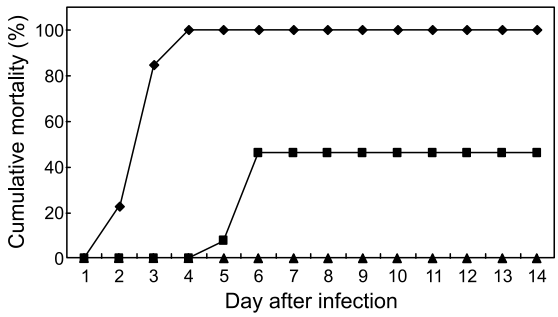

Fig. 2. Cumulative mortality of striped beakperch experimentally infected with *P. anguilliseptica* by intraperitoneal infection at 18°C. ♦, 4.39×10^7 CFU/fish; ■, 4.39×10^5 CFU/fish; ▲: PBS control.

reached 30% at some fish farms. In the case of eels in the Netherlands, mortality varied from 3% to 20% within 2-3 weeks of the initial outbreak (Haenen and Davidse, 2001). In our study, 20-30% of Striped beakperch with bacterial infections died. Water

temperature was approximately 16-18°C when mortality increased, and mortality began to decrease

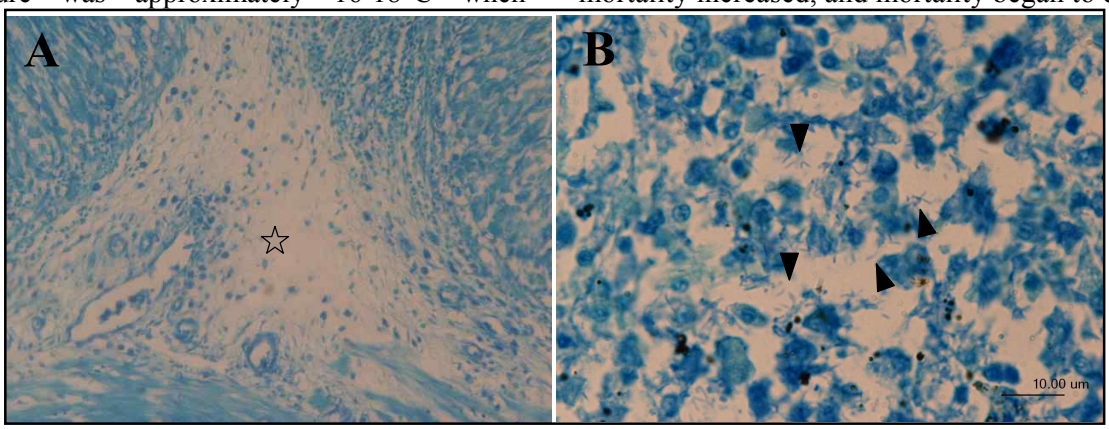

Fig. 3 Intestine (A) and spleen (B) of rock bream with *P. angquillseptica* infection showing surrounding inflammation (☆) and bacteria (head arrows).

as the water temperature exceeded 20°C. Earlier studies have shown relationships between bacterial diseases in several fish species and water temperature (Tort et al., 1996; Domenech et al., 1997, 1999; Haenen and Davidse, 2001; Ferguson et al., 2004). In the case of sea bream in Spain, water temperatures were below 12°C during outbreaks, and the disease persisted until the water temperature increased to 18-20°C (Doménech et al., 1997, 1999). For eels in the Netherlands, mortality ceased when the water temperature increased to 26-27°C (Haenen and Davidse, 2001). The optimum temperature for striped beakperch was 20-22°C (Oh et al., 2006). This study found that the optimum growth temperature for *P. anguilliseptica* ranged from 15 to 18°C. Under stressful conditions such as water temperature changes, the immune systems of fish become partially depressed and are more susceptible to infections by ubiquitous or opportunistic pathogenic bacteria such as *P. anguilliseptica* (Tort et al., 1996). Temperature is a well-known principal environmental cue in fish. To control a disease like 'winter disease,' we know that raising the water temperature above 26°C has a significant effect on mortality (Muroga et al., 1973).

Upon microscopic examination, the infected fish were found to have hemorrhagic lesions on their intestines, although no apparent external clinical lesions were detected on any of the dead or surviving fish examined. Also, bacteria were re-isolated from the brains, spleens, and kidneys of the dead fish in large numbers. Histopathologically, inflammatory cells had infiltrated the intestines, and bacterial infiltration was shown within the intestines, spleens, and kidneys of the affected fish (Fig. 3). The pathogenicity test and histopathological findings strongly suggested that the disease was caused by *P. anguilliseptica*.

This is the first study of *P. anguilliseptica* infection-caused mortality in farmed striped beakperch. Further studies should be performed to evaluate the pathogenicity and infectivity of *P. anguilliseptica* in rock bream at different temperature ranges.

Acknowledgements

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