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# Pathogenic *Photobacterium* sp. induces mortality in the lined seahorse (*Hippocampus erectus*): China's first case report

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**Abstract**

In August 2019, an unknown disease was first reported in farmed seahorses (*Hippocampus erectus)* in Binhai New Area in Tianjin city, China, with a cumulative mortality rate of 25% within seven days of onset. The main symptoms of the affected seahorses were discoloration of the body surface, abdominal distention, kidney erosion, and intestinal enlargement. Histopathological observation revealed damage to the diseased seahorses' intestines, liver, and kidneys. Strains HM-2019-5 and HM-2019-6 were isolated from diseased kidneys and identified as *Photobacterium* sp. based on physiological and biochemical tests and 16S rDNA sequencing analysis. Artificial infection experiments demonstrated that strain HM-2019-5 could cause a 90% morbidity rate in healthy seahorses. Antimicrobial susceptibility tests showed that HM-2019-5 and HM-2019-6 were resistant to 13 of 16 antimicrobial agents tested. This is the first report of photobacterial disease in a seahorse to the best of our knowledge.

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## Introduction

Seahorses (*Syngnathidae Hippocampus*) belong to the *Syngnathidae* family and the *Hippocampus* genus. They are named for their head, which resembles a horse's head and has high medicinal and ornamental value (Lin, Lin & Huang., 2010; Lourie et al., 1999). Seahorses require a highly-specific living environment, limited to a very narrow range of seagrass, mangroves, and corals in temperate and tropical shallow coastal zones. Due to the destruction of the ecological environment of coastal waters and predatory fishing in recent years, the population of seahorses as a natural resource has declined sharply. At present, seahorses are listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora as a protected species (Yang et al., 2006). Therefore, artificial breeding and farming are important for restoring seahorse stocks and mitigating the sharp decline in wild seahorse populations. However, pathogenic diseases significantly hinder the development of seahorse farming (Koldewey & Martin-Smith, 2010). Several pathogenic infections observed in seahorse farms have been reported, including bacterial pathogens like the *Vibrio* spp. *V. fortis* (Wang et al., 2016), *V. alginolyticus*, *V. splendidus* (Balcázar et al., 2010), *V. harveyi* (Alcaide et al., 2001; Qin et al., 2017; Tendencia, 2004), *V. rotiferianus* (Yang et al., 2017), *V. tubiashii* (Shao et al., 2020), *V. parahaemolyticus* (Lin et al., 2016), *Mycobacterium* sp. (Balcázar, Planas & Pintado, 2011; Fogelson et al., 2017), *Nocardia nova* (Dill et al., 2017), and *Tenaciba culumaestuarii* (Declercq et al., 2014), and parasites such as *Uronema* (Declercq et al., 2014), marine leeches, ciliates (Meng & Yu 1985), microsporidians (Vincent & Clifton-Hadley, 1989), and fungi (Blazer & Wolke, 1979).

In August 2019, a disease outbreak occurred in a seahorse farm located in Tianjin Municipality. The cumulative mortality of this disease was around 25% on the 7th day after the disease was first observed. Here we report the pathogenesis of this disease. The causative pathogen was isolated and identified as *Photobacterium* sp. based on morphological and biochemical characteristics and phylogenetic analysis of 16S rDNA gene sequences. This is the first report of Photobacterium sp. as a pathogen causing disease in seahorses to the best of our knowledge.

## Materials and Methods

Seahorse fish. Ten diseased seahorses (body length 10–12 cm) were collected from an artificial fish farm in Tianjin Municipality. Dying seahorses were loaded into oxygen bags and quickly sent to laboratories for diagnosis and pathogen isolation. Healthy seahorses (body length 8–10 cm) without observational signs of disease were bought from another fish farm in Zhanjiang, Guandong province of China.

Parasite check and pathogen isolation. Samples of fins, gill, mucus and visceral tissue from five diseased seahorses were examined under a microscope for parasites. After repeatedly wiping the surface of each seahorse with 75% alcohol, another five diseased seahorses were dissected under aseptic conditions, and small samples from the kidney, liver, and spleen from each seahorse were streaked and inoculated on 2216E and TCBS plates. The samples were cultured at 28°C for 48 h, before single colonies of the dominant bacteria were selected for further purification. Two strains were isolated and tentatively named HM-2019-5 and HM-2019-6.

Pathogen identification. Purified single colonies were plated on 2216E and TCBS plates to enable observations of colony morphology. Physiological and biochemical assays were performed using standard methods, including Gram staining, motility, and indole production. Genomic DNA extracted from strains HM-2019-5 and HM-2019-6 were used as templates for PCR, and the 16S rRNA sequences were PCR amplified using general primers 27F and 1492R (Lane, 1991). The reaction procedure was as follows: pre-denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 30 s; renaturation at 55 °C for 30 s; and extension at 72 °C for 90 s. The procedure was repeated for 35 cycles, followed by incubation for 10 min at 72 °C. The amplified products were sequenced by Sangon Biotech (Shanghai, China). The sequencing results were compared with the gene fragments in the

NCBI database registered in GenBank for homology, and a phylogenetic tree was constructed using MEGA 4.1.

Pathological investigation. To observe any pathological changes in the tissues and organs of diseased seahorses, the intestine, kidney, and liver were removed from dead seahorses and fixed with 10% neutral formalin. Fixed tissues were processed by routine histological procedures whereby 5-μm-thick tissue sections were stained with hematoxylin and eosin and examined under a light microscope. Tissues from healthy fish were used as controls.

Artificial infection. In total, 60 healthy seahorses were acclimated to our laboratory for two weeks. The water temperature, salinity, and dissolved oxygens were 24–26 °C, 30, and 6.3–8.5 mg/L, respectively. The bacterial strain HM-2019-5 was cultured for 24 h at 28 °C and 150 r/min followed by centrifugation at 6,000 × *g* for 10 min. The bacteria were then collected, and their concentration adjusted to $5.2 \times 10^7$ CFU/mL using sterile PBS. The 60 healthy seahorses were randomly divided into two groups, and the test group was intraperitoneally injected with 0.05 mL of the bacterial suspension.

In contrast, the control group was injected with 0.05 mL of sterile PBS. The seahorses were closely observed for 14 days, and any dead fish were removed immediately. Bacteria were re-isolated from the kidneys of dying seahorses. Physiological and biochemical reactions then identified the isolates.

Antimicrobial susceptibility testing. The susceptibility pattern of isolates HM-2019-5 and HM-2019-6 were tested using a previously-published method (Bauer et al., 1966). Briefly, 0.1 mL of a $10^8$ CFU/mL HM-2019-5 suspension was plated onto Mueller Hinton agar plates. Then, 16 types of drug susceptibility test papers (neomycin, ampicillin, Polymyxin B, streptomycin, norfloxacin, kanamycin, doxycycline, Levofloxacin, etc. gentamycin, ciprofloxacin, erythromycin, roxithromycin, tetracycline, florfenicol, vancomycin, and tobramycin) were positioned on each plate. The inhibition zone diameter was then measured after the vessels had been cultured for 48 h at 28 °C to determine the sensitivity of HM-2019-5 and HM-2019-6 to these drugs.

## Results

The main symptoms of the diseased seahorses were loss of appetite, slow response to external stimuli, abnormal swimming, and frequent floating on the water surface. Body color faded to brownish-black (**Figure 1B**). The abdomen was distended, with ascites in the abdominal cavity. No food was observed in the intestinal tract, swollen, loose and inelastic. In addition, eroded kidneys were observed (**Figure 1C**).

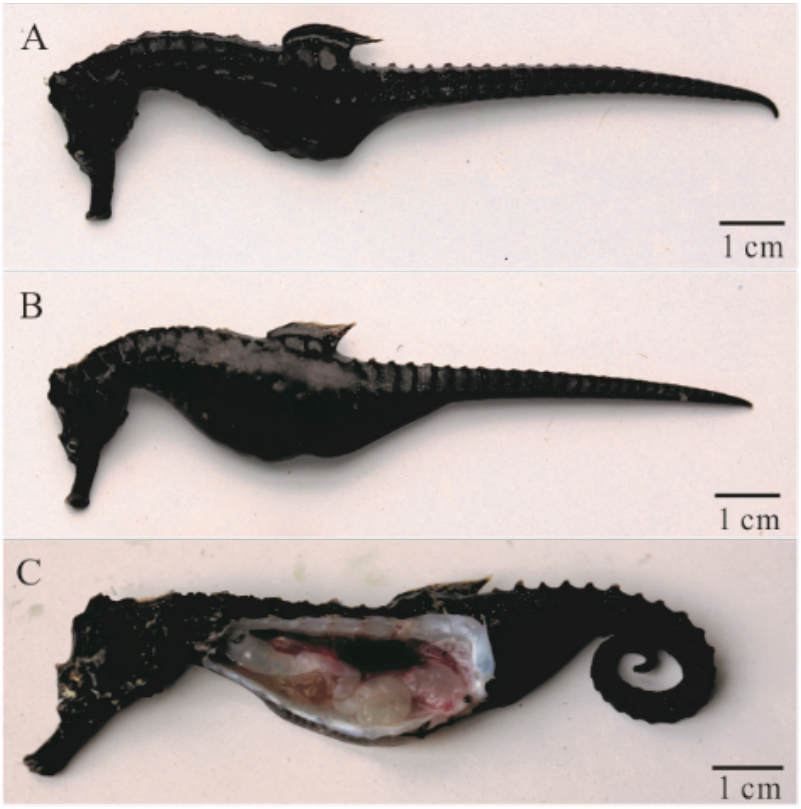


**Figure 1** Clinical signs of a diseased seahorse. Healthy seahorse (A). Body color faded, distended abdomen. (B). Loose intestinal tract and eroded kidneys (C).

Pathological examination of tissue sections suggested that some liver cells in the diseased seahorses appeared to have undergone fatty degeneration and necrosis. Liver cells were replaced by fat cells; blood vessels were congested with red blood cells (**Figure 2B**). Increased leukocytes were observed in the kidneys, together with tubular necrosis (**Figure 2D**). The intestinal muscle layer was thinned, the muscle and mucosal layers had become separated, and the intestinal villi and affected intestinal mucosa had necrotic changes and sloughed (**Figure 2E**).

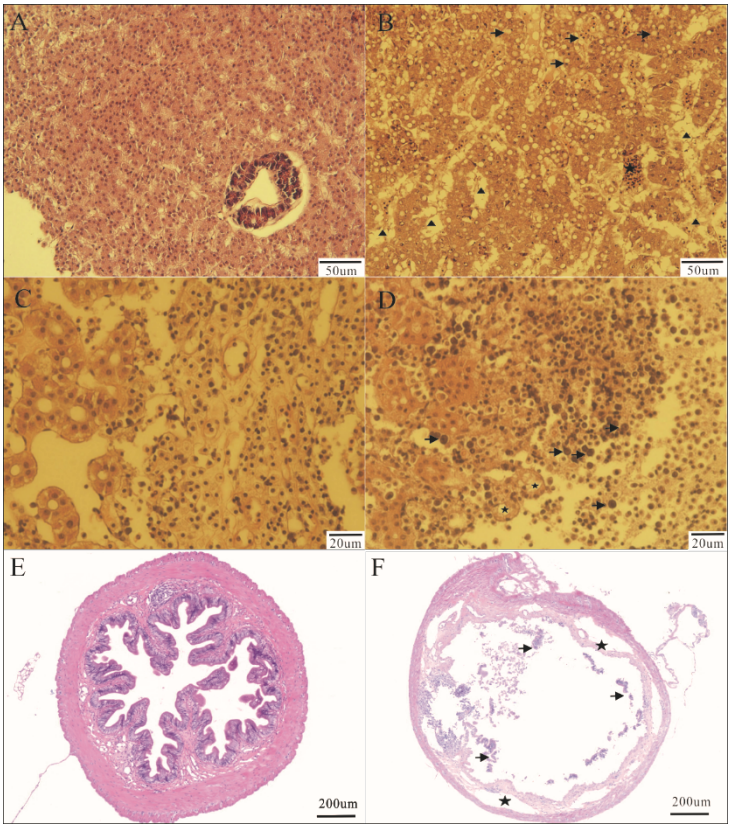


**Figure 2** Histological changes in the liver, kidney, and intestinal tract of a *Photobacterium* sp.-infected seahorse. (A) Normal parenchymatous appearance. (B) Diseased liver, showing many irregular vacuolations (➝). Liver cells were replaced by fat cells (▲), with large numbers of red blood cells accumulated in blood vessels (★). (C) Normal kidney appearance. (D) Affected kidney, showing vacuolar degeneration and necrosis of epithelial cells of the renal tubule (★). Increased numbers of leukocytes (➝). (E) Normal intestinal tract. (F) The intestinal tract, showing intestinal villi and mucosal tissue largely decayed and separated from the intestine lining (★), and severely degenerated mucosal epithelial cells (➝).

Ciliates were detected from gills and body surface mucus, while no parasites were observed in visceral tissues. The dominant colonies were isolated from the kidneys of the diseased seahorses, with two strains (HM-2019-5 and HM-2019-6) randomly selected for purification. After culturing on 2216E plates at 28°C for 48 h, the colonies could be described as round, moist, smooth, pale yellow, opaque, with neat edges, with a colony diameter of about 1.5–2 mm—blue-green colonies formed on TCBS medium.
Physiological and biochemical tests suggested that strains HM-2019-5 and HM-2019-6 were Gram-negative bacilli that had no capsule, were motile, had no hemolysis, were positive for oxidase and methyl red, and did not produce indole or hydrogen sulfide. In addition, they were able to utilize glucose, fructose, and mannose, but not sucrose, arabinose, rhamnose, mannitol, or melibiose (**Table 1**).

**Table 1** Physiological and biochemical identification of isolates HM-2019-5 and HM-2019-6

| Items | Result | | Items | Result | |
|---|---|---|---|---|---|
| | HM-2019-5 | HM-2019-6 | | HM-2019-5 | HM-2019-6 |
| Gram stain | - | - | Glucose | + | + |
| Motility | + | + | Mannose | + | + |
| Hemolysis | γ | γ | Fructose | + | + |
| Oxidase | + | + | Arabinose | - | - |
| Methylation | + | + | Rhamnose | - | - |
| Indole production | - | - | Mannitol | - | - |
| $H_2S$ production | - | - | Melibiose | - | - |
| Citrate utilization | - | - | Sorbitol | - | - |
| Spore production | - | - | Sucrose | - | - |

Note: "+"positive; "-"negative; "γ" hemolysis.

PCR amplification results showed that the 16S rDNA sequences of strains HM-2019-5 and HM-2019-6 were completely identical. Compared with the NCBI data library, the results showed that similarity with the *Photobacterium* sp. strain SJS7 (MG383477) was the highest at 99%. A phylogenetic tree was constructed by matching the registered 16S rDNA gene of *Photobacterium* in GenBank. The results showed that strains HM-2019-5 and HM-2019-6 converged into one branch and merged into a larger group with *Photobacterium galatheae* S2753, *Photobacterium halotolerans* BDH29, *Photobacterium ganghwense* ZR07, and *Photobacterium* sp. SJS7 (**Figure 3**). These findings, combined with physiological and biochemical characteristics, led to the identification of HM-2019-5 and HM-2019-6 as *Photobacterium* sp.

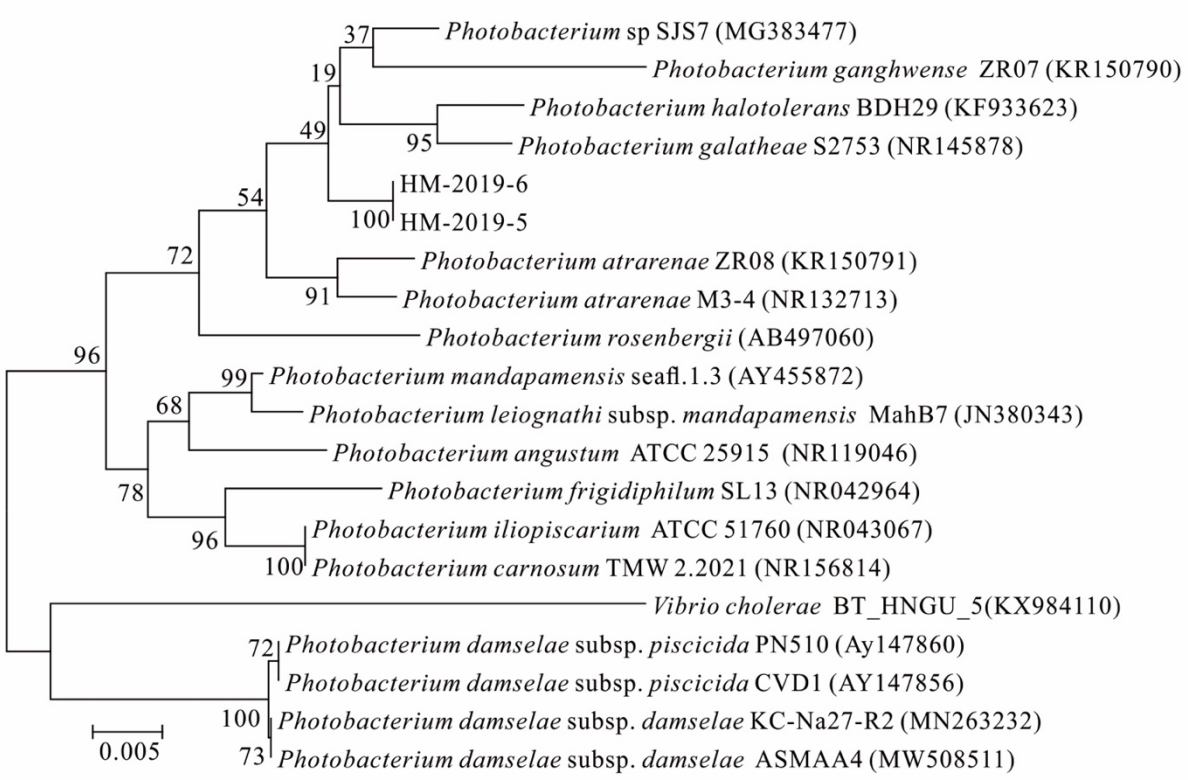


**Figure 3** Phylogenetic tree based on 16S rDNA gene sequences available in GenBank. Accession numbers of all strains are given in brackets.

Seahorses began to show disease and death from the 3rd day after artificial infection. The cumulative mortality rate was 90% by the 8th day (**Figure 4**). The main symptoms of affected seahorses were anorexia, discoloration, and abdominal distention. Bacteria isolated from dying hippocampal kidneys had the same physiological and biochemical features as strain HM-2019-5. The drug resistance of strain MH-2019-6 was consistent with that of HM-2019-5. Among the 16 antibiotics tested, the strain HM-2019-5 showed variable resistance to 13 of the antibiotics except for streptomycin, roxithromycin, and florfenicol (**Table 2**).

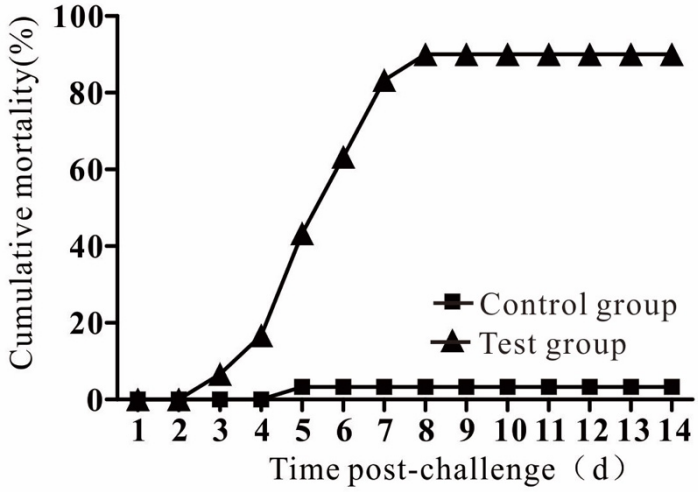


**Figure 4** Cumulative mortalities of healthy seahorses experimentally infected by isolate HM-2019-5.

**Table 2** Sensitivity of strains HM-2019-5 and HM-2019-6 to 16 different antibiotics.

| Antibiotics | Sensitivity | | Antibiotics | Sensitivity | |
|---|---|---|---|---|---|
| | HM-2019-5 | HM-2019-6 | | HM-2019-5 | HM-2019-6 |
| Tetracycline | I | I | Ampicillin | I | I |
| Neomycin | R | R | Norfloxacin | R | R |
| Streptomycin | S | S | Ciprofloxacin | R | R |
| Polymyxin B | R | R | Florfenicol | S | S |
| Doxycycline | R | R | Vancomycin | R | R |
| Kanamycin | I | I | Roxithromycin | S | S |
| Tobramycin | R | R | Gentamycin | R | R |
| Levofloxacin | R | R | Erythromycin | R | R |

Note: "S"sensitive; "I"moderately sensitive; "R"resistant.

## Discussion

From a taxonomic perspective, the genera *Photobacterium* and *Vibrio* are closely related and belong to the *Vibrio* family. Many *Vibrio* sp. bacteria, such as *V. anguillaru*, *V. parahaemolyticu*s, *V. alginolyticus*, *V. vulnificus,* and *V. harveyi*, can cause fish diseases, resulting in significant economic losses (Dang et al., 2021; Lazarte et al., 2021; Sony et al., 2021). However, except for *Photobacterium damselae*, there are few reports of the genus *Photobacterium* as a fish pathogen (Liu et al., 2003; Wang et al., 2007).

Recently, *Photobacterium ganghwense*, another bacterium belonging to the genus *Photobacterium*, was reported as a crab pathogen (Xu et al., 2012). In our study, the 16S rDNA gene sequences of HM-2019-5 and HM-2019-6 had a similarity of up to 98% with *P. ganghwense* and were clustered into one branch in the phylogenetic tree. This suggested that they have a very close kinship. This further increased the possibility that *P. ganghwense* was a pathogen of aquatic animals. The isolated strain HM-2019-5 was highly

pathogenic to healthy seahorses. Based on the physiological and biochemical tests and the 16S rDNA gene sequencing, strain HM-2019-5 was identified as belonging to the genus *Photobacterium*. This is the first report of *Photobacterium* causing seahorse disease and will inform the necessity for preventing and controlling seahorse diseases. In addition, more attention should be paid to other bacteria of the genus *Photobacterium* that could potentially be harmful to aquatic animals.

In addition to liver and kidney lesions in the affected seahorses, severe lesions were observed in intestinal tissue. Artemia (brine shrimp) was used to feed seahorses in the seahorse farms where the disease was identified. We found that *Artemia* contained large numbers of *Photobacterium* (data not shown). Therefore, we suspect that this disease may be initialized by intestinal infection originating from the food containing *Photobacterium* bacteria. Further investigation is needed to confirm the causality and identify suitable mitigation strategies such as disinfecting biological bait before feeding.

Drug susceptibility testing showed that strains HM-2019-5 and HM-2019-6 showed resistance or moderate resistance to 13 of the 16 drugs. This may be related to the heavy use of antibiotics in the aquaculture industry. To reduce the use of antibiotics, other methods, such as biological control and vaccines, should be given more attention.

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