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Describing factors behind the mortality of juvenile seahorses, *Hippocampus kuda* (yellow seahorse), in captive condition

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Abstract

Seahorse aquaculture succumb to a variety of drawbacks such as infections and diseases. Mortality rate and cause of diseases of juveniles remained a dilemma. In this study, cause of mortality of healthy 4-day old juvenile seahorses in captivity, *Hippocampus kuda*, were investigated. Pregnant male *H. kuda* donated from fishermen and brought to the laboratory gave birth to juveniles which contacted an infection leading to mass mortality. The infection was characterized by loss of motility and occurrence of thick bacterial sludge around whole bodies of seahorses, amassing in the base of the raising tanks. Dead juvenile seahorses (n=266) were subjected to postmortem examination. Morphological microscopic examination of deceased bodies, characterization and identification of bacterial isolates from water samples in rearing tanks were done. Morphological examination revealed no unusual noticeable signs in the bodies. However, bacterial samples collected, isolated and sub-cultured from water samples of rearing tanks revealed two distinct bacterial isolates and both tested as gram-negative with circular, smooth, and creamy-colored colonies. Moreover, DNA extraction and barcoding based on 16s rDNA gene sequencing identified both isolates as *Halomonas* sp. and *Cobetia* sp. respectively, and may be responsible for the infection and high mortality as observed. This study takes precedence in documenting and identifying bacteria aside from the genus Vibrio as stated in literatures, behind the mortality of juvenile seahorses in captivity.

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Introduction

Seahorse aquaculture had gained popularity due to increase market demand, especially for Traditional Chinese Medicine (TCM). The common seahorse (*Hippocampus kuda*) also known as the yellow seahorse or spotted seahorse is one of the several species of seahorses in its family that have attracted public attention for decades (LePage *et al.*, 2015). Basically, yellow seahorses have higher market value in aquarium trading and is one of the most traded seahorses in the Philippines. The high demand for this species in the use for traditional medicines, as ornaments, live aquarium pets, and even food resource is due to its great proportions, even texture, and light-colored pigmentation (Vincent, 1996). Its smooth appearance and pale yellow complexion are preferred by overseas markets of both traditional Chinese medicine and patent medicinal preparations (Celino *et al.,* 2012). While researchers have learned much about seahorse biology and ecology, a proposed resolution for many cases in marine conservation is captive culture or syngnathid aquaculture and conservation (LePage *et al.*, 2015). Although, captive-breeding and rearing of seahorse in conservation facilities has proven challenging to enthusiasts since high susceptibility of diseases from various pathogens, unsuitable nutrition and environmental conditions are encountered (Raj *et al.*, 2010; LePage *et al.*, 2015). Disease outbreak in aquaculture is a final product of a progression of connected events which includes association between host, the environment and the pathogen (Snieszko, 1974). As reported by Vincent and Clifton – Hardley (1989), captive seahorses experience ill effects due to fungal, bacterial, for example, *Vibrio harveyi* (Alcaide *et al.,* 2001; Koldewey, 2005) and parasites (e.g. *Glugea heraldi*).

Herewith, determining factors behind mortality of juvenile seahorses could be of great aid in seahorse aquaculture and conservation. The results can be a useful tool for the recovery of wild seahorse populations in such a way that it can set up a standardized and improved rearing procedure to ensure effective growth and survival rates in the early development of newborn. Thus, this study is important.

As of today, there has been developing interest for rearing seahorses (Job *et al.*, 2002). However, one of the bottlenecks for seahorse aquaculture is the low juvenile survival that is frequently experienced in the initial months of raising (Payne and Rippingale, 2000). As indicated by Moreau *et al.* (2000), seahorse culture has demonstrated in fact challenging fundamentally as a result of issues with eating routine and infections. The multiple causes behind mortality may include environmental conditions, stress, parasitic infection, and bacterial infection. Recorded literature show Vibrio bacteria commonly found on aquatic animals. Some Vibrio species that are pathogens on marine fish species include *Vibrio anguillarum, V. ordalii, V. harveyi, V. splendida, V. orientalis, V. fischeri.* Several studies reported Vibrio that causes disease in seahorses, the first signs were anorexia, lethargy swim, pale tail and fin, white patch, deep skin ulcers (Vincent and Clifton-Hadley, 1989; Austin and Austin, 1993; Alcaide *et al.,* 2001).

Yet, there is still a dearth of information that remained to be unraveled. Hence, the significance and imperativeness of achieving a stable rearing condition promotes for the advancement of growth development and for the improvement of disease resistance and survival rate.

Moreover, as indicated by LePage *et al.*, (2015) in their investigation of certain seahorse populace, the three noteworthy reasons for morbidity and mortality were bacterial dermatitis, bilaterally symmetrical myopathy and mycobacteriosis. Further research associated with syngnathid condition are gradually emerging however, there are just practically little to no distributed writings and studies up to date regarding certain microbes under family Halomonadaceae causing mortality particularly in reared juvenile seahorses.

Hence, this study was conducted to determine factors behind mortality of juvenile seahorse, *H. kuda*, under

captive condition. This research provides qualitative description of possible sources of diseases and mortality of juvenile seahorse, in captivity. Important findings help in improvement of disease resistance, survival rate which is necessary for success in aquaculture for conservation purposes and recovery of populations.

Materials and methods

Sampling area and collection of juvenile seahorses

Seahorses, *Hippocampus kuda*, were readily available in the Premiere Research Institute of Science and Mathematics (PRISM) of MSU-IIT, Iligan City, Philippines which were reported to come from the coastal waters of Tubod, Lanao del Norte (Fig.1) donated as live and bycatch samples from fishermen. Hence, such opportunity was taken advantage to study factors behind mortality of juvenile seahorses.

Fig. 1. Geographical location of the coastal areas of Tubod, Lanao del Norte where the samples were collected. (Sources: https://www.google.com/maps).

Adult pregnant male seahorse was maintained in a rearing tank where it was fed once a day with *Artemia* sp. After being released from the brood pouch of the male seahorse, all juveniles were moved into separate rearing tanks and was fed for once a day with *Artemia* sp. and zooplankton from the seawater. Upon regular cleaning of the holding tanks, deceased juvenile seahorses were collected and were used in the study. Samples were preserved in aseptic containers (vials) with ethanol for preservation.

Microscopic examination of deceased juvenile seahorses

The specimens were brought to the laboratory to be examined under the stereo optical microscope in 10x and 30x magnification. The morphological characteristics of the deceased juvenile seahorses were noted and images were taken. Diagnosis and descriptions were made based on related literatures.

Isolation of bacteria from water sample

To avoid a dense culture of cells from the water sample, serial dilution was used to reduce it to a more usable concentration. At least six (6) test tubes containing 9ml of filtered seawater were prepared. Using a sterile pipette with sterile tips, 1ml of the water sample was added in the first test tube of the set which were then mixed by swirling upside down few times. From the first tube, 1ml of the sample was transferred to the second tube. The procedure was repeated with all the remaining tubes.

From the dilution series, 0.1ml of the solution was pipetted out onto the center of the surface of the marine agar plate. Employing aseptic technique, an L-shaped glass rod was used for the spread plate method to evenly distribute a small volume of the sample on the agar surface. The plates were inverted and wrapped with paper for incubation 24 hours at room temperature.

To prepare for agar plates, desired amount of marine agar and filtered seawater was measured and cooked into a clean flask for 1 minute. Flasks were covered with aluminum foil and was sterilized at 121°C for 15 minutes. The medium was cooled before pouring onto the plates.

After 24 hours, bacterial colonies were visible on the surface of the agar plates. Individual colonies were examined under the stereo microscope and manually picked using sterilized loop for pure culture. Marine broth was prepared using nutrient broth and filtered seawater to cultivate the isolated marine bacteria.

After 24 hours, the cultivated pure culture of bacteria in the broth were spread over the agar surface of prepared agar slants for identification.

Morphological Cell Characterization and Colonies

Colony morphology characterization was based on form, margin, color, elevation and surface of the whole colony (Fig. 2) (Reynolds, 2011). Descriptions were compared to Bergey's Manual of Determinative Bacteriology (usually named Bergey's Manual) that portrays most of bacterial species.

Moreover, Gram stain technique or Gram staining, (Gram's method), was also used to distinguish and classify bacterial species into Gram-positive or Gram-negative bacteria by applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture from the agar slant to the microscopic slide followed by the addition of iodide, which binds to crystal violet and traps it in the cell. Rapid decolorization with ethanol or acetone and lastly counterstaining with safranin.

All procedures were done in the laboratory at the Premiere Research Institute of Science and Mathematics (PRISM) of MSU-IIT, Iligan City, Philippines.

DNA Extraction and Barcoding

Bacterial DNA was extracted using Quick Bacteria Genomic DNA Extraction Kit (Dongsheng BIOTECH) and following the manufacturer's procedures. The extracted genomic DNA was then sent to Macrogen, Inc. Laboratory in Korea for DNA barcoding using unidirectional 16s rDNA gene sequencing. PCR amplification was done using the paired primers 27F (5'-AGATTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Deobagkar *et. al*, 2012). PCR amplification was carried out within the standard PCR conditions: Pre-denaturation of 94°C for 1 min; followed by 30 cycles of denaturation at 94°C for 30 sec; Annealing at 55°C for 30 sec; Extension at 72°C at 1 min.; Termination of 72°C for 10 min. and cooling temperature at 4°C. Obtained 16s gene sequences were aligned by Sequencer 4.1.4 and results were compared with GenBank references sequences using BLAST.
(www.ncbi.nlm.nih.gov/blast/).

Results and discussion

Seahorse aquaculture is of main significance and could be a potential answer for assurance of wild populace while giving an economically suitable exchange (Vincent, 1996). However, infections are a noteworthy concern in the rearing and support of captive reared seahorses especially juveniles because of their vulnerability to various pathogenic microorganisms in research facility and aquaculture restriction (Koldewey and Martin-Smith, 2010).

Morphological characteristics of deceased juvenile seahorse

Deceased juvenile seahorses, *Hippocampus kuda*, (n=266) were examined under stereo microscope for postmortem morphological characteristics. Based on Fig. 3 microscopic examination of juvenile seahorses showed no unusual clinical signs or any physical deformations linked to the infection. The trunk rings

were progressively articulated. The entire body was coated in hard plates and was intensely pigmented. Also, the head was moderately huge in extent to the entire body, while the tail was the opposite. The nose was very much developed and functional before the juveniles got infected (Choo and Liew, 2006). Deceased juvenile seahorses have experienced an asymptomatic condition where the infected fish may die before any obvious lesions appear. There were no changes in the postmortem morphological characteristics of juvenile seahorses. As indicated by Francis-Floyd and Yanong (2002) younger fish may be infected with disease but not showing any actual physical signs of infection may be largely related to the slow progression of the disease. However, they may develop a recurrent progressive disease as they mature and become much more susceptible or get increasingly inclined to stress and pressure. Hence, further analysis on the water samples, isolating bacterial colonies and barcoding were necessary to fully describe and determine the sudden cause of mass mortality of juvenile seahorses.

Table 1. Morphological characteristics of isolated pure bacterial colonies from water samples in the rearing tanks of the deceased juvenile seahorses.

Bacterial Isolates	Characteristics			
	Form	Elevation	Margin	Color
I-1	Circular	Convex	Entire	Creamy-white
I-2	Circular	Convex	Entire	Creamy-white

Mass mortality of juvenile seahorses, *Hippocampus kuda*, have occurred. The infection was characterized by loss of motility and occurrence of thick bacterial sludge around whole bodies of seahorses, amassing in the base of the raising tanks. Deceased juvenile seahorses were often found recumbent at the bottom of the rearing tank days after they were released from the brood pouch of a pregnant male seahorse, *Hippocampus kuda*, covered with a dense biofilm.

Characterization and Identification of bacteria from water sample

Table 1 and Fig. 4 shows the morphological characteristics and gram-stain reaction of the bacterial isolates from the water samples obtained from the rearing tanks. Herewith, two distinct bacterial isolates were identified, both tested as gram-negative with circular, smooth, and creamy-colored colonies that somehow resembles and presumptively belong to the genus Vibrio. However, further examination, the two distinct colonies isolated and characterized were identified through DNA barcoding based on unidirectional 16s rDNA gene sequencing and was found out to belong to family Halomonadaceae. Resulting sequences were blasted to NCBI database and bacterial isolates, I-1 and I-2, were confirmed to be linked to the following genera Halomonas and Cobetia, respectively, the genus *Halomonas* (Vreeland *et al.*, 1980) and the genus Cobetia that was initially depicted by Cobet *et al.* (1970) as *Arthrobacter marinus*. Previously, it was also assigned as *Pseudomonas marina* (Baumann *et al.*, 1972), *Deleya marina* (Baumann *et al.*, 1983) and *Halomonas marina* (Ibacache-Quiroga *et al.*, 2017).

The genus Cobetia is a marine bacterium accommodated under the class Gammaproteobacteria. This marine bacterium is an aerobic, Gram-negaive, straight, rod-shaped cells. The morphological characteristics of this bacteria isolate were similar to those reported previously by Arahal *et al.* (2002). Colonies are round in shape, bright, with smooth texture and cream pigmented. However, there are only limited studies regarding on its possible pathogenic potential to marine organisms.

A study by Kim *et al.* (2010) represents a slightly halophilic bacterium strain belonging to the genus *Cobetia* based on analysis of molecular biology and biochemical and phenotypic properties. *Cobetia crustatorum* sp. was isolated from Jeotgal, a traditional Korean fermented seafood made from thin-shelled surf clams. Strain of strongly associated

organisms could be similar to a variety of features including pathogenicity.

Moreover, Halomonas is also a member from the class Gammaproteobacteria portrayed as an aerobic, Gram-negative rod-shaped cell, halotolerant microorganisms and are generally circulated in saline environments (Romanenko *et al.*, 2002). When cultured in a solid medium, colonies are viewed as white or yellow, ending up light brown after prolonged brooding. The first report of a pathogenic potential action of Halomonas species was on scallop hatchlings by Rojas and Miranda (2009) as biofilm-producing bacteria infecting Chilean scallop incubation facilities. A bacterial strain was described biochemically and was distinguished by polymerase chain response enhancement of 16S rRNA as *Halomonas* sp. (Accession number DQ885389.1).

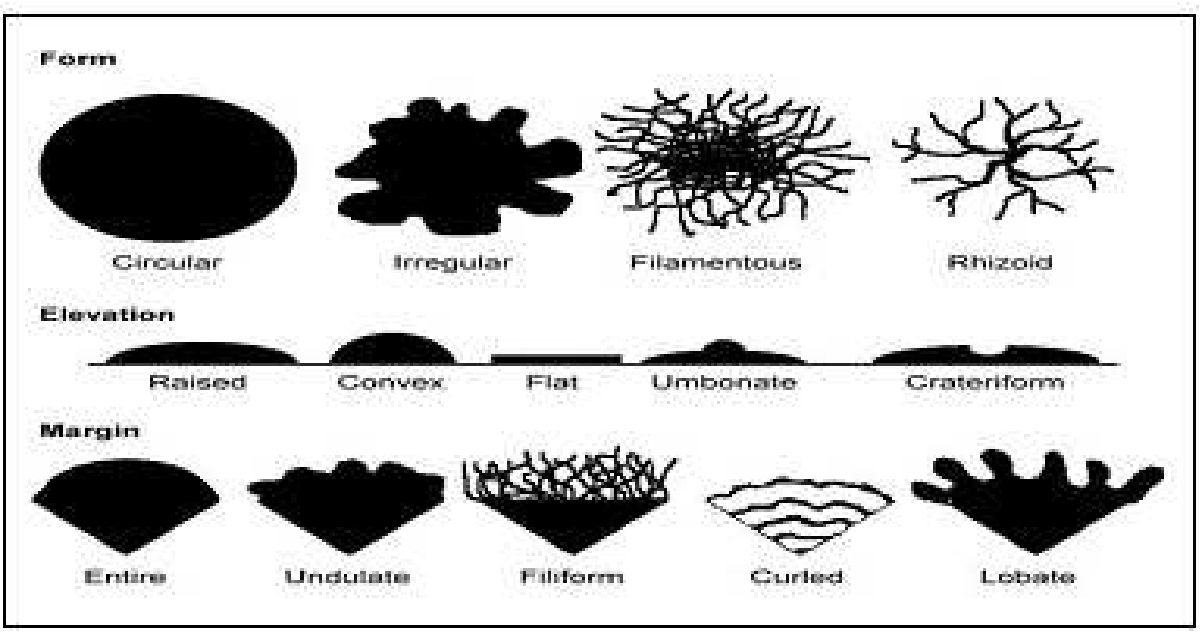

Fig. 2. Morphological Characteristics of bacterial colonies.

In addition, three species with conceivable pathogenic capability of *Halomonas* sp. were accounted for to have been isolated from marine spineless creatures as their symbionts (Kim *et al.*, 2013). The marine bacterium strain *Halomonas halocynthiae* was isolated from gill tissue of the ascidian *Halocynthia aurantium* which inhabits the coastal waters of the Sea of Japan (Romanenko *et al.*, 2002), *Halomonas zhanjiangensis* sp. nov. is a halophilic bacterium that was isolated from a sea urchin (*Hemicentrotus pulcherrimus*) collected from the South China Sea (Chen *et al.*, 2009), and *Halomonas profundus* sp. nov., a new polyhydroxyalkanoates (PHA) producing bacterium isolated from a deep-sea hydrothermal vent shrimp (Simon Colin *et al.*, 2008).The strain *Halomonas halocynthiae* was isolated from the gill tissue of an ascidian *Halocynthia aurantium* from the coastal sea water in Troista Bay as indicated by Romanenko *et al.* (2002). This marine bacterium strain belongs to the *Halomonas* cluster, phylogenetically (Ventosa *et al.*, 1989) however according to Dobson and Franzman (1966) it is different from the *Halomonas* description to some extent due to its phenotypic and chemotaxonomic characteristics.

Halomonas zhanjiangensis has also been documented to have pathogenic potential causing an infection to a sea urchin (*Hemicentrotus pulcherrimus*) gathered from a salt marsh of Naozhou Island in the South China Sea, close to a southern city, Zhanjiang (Chen *et al.*, 2009). The cells of this bacterial strain are presented as slightly halophilic, aerobic, Gram-negative, non-sporulating rods that occurs individually or as doublets and motile with peritrichous flagella.

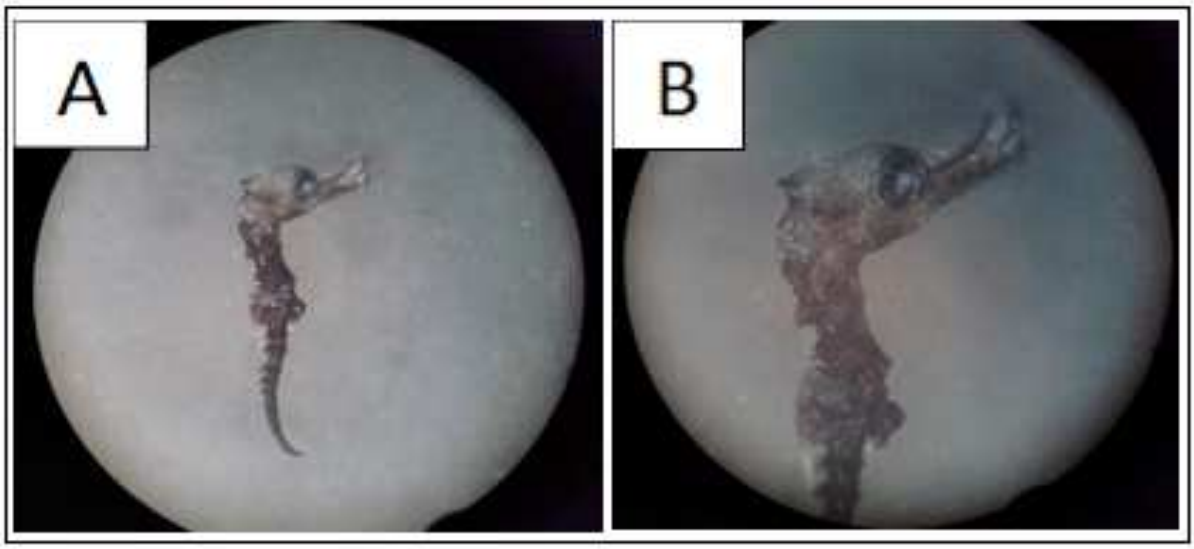

Fig. 3. Postmortem morphological characteristics of a representative juvenile seahorse viewed under stereo optical microscope in (A) fixed and (B) zoom or pancratic magnification.

Halomonas profundus was a recent marine pathogen producing PHA extracted by Simon Colin *et al.* (2008) from a deep-sea hydrothermal vent shrimp. This marine bacterium strain is characterized as motile, mesophilic, aerobic, Gram-negative, and mildly halophilic rod.

There were only several studies conducted to validate the pathogenicity of *Halomonas* sp. to numerous marine organisms. On the other hand, bacterial strain from the genus Cobetia was isolated from the water samples in the rearing tanks of the deceased juvenile seahorse, *Hippocampus kuda*, for the first time.

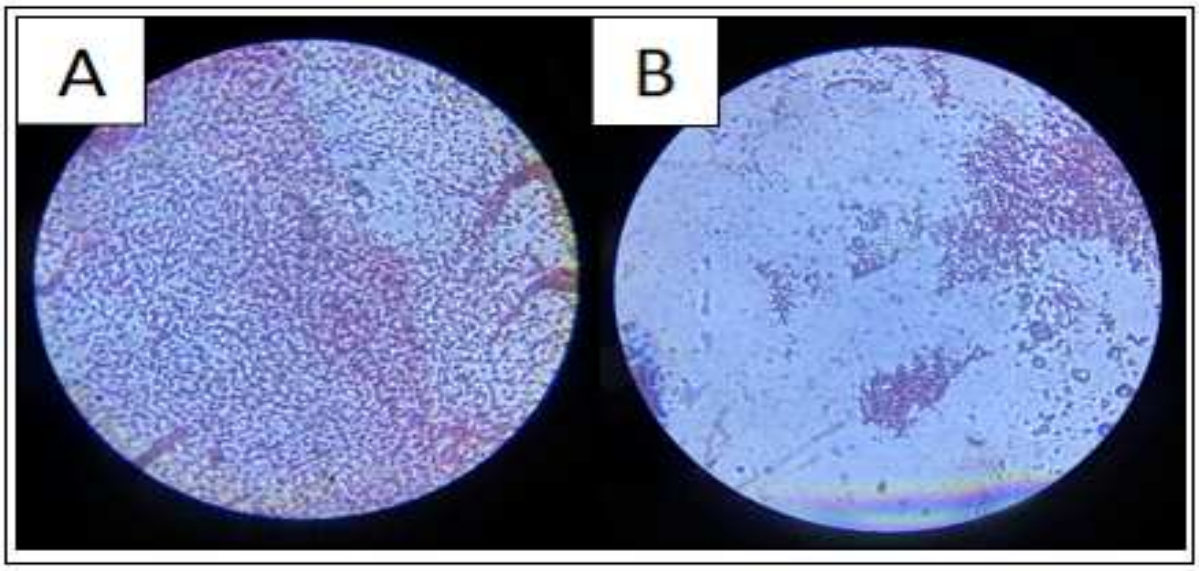

Fig. 4. Gram-negative rod-shaped cells (A) *Halomonas* sp. and (B) *Cobetia* sp. isolated from water samples in the rearing tanks of the deceased juvenile seahorses.

As a result of inadequate literature on its probable pathogenic action, there would be poor survival level of reared seahorses. However, since the genera Halomonas and Cobetia are under the same family Halomonadaceae, it is possible that both have a degree of similarity on pathogenicity which was a possible cause behind the mortality of the juvenile seahorses in captive condition. Various species of syngnathids are presently effectively raised in culture, some even on a commercial scale (Forteath, 1997).

Although no clarifications were given for these practices, Thampi Raj (2002) thought that environmental parameters such as temperatures could be the one plausibility that might be able to encouraged bacterial development in the rearing tanks and animals in overloaded tanks may be inclined to create infections.

Noteworthy, in the past different examinations about seahorse condition proposed Vibrio to be the main culprit with high mortality causing white patch diseases (Thampi Raj, 2002) and vibriosis (Alcaide *et al.*, 2001) with noted symptoms such as external white patches and anorexic conditions, and external haemorrhages and haemorrhagic liver, respectively.

Balcázar *et al.* (2010) also reported it as an opportunistic pathogen to a wide range of marine organisms. However, for this study, the two bacterial strains identified in deceased juvenile seahorses under captive condition does not belong to the genus Vibrio contrary to what is commonly recorded in literature.

Conclusion

From postmortem microscopic examination, juvenile seahorses showed no unusual clinical signs or any physical deformations linked to the infection. Based on the morphological characteristics of the colony and further species identification, two distinct bacterial isolates from the water sample of the rearing tanks of the deceased juvenile seahorse, *Hippocampus* kuda, were successfully barcoded to belong to *Halomonas* sp. and *Cobetia* sp. under family Halomonadaceae for the first time which could be a factor behind the mortality of the juvenile seahorses under captive condition.

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