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Experimental İnfection and Treatment of *Vibrio alginolyticus* and *Vibrio splendidus* in Captive Bred Spotted Seahorse, *Hippocampus kuda* Larvae

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Abstract

Due to their distinctive look, seahorses are famous in the aquarium trade and traded worldwide for their alleged therapeutic benefits. The present study was conducted to identify the causative agents for larval mortality of *Hippocampus kuda* and to find a treatment to reduce larval mortality at an ornamental fish hatchery in Sri Lanka. Bacteria causing vibriosis in *H. kuda* larvae were isolated and identified using morphological and biochemical tests. The disc diffusion method was used with 30 µg of chloramphenicol, streptomycin, tetracycline and ampicillin to evaluate antibiotic susceptibility of isolated bacteria. As pathogenic vibrios were more sensitive to tetracycline, the experimentally infected *H. kuda* larvae were exposed to 20-50 µg/L tetracycline for 30 days. *V. alginolyticus*, *Vibrio splendidus*, *V. parahaemolyticus*, *V. mimicus*, *Aeromonas hydrophila* and *Plesiomonas shigelloides* were found in *H. kuda* larvae, while *V. alginolyticus* and *V. splendidus* were encountered repeatedly. *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, *Aeromonas* sp., *Micrococcus* spp., *M. leteus* and *Bacillus circulans* were identified from the larval culture water. *V. alginolyticus* was sensitive to chloramphenicol and tetracycline, while *V. splendidus* was sensitive to tetracycline. Tetracycline, 30 mg/L as a bath treatment, effectively treats vibriosis in larval rearing tanks of *H. kuda*.

Introduction

Seahorses (*Hippocampus* spp.) are strictly marine species widely distributed throughout the Inodo-Pacific regions, from the Indian Ocean to the Northwestern, Western Central and Eastern Central areas of the Pacific region (Foster et al., 2004; Koldewey, 2005; Perry et al., 2020; Zhang et al. 2022). Most of the *Hippocampus* spp. coexist with macroalgae, seagrasses, mangroves, sponges, and corals in shallow waters, with some living in deeper oceanic habitats (Perry et al., 2020). The genus *Hippocampus* currently includes 48 valid species (Koning and Hoeksema, 2021). They exhibit an elongated snout with a small terminal mouth, fused jaws, a vertical body axis, a prehensile tail and an extended body cover of bony plates rather than scales (Lourie et al., 2004). Due to this unusual appearance and alleged medicinal properties, *Hippocampus* spp. are traded worldwide (Job et al., 2002; Tendencia, 2004) and five seahorse species (*H. trimaculatus*, *H. spinosissimus*, *H. kelloggi*, *H. algiricus* and *H. kuda*) account for over 90% of the world's seahorse trade (Foster et al., 2016). Number-wise, *H. kuda* was the most traded *Hippocampus* species, followed by *H. reidi* and *H. comes* (Koning and Hoeksema, 2021).

It is estimated that one million seahorses are captured annually from the oceanic waters of 65 countries, with less than 10% surviving more than six months and most dying within six weeks of captivity (Woods, 2000). This results in a worldwide population

decline of seahorse species (Woods., 2000; Job et al., 2002; Xie et al., 2020; Koning and Hoeksema, 2021; Zhang et al., 2022) and Asian seahorse fishers repeatedly cited the rapid decline of the catch due to the reduction of wild stocks (Job et al., 2002; Perry et al., 2020). Aquaculture of seahorses has been proposed as one solution to the seahorse trade in traditional medicine, aquarium fish and curios (Foster et al., 2004; Koldewey and Martin-Smith, 2010; Novelli et al., 2015; Foster et al., 2016; Zhang et al., 2022). Out of 27 seahorse species that are represented in the international aquarium trade (Koning and Hoeksema, 2021), at least 13 species are used in commercial culture or are being researched for their potential for culture (Koldewey and Martin-Smith, 2010). After being listed in Appendix II of the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) in 2002, the proportion of cultured seahorses in the aquarium trade increased rapidly (Novelli et al., 2015). Most of the specimens of *H. kuda*, *H. reidi*, *H. comes*, *H. abdominalis,* and *H. ingens* in the global trade come from cultivation.

The low survival rate of juvenile seahorses in the first few months is considered one of the major bottlenecks in the further development of commercial seahorse aquaculture (Koldewey and Martin-Smith, 2010; Novelli et al., 2015; Perry et al., 2020; Xie et al., 2020). Poor water quality, nutrient deficiencies and pathogenic or parasitic diseases may all contribute to the low survival of sea horse larvae. As such, disease diagnostic information is becoming increasingly relevant in sea horse larval health management and disease control. Like most cultivated fishes, seahorses become stressed when reared in high densities, making them more susceptible to infections (Sanaye et al., 2013; Zhang et al., 2022). They are prone to bacterial (Alcaide et al., 2001; Koning and Hoeksema, 2021; Zhang et al., 2022) and some fungal infections that may be brought about by stress associated with poor water quality, nutrition and handling (Novelli et al., 2015; Koning and Hoeksema, 2021). Bacterial infections have recently resulted in many sea horse deaths, resulting in significant financial losses for sea horse farmers (Tendencia, 2004; Zhang et al., 2022). Vibriosis affects syngnathids and a *Photobacterium* sp. from the Vibrionaceae family killed 25% of the sea horses in a Chinese sea horse farm in seven days (Zhang et al. 2022). Sanaye et al. (2013) reported *Vibrio* and *Photobacterium* coinfection in *H. kuda* in India and *V. harveyi*. *V. alginolyticus* and *V. vulnificus* were recorded from Vietnam in 2016 (Binh et al., 2016). Therefore, a better understanding of disease signs, pathogenicity, and control is required for large-scale production of sea horse larvae in captivity. The current study was designed to isolate and identify pathogenic bacteria linked to mass mortality in captive-bred *H. kuda* larvae in Sri Lanka. The pathogenicity of isolated virulent bacteria was tested on healthy *H. kuda* larvae, and their antibiotic sensitivity was investigated to find a suitable dosage to treat the larval infection.

Materials and Methods

Sample Collection and Parasitological Survey

Captive rearing of seahorses in Sri Lanka is still under experimental conditions. An ornamental fish farm in Pitipana, Negombo, successfully breeds and grows *H. kuda* larvae up to the marketable size (Figure 1) in a hatchery. According to the hatchery owner, the broodstock was acquired from marine fishermen in Mannar, Sri Lanka, who accidentally caught seahorses as bycatch during their shrimp fishing activities. Adult seahorses were stocked in fiberglass tanks (500 L) filled with seawater filtered through a double sand filter, with 25% of the water replaced daily. The larvae were reared in 50 L glass tanks at two larvae/L density. The larvae were fed *Artemia salina* nauplii and kept in natural temperature and photoperiod ranges (26-27 °C; 12D:12N). Following the discovery of many dead larvae in several rearing tanks, the larvae were observed for behavior and moribund larvae were collected and examined for abnormalities such as lesions, necrotic areas, eroded areas, blisters and fin rots. Then, using a compound light microscope (Olympus CX21FS1) at 10 and 40 magnification, wet mounts of scrapings, excised gills, lesions, and intestines) of freshly euthanized larvae were examined for parasites. Tricaine methane-sulfonate (MS-222) was used to anesthetize fish before euthanizing (Figure 1**)**.

Three weeks old moribund larvae and rearing water samples were transported aseptically to the Faculty of Science, University of Kelaniya, for bacteriological and histological studies. The standard length of the sea horse larvae was measured according to Lourie et al. (2004) using a vernier caliper (Dasqua, 0-150 mm).

Collection of Samples for Microbiological Studies

All the water samples were collected under aseptic conditions and were taken from two selected tanks, which showed larval mortality. Altogether, four samples representing two samples from each tank were collected. Ten moribund *H. kuda* larvae 21 days old were sampled aseptically from two tanks to conduct bacteriological studies.

Histology of *H. kuda* Larvae

Whole moribund *H. kuda* larvae (30.2±0.37 mm, SEM) and random samples of gills were fixed in 10% phosphate-buffered formalin. Samples were processed following standard procedure and 5 µm thick sagittal and transversal tissue sections (Leica microtome RM2235) were stained with Hematoxylin and Eosin stains (Camargo and Martinez, 2007). The specimens were observed under a high-power microscope (Olympus, CX21 FS1). Histological examinations on healthy *H. kuda* larvae were carried out in the same manner as the negative controls.

Isolation of Bacteria from the Water

Bacteria were isolated from water using thiosulphate citrate bile sucrose agar (TCBS), Vibrionaceae selective agar, tryptone soy agar (TSA) and nutrient agar (NA). Petri dishes were incubated at room temperature for 24-48 hr. Pure colonies were obtained after several isolation steps. Isolated bacterial cultures were maintained in slope cultures on TSA and NA in the refrigerator at 4°C (Frerichs, 1984).

Isolation of Bacteria from Moribund *H. kuda* Larvae

The larvae were surface sterilized by immersing them in a 70% alcohol solution and then rinsing them in sterilized seawater. After surface sterilization of larvae, they were homogenized in alkaline peptone water using a sterilized mortar and pestle. Homogenate was added into a flask containing alkaline peptone water and topped up to 200 mL. Then the flasks were shaken overnight at room temperature (25-27°C) in a mechanical shaker (Orbital shaker-SHK O0310III, Infitek, China). For the isolation of bacteria from each larval sample streak plate technique was carried out on TCBS, TSA, NA and PDA. Plates were then incubated overnight at room temperature. Following several subculturing steps, pure bacterial cultures were obtained.

Identification of Bacteria Using Morphological and Biochemical Methods

The bacterial identification process was based on morphological and biochemical tests following the methods described in Bergey's Manual of Systematic Bacteriology (Holt, 1984) and Cowan and Steel's Manual for the Identification of Medical Bacteria (Cowan, 1993). As the morphological characteristics, Gram's reaction of cells, arrangement of cells, motility and presence of endospores were observed in each isolated colony. All the biochemical tests were performed along with test controls without inoculum. The preliminary tests such as Gram staining, motility, oxidase, catalase, anaerobic growth, Huge and Leifson's test and glucose acid test were performed to identify bacterial strains up to genus level. Further identification up to the species level was carried out based on biochemical tests such as growth under anaerobic conditions, growth in 6% NaCl, indole test, growth in KCN medium, nitrate reduction, Voges-Proskauer test, urease activity, arginine hydrolysis, gelatin hydrolysis, starch hydrolysis, digestion of casein, lysine decarboxylase test, ornithine decarboxylase test, lipase activity and utilization of citrate.

Pathogenicity Tests

Among the identified bacteria, *V. alginolyticus* and *V. splendidus* were most common on *H. kuda* larvae and those two species were used for an experimental challenge in the present study. The challenge test for bacterial pathogens was performed on 30 day old *H. kuda* larvae (56.6±0.8 mm, SEM). *V. alginolyticus* and *V. splendidus* cultures were used to prepare three bacterial suspensions. The first and second suspensions were made with *V. algynolyticus* and *V. splendidus*, respectively. The third suspension was made by combining both *Vibrio* spp. The infection dose in the immersion challenge was maintained at 1×10^6 - 1×10^7 CFU /ml (Marudhupandi et al., 2017).

The *H. kuda* larvae were maintained in four sets of randomly arranged 5 L plastic buckets, each with 2 L of water. The experimental setup was in three replicates for three suspensions and control. Each bucket contained five healthy larvae and the bacterial suspensions were directly added to the water for the inoculation. No bacteria were added to the control buckets. Also, treatment and control buckets were given the same conditions during the experimental period, e.g., 24 hr. aeration. The larvae were fed with 0.5 g hatched *Artemia* thrice daily at 8 am, 4 pm and 12 pm (10-12 individuals /mL in culture water).

The challenged *H. kuda* larvae were sampled to reisolate the bacteria to verify Koch's postulate (Cvitanich et al., 1991). Two *H. kuda* larvae from challenged and control buckets were carefully picked up with sterilized forceps. The larvae were homogenized in alkaline peptone water using a motor and pestle and inoculated on TCBS agar and identified using biochemical tests, as mentioned before.

Antibiotic Sensitivity Tests for *V. algynolyticus* and *V. splendidus*

Drug resistance patterns of *V. algynolyticus* and *V. splendidus* were determined by disc diffusion test on Mueller-Hinton agar (Merck™) supplemented with 1% NaCl. One loopful from the overnight growth of each bacterial culture was separately transferred into a sterile phosphate buffered saline solution (10 ml) and homogenized. Each Mueller-Hinton agar plate was seeded with 0.1 ml of the above solution. The commercially available chloramphenicol (30 µg), streptomycin (30 µg), ampicillin (30 µg) and tetracycline (30 µg) discs were placed on each of the seeded agar plates and incubated overnight at room temperature. After the incubation period, the diameters of the inhibition zones were measured in millimeters using a vernier caliper. Based on those data, strains were categorized as susceptible, moderately sensitive or resistant as described by NCCLS (1997).

Determination of Antibiotic Dosage

As both *V. algynolyticus* and *V. splendidus* were sensitive to tetracycline, the right dosage for treatment was tested. Four tetracycline concentrations, 20, 30, 40 and 50 mg/L, were tested. On TSA plates supplemented with 1% NaCl and seeded with the bacterial species, filter paper dipped in each antibiotic solution was placed. After two days of inoculation, zone diameters were measured using a vernier caliper. The dosage that

gave the largest zone was selected to treat the infected *H. kuda* larvae.

Use of Tetracycline to Treat Vibriosis in *H. kuda* Larvae

The experiment was conducted in two sets of 7 L plastic buckets with three replicates per treatment and the control. Four weeks old, five healthy *H. kuda* larvae (52.6±0.4 mm, SEM) from the same batch were stocked in each bucket. The larvae were immersed for 15 min in the *V. algynolyticus* and *V. splendidus* suspension and the infection dose was maintained at 1×10^6 - 1×10^7 CFU /ml. *H. kuda* larvae in the treatment buckets were exposed weekly to 30 mg/L tetracycline solution for five weeks. Buckets were well aerated and nylon holdfasts were provided for the attachment of larvae. The larvae were fed with 0.5g hatched *Artemia* thrice daily at 8 am, 4 pm and 12 pm (10-12 individuals /ml in culture water). Feed was adjusted daily according to the number of individuals who survived in each bucket. Buckets were siphoned out daily to remove excess feces and the wastewater was exchanged twice a week. Buckets were checked daily for any mortality and the survival rate of *H. kuda* larvae was recorded.

Statistical Analysis

Anderson and Darling's normality test was conducted to find the normal distribution of data. One-way ANOVA followed by Tukey's test was carried out to find significant differences between the survival rates of *H. kuda* in antibiotic treated and control experiments. The statistical tests were conducted using the Minitab 14 version.

Results

The Symptoms of Infected *H. kuda* Larvae

Most of the individuals were located on the surface of the water or near the aerators rather than hanging near the holdfasts. Losses of appetite, erratic swimming, rotational swimming and skin color changes were common among the infected *H. kuda* larvae during the present study. *Vorticella* sp. was the only external parasite found attached to the body armor of moribund larvae. The light-colored putrid areas and heavy secondary infections due to fungus were observed in the tail end of some of the moribund larvae (Figures 2A and B).

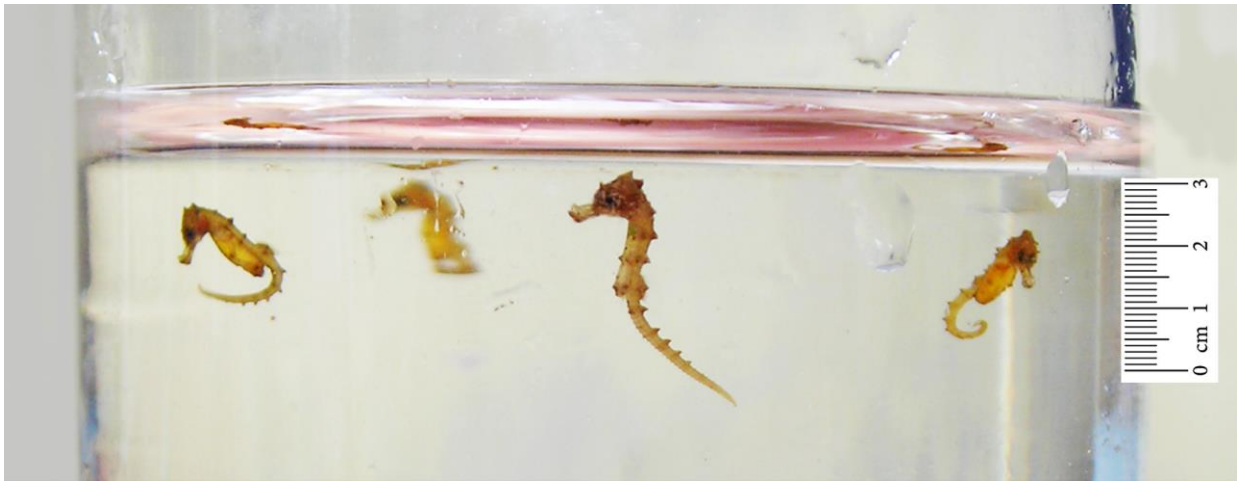

Figure 1. *H. kuda* larvae captive bred at an ornamental fish hatchery in Pitipana, Negombo, Sri Lanka

Figure 2. External symptoms observed in the moribund *H. kuda* larvae. 2A - Initial stage of tissue necrosis in the tail end (10 x 10), 2B – Secondary fungal infections in the putrid tail end (10 x 10)

Histological Observations

Necrotic areas and hemorrhages in the periorbital tissue, soft tissue around the snout, anus and gills were noticed in the moribund larvae (Figures 3A and 3B). The hemorrhagic areas were irregular in shape and appeared as dark patches. The tissues of necrotic areas contained large rod-shaped bacterial aggregates, indicating infiltration of bacteria into the internal body parts (Figure 3C).

Bacteria Identified from *H. kuda* Larvae and Rearing Water

The *Vibrio* species isolated were *V. alginolyticus, V. splendidus, V. paraheamolyticus* and *V. mimicus*. In addition, nonvibrios, *P. shigelloides* and *A. hydrophila* were encountered in moribund larvae (Table 1). *H. kuda* larval rearing water contained gram-negative and positive bacteria including, *V. alginolyticus, V. mimicus, V. fluvialis, Aeromonas* sp., *M. leteus, Micrococcus* spp. and *B. circulans* (Tables 2 and 3).

Pathogenicity Test

Up to two days after the challenge test, none of the *H. kuda* larvae died, irrespective of the different treatments. The first sight of dead larvae was due to the combined infection of *V. alginolyticus* and *V. splendidus* after two days of experimental infection. The mortality rate of *H. kuda* larvae in three treatments increased to 100% on the seventh day of infection (Table 4). The pathogenic agents *V. splendidus* and *V. alginolyticus* were re-isolated from the dead larvae in this challenging experiment.

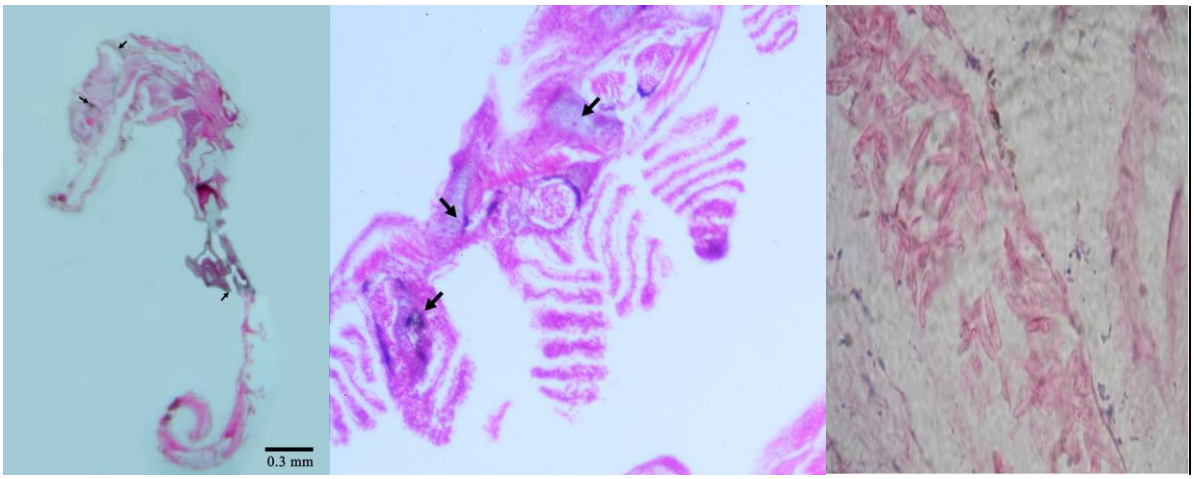

Figure 3. A - The histological section of the whole mount (sagittal section) of *H. kuda* larvae showing bacterial necrosis (arrow; 10 x 40); B – Hemorrhagic areas and necrotic tissue damages in transverse section of gill tissues (arrow; 10 x 40); C – Rod-shaped bacterial cells observed in the histological section of the soft tissues in the snout (10 x 40, oil immersion lens)

Table 1. Bacteria isolated from captive bred *H. kuda* larvae

	First stage identification															Second stage identification										Genus /species
Code No	Gram's reaction	Motility	Oxidase	Catalase	Growth under anaerobic conditions	Growth in air	OF test	Glucose(acid)	Growth in 6% NaCl	Growth in KCN	Citrate as a carbon source	Gas from glucose	Growth at 37^{0}C	Acid from inositol	Acid from lactose	Acid from sucrose	Voges-Proskauer test	Starch hydrolysis	Reduction of NO_3 to NO_2	Indole test	Gelatin hydrolysis	Casein hydrolysis	Arginine hydrolysis	Lysine decarboxylase test	Ornithine decarboxylase test	Lipase activity	
A*	S	+	+	+	+	+	F	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	*Vibrio alginolyticus*
A2*	S	+	+	+	+	+	F	-	+	-	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	*Aeromonas hydrophila*
A2**	S	+	+	+	+	+	F	+	+	+	+	-	+	-	-	+	+	+	-	-	+	-	-	-	-	+	Unidentified I
B1*	S	-	+	+	+	+	F	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	-	+	+	+	*Vibrio splendidus*
B2*	S	+	+	+	+	+	F	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+	*Plesiomonas shigelloides*
B3**	S	+	+	+	+	+	F	+	+	-	+	-	+	-	-		-	-	-		-	-	-	+	+	-	Unidentified II
C1*	C	+	+	+	+	+	F	+	+	-	+	-	+	-	-	-	-	+	-	+	+	-	-	+	+	+	*Vibrio paraheamolyticus*
C4**	S	+	+	+	+	+	F	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	-	+	+	+	*Vibrio mimicus*
b1	S	+	+	+	+	+	F	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+	Unidentified III

Note: S – short rod, P - plump rod, L – long rod, C- curved rod, X-C2 culture died after few weeks hence could not perform the tests denoted as x.

Antibiotic Sensitivity Test for *V. splendidus* and *V. Iginolyticus*

V. alginolyticus was sensitive to chloramphenicol and ampicillin and resistant to tetracycline and erythromycin. *V. splendidus* was sensitive to tetracycline and erythromycin and resistant to chloramphenicol and ampicillin. Antibiotic susceptibility patterns of *V. alginolyticus* and *V. Splendidus* are shown in Table 5.

Determination of the Antibiotic Dosage for Vibriosis

According to the tetracycline dose selectivity test, the best inhibition zone diameter was given by 30 mg/L (Table 6).

Use of Tetracycline to Treat Vibriosis in *H. kuda* Larvae

As both *V. alginolyticus* and *V. splendidus* showed resistance to tetracycline, it was selected for the antibiotic treatment. *H. kuda* larvae exposed to 30 mg/L tetracycline gave promising results with higher survival rates than the control fish ($P<0.05$). Survival rates of *H. kuda* larvae with respect to the antibiotic treatment are presented in Figure 4.

Discussion

H. kuda, which the International Union for the Conservation of Nature (IUCN) lists as vulnerable, is a favored fish in the aquarium trade. In the present study, vibriosis was identified as the primary cause of mass mortalities in *H. kuda* larval rearing tanks of a commercial hatchery in Sri Lanka. Infected larvae became sedentary, exhibiting signs of disease such as irregular movement, sluggish behavior, and a loss of appetite, and lay listlessly on the tank bottom. After this state, death quickly followed and their bodies were broken into pieces within a day. According to the literature, most of the bacteria isolated from cultured seahorses on the conventional media are known to belong to the genus *Vibrio* (Tendencia, 2004; Koldewey, 2005; Martins et al., 2010; Xie et al., 2020; Koning and Hoeksema, 2021; Kang et al., 2022). *V. alginolyticus*, *V. splendidus*, *V. paraheamolyticus* and *V. mimicus* were the vibrio species encountered in the present study in the *H. kuda* larvae. *V. harveyi*, *V. fortis*, *V. parahaemolyticus*, *V. splendidus*, *V. alginolyticus* (Wang et al., 2016) and V. *vulnificus* (Qin et al., 2018) were isolated previously from the cultured sea horses. In the present study, *V. alginolyticus* and *V. splendidus*

Table 2. Identification of gram-negative bacteria from larval rearing tanks of *H. kuda*

	First stage identification													Second stage identification															Genus
Code No	Shape	Motility	Oxidase	Catalase	Growth under anaerobic condition	Growth in air	OF test	Glucose(acid)	Growth in 6% NaCl	Growth in KCN medium	Citrate as a carbon source	Gas from glucose	Growth at 37^{0}C	Acid from inositol	Acid from lactose	Acid from sucrose	Voges-Proskauer test	Starch hydrolysis	Reduction of NO^{-3} to NO^{-2} NO^{-}_2	Indole test	Gelatin hydrolysis	Casein hydrolysis	Arginine hydrolysis	Lysine decarboxylase test	Ornithine decarboxylase test	Lipase activity	
I1	SR	+	+	+	+	F	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	*Vibrio alginolyticus*
X1*	SR	+	+	+	+	F	+	+	+	-	+	-	+	-	-	+	-	+	+	+	+	+	-	+	+	+	*Vibrio mimicus*
Y1*	PR	+	+	+	+	F	+	+	+	+	-	-	+	-	-	+	-	+	+	-	-	+	+	-	-	+	*Vibrio fluvialis*
Y1**	SR	+	+	+	+	+	F	+	+	+	-	-	+	-	-	+	-	+	+	-	-	+	+	-	-	+	*Aeromonas* sp.

Table 3. Identification of gram-positive bacteria from larval rearing tanks of *H. kuda*

	First stage identification												*Second stage identification*												*Genus/Species*
Code no	*Shape*	*Spores*	*Motility*	*Oxidase*	*Catalase*	*Acid-fast*	*Growth in air*	*OF test*	*Glucose(acid)*	*Spore shape*	*Spore position*	*Citrate as a carbon source*	*Arginine hydrolysis*	*Pigment production*	*Acid from arabinose*	*Acid from xylose*	*Urease test*	*Voges-Proskauer test*	*Starch hydrolysis*	*Reduction of NO^{-}_3 to NO^{-}_2*	*Indole test*	*Gelatin hydrolysis*	*Casein hydrolysis*	
N2	*S*	-	-	-	+	-	+	*O*	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	*Micrococcus leteus*
N3	*R*	+	+	+	+	+	+	*F*	+	*oval*	*Central*	+	-	-	+	+	-	+	+	+	-	-	-	*Bacillus circulans*
X1	*S*	-	-	-	+	+	+	*O*	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	*Micrococcus sp.*

were encountered repeatedly in moribund *H. kuda* larvae. They were identified as two of the most virulent species that caused larval mortalities in the hatchery. *V. alginolyticus* is a curved, gram-negative, oxidase-positive bacteria that was before associated with high mortality in the aquaculture systems in temperate and tropical countries (Koldewey, 2005; Mejdi et al., 2008; Sharma et al., 2013). It can grow in high salt concentrations and form large, yellow (sucrose fermenting) colonies on TCBS medium. According to Koldewey (2005), under aquarium conditions, *V. alginolyticus* is the most frequently isolated species from the internal organs of Syngnathidae fishes.

In the present study, according to the histological examination, necrotic regions and hemorrhages were common in the moribund *H. kuda* larvae. Similarly, Jiang et al. (2020) and Xie et al. (2020) observed necrotic muscle tissue underneath the necrotic epidermis in places where bacterial invasions were seen in cultured *H. kuda*. Similarly, when *V. alginolyticus* infected adult *H. reidi*, it caused external hemorrhages, loss of skin color, depressed abdomen, hemorrhagic liver, pale kidney and ascitic fluid in the intestines (Alcaide et al., 2001; Martins et al., 2010). *V. alginolyticus* produces toxins as serum proteases, which may be responsible for tissue alterations and necrosis in infected fishes (Sharma et al., 2013). These events might cause organ dysfunction affecting metabolic and osmotic activities, culminating in *H. kuda* larval deaths. Mortality of cultured *Sepia* spp. (Sangster and Smilowitz, 2003) and *Lates calcarifer* (Sharma et al., 2013) infected with vibrios have also been confirmed due to the pathogenicity of *V. alginolyticus*.

The other most virulent species identified was *V. splendidus,* which is a planktonic organism that commonly infects marine fish (Gatesope et al., 1999; Koldewey, 2005; Mejdi et al., 2008; Kang et al., 2022) and mollusks larvae (Sugumar et al., 1998). Tendencia (2004) previously recorded *V. splendidus* infection in cultured *H. kuda* in the Philippines. *V. splendidus* caused seahorse gas bubble disease (Kang et al., 2021) and was pathogenic to rainbow trout and turbot (Austin et al., 2007). The combined infection of *V. alginolyticus* and *V. splendidus* in the present study might have accelerated larval deaths as these hemolytic bacteria can break down blood cells in infected animals by hemolysis. Balcázar et al. (2010) also reported combined infection of *V. alginolyticus* and *V. splendidus* in seahorses and those infected showed lethargy, loss of appetite, white spots on the skin, and necrotic tail lesions similar to the observations of the present study.

V. parahaemolyticus, which is pathogenic for fish and is a leading seafood-borne pathogenic bacterium, was detected in small numbers in the moribund *H. kuda* larvae. *V. parahaemolyticus* is involved in causing natural disease outbreaks of marine and brackish water aquaculture animals (Sony et al., 2021). To our knowledge, this is the first time the infection of *V. mimicus, Plesiomonas shigelloides* and *Aeromonas hydrophila* on seahorse larvae has been recorded. *V. mimicus* infection caused high mortalities in yellow catfish, southern catfish, and Zhengchuan catfish in China (Geng et al., 2014). *P. shigelloides* can be found in freshwater and marine environments and in animals that live in such environments. *A. hydrophila* is a freshwater fish pathogen but is occasionally found in

Table 4. Survival of captive bred *H. kuda* larvae challenged with *V. alginolyticus* and V. *splendidus*

Infected Bacteria	Number of larvae that survived after infection						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
V. alginolyticus	5	5	5	4	3	2	0
V. splendidus	5	5	4	4	3	1	0
V. alginolyticus and *V. splendidus*	5	5	3	3	2	0	0
Control	5	5	5	5	5	5	5

Table 5. Antibiotic susceptibility patterns of *V. alginolyticus* and *V. splendidus*

Antibiotic	Amount of antibiotic/disc (µg)	Measured zone diameter(cm) and susceptibility status of isolates	
		V. alginolyticus	*V. splendidus*
Ampicillin	30	0.9 (R)	1.0 (R)
Chloramphenicol	30	1.7 (MS)	1.1 (R)
Erythromycin	30	1.0 (R)	1.7 (MS)
Tetracycline	30	1.4 (MS)	3.2 (S)

R - Resistant; MS - Moderately sensitive; S - Sensitive

Table 6. Mean zone diameter (mm) for different Tetracycline concentrations

Tetracycline dosage (mg/L)	Mean zone diameter (mm)
20	8.0±2.81
30	25±7.5
40	Unclear margin

marine fish species (Austin et al., 2007). *A. hydrophila* has previously been isolated from marine fish species, including *Amphiprion sebae* (Dhayanithi et al., 2012), *Epinephelus tauvina*, *Hilsa ilisha* and *Lethriuns nebulosus* (Al-Maleky, 2013).

The virulent bacterial isolates were utilized to infect healthy seahorse larvae using the immersion-challenged method to confirm the pathogenicity and their effect on larval mortality. Both *V. alginolyticus* and *V. splendidus* were reisolated from the infected *H. kuda* larvae. The results showed that combined infection of *V. alginolyticus* and *V. splendidus* caused 100% mortality in cultured *H. kuda* larvae within five days. *V. alginolyticus* appeared to be more virulent than *V. splendidus*. *V. splendidus* infection in seahorses could be a secondary infection in the already immunocompromised fish (Kang et al., 2022). When *H. reidi* was submitted to an immersion bath in a solution containing 1.0×10^7 CFU of *V. alginolyticus*/mL for 15 minutes, 100% mortality was observed within twenty-four hours in the infected animals (Martins et al., 2010). In the present study, 100% mortality of 30 days old *H. kuda* larvae were observed after six days when they were challenged in an immersion bath of 1×10^6 - 1×10^7 CFU of *V. alginolyticus* and V. *splendidus*/mL for 15 min. However, in uninfected fish, neither mortality nor bacterial lesions were observed in the present study, as reported by Alcaide et al. (2001) and (Martins et al., 2010) after experimental infection with *V. harveyi* and *Vibrio alginolyticus* in the *Hippocampus reidi*. The high mortality rates in larval *H. kuda* observed in the present study could partly be attributed to the high water temperature in tank water because of the tropical weather conditions in Sri Lanka, which may have fostered the bacteria's rapid proliferation (Manchanayake et al., 2023). *Vibrio* becomes more virulent at higher temperatures (Sangster and Smilowitz, 2003; Tendencia, 2004).

According to the antibiotic susceptibility patterns, *V. alginolyticus* is susceptible to chloramphenicol and tetracycline, while *V. splendidus* is relatively more vulnerable to tetracycline. The present study recommends a 30 mg/mL tetracycline solution as a bath treatment to depress vibriosis *H. kuda* larvae cultured in hatcheries. Tetracycline, a broad-spectrum bacteriostatic drug widely used in aquaculture (Qin et al., 2018; Sony et al., 2021), is produced by the *Streptomyces* species and inhibits protein synthesis in bacteria. Xie et al. (2020) also recommended tetracycline to control vibriosis in seahorse culture as it is highly efficient in controlling *V. alginolyticus*.

In the present study, the isolation of three *Vibrio* spp. from the water shows that the microbial fauna of seahorses depends to a large extent on the microbial fauna of the rearing water. *V. alginolyticus* and *V. mimicus* were encountered from both rearing water and moribund larvae. Therefore, the rearing water may be the transmission route of these bacteria. As seahorse larva has an open mouth and anus when they leave the brood pouch (Novelli et al., 2015), they can be easily infected by the pathogenic vibrio present in the environment. In addition to *Vibrio*, *Bacillus*, *Micrococcus*, and *Aeromonas* species were isolated from the water.

The virulence of *Vibrio* species varies based on the host's immune condition, and the diseases can be avoided by managing water quality and improving the culture environment (Kang et al., 2022). As significant losses occur in the larval stages of *H. kuda* and water acts as a transmitting medium of pathogens, the industry needs to shift to bacteria-free culture systems. *V. alginolyticus* and *V. splendidus* were found to be

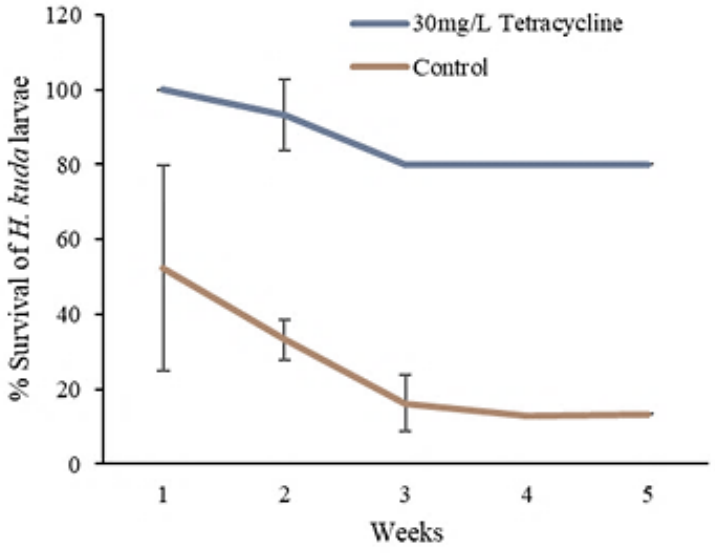

Figure 4. Percentage survival of *H. kuda* larvae challenged with *Vibrio alginolyticus* and *Vibrio splendidus* and treated with 30 mg/L tetracycline

associated with commercial grade artemia used to feed fish larvae, a fact that should be taken into consideration when husbandry practices further improve *in H. kuda* larval rearing. Koldewey (2005) recommended installing UV sterilizers through which water from the live food holding tanks can be recirculated to minimize food-borne vibriosis. Seahorse farmers must take strict precautions and adopt biosecurity measures to reduce bacterial loads in water and live food before they opt for antibiotics to treat bacterial diseases. Antibiotics in aquaculture should not be encouraged and should only be recommended as a last option to control vibriosis in *H. kuda* larval rearing tanks in hatcheries.

Conclusions

V. alginolyticus, *V. splendidus*, *V. paraheamolyticus* and *V. mimicus* were the vibrio species isolated from *H. kuda* larvae. Nonvibrios, *P. shigelloides* and *A. hydrophila* were also found in moribund larvae. The presence of *V. mimicus*, *P. shigelloides*, and *A. hydrophila* on seahorse larvae grown in captivity was recorded for the first time in this study. The most frequent vibrio species infected in *H. kuda* larvae were *V. alginolyticus* and *V. splendidus*. Larval culture water contained several bacterial species, including *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, *Aeromonas* sp., *Micrococcus* spp., *M. leteus* and *B. circulans*. Both *V. alginolyticus* and *V. splendidus* were sensitive to tetracycline out of chloramphenicol, streptomycin, and tetracycline. Tetracycline, 30 mg/L as a bath treatment, effectively treats vibriosis in larval rearing tanks of *H. kuda*.

Ethical Statement

This study was carried out in strict accordance with the recommendations Research Ethics Committee (ERC), University of Kelaniya. The protocol was approved by the ERC on 15.12.2017 (Project: Breeding and larval rearing of marine ornamental fish in Sri Lanka, Protocol Number: 2017/Sc/12/06). All surgery was performed under MS222 anesthesia, and all efforts were made to minimize the number of fish sacrificed and suffering.

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Author Contribution

Conceptualization: UPKE, DKG, PR, VCB, Data Curation: UPKE, GKG, PR, VCB, Formal Analysis: PR, VCB, Investigation: PR, VCB, Methodology: UPKE, DKG, PR, VCB, Project Administration: UPKE, DKG, Resources: UPKE, DKG, Supervision: UPKE, DKG, Writing-original draft: PR, VCB, UPKE, Writing-review and editing: UPKE, DKG, PR, VCB.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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