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Characterization and infectivity evaluation of *Vibrio harveyi* causing white patch disease among captive reared seahorses, *hippocampus kuda*

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Pathogenic bacterium was isolated from infected parts of the captive reared seahorse. During the present investigation, high mortality with symptoms such as external white patches on the body and anorexic conditions were noted among laboratory-cultured seahorses, *Hippocampus kuda*. Bacteria isolated from internal organs of infected fish were biochemical homogenized and identified as *Vibrio harvei*. In infectivity studies with Tilapia, *Oreochromis mossambicus*, symptoms such as tail rot and erythemia were noted apart from the white skin. The lethal dose for Tilapia with average body weight of 8.8g was 8×10^6 cells/fish, while for seahorse with an average body weight of 6.2 g, it was 4×10^4 cells/fish.

[Keywords: *Vibrio harveyi*, Seahorse, *Hippocampus kuda*, Pathogenic bacteria, White patch disease, Infectivity of bacterial isolates**]**

Introduction

Over-exploitation of fishes for non-food use is now causing concern, particularly for some of the species used as traditional medicines[1-6]. Seahorse, used in traditional Chinese medicine is one such specie. The information on their biology as well as the culturing technique and their disease problems is essential in the above scenario. Seahorse culturing has proven technically challenging primarily because of problems with diet and disease. Seahorses are strict carnivores with voracious appetites for live foods, and succumb very rapidly to a variety of parasitic, fungal and bacterial ailments. Captive seahorses suffer from ailments caused by fungal, bacterial and parasites (e.g. *Glugea heraldi*) as reported by Vincent and Clifton – Hardley, 1989[7]. They are classified as threatened species. Captive breeding had failed in many instances due to their susceptibility to disease. Among the bacterial pathogens, different species of *Vibrio* are considered[8]. In general, strains of *V. harveyi* are implicated in luminous vibriosis[9] and thus constitute an important pathogen of the penaeid shrimps in farming system. It was also reported as an opportunistic pathogen of the common snook[10] and has been isolated form diseased marine fish such as *Acanthopagrus cuvieri*[11], Sea bream, *Sparus aurata*[12], and Dentex, *Dentex dentex*, cultured on the Mediterranean coast of Spain. Present study consists the isolation, characterization and transmission of disease to apparently healthy *Hippocampus kuda* and *Oreochromis mossambicus*.

Materials and Methods

Isolation of the bacterial pathogen (bacterial isolates) form seahorse

Infected seahorses were segregated and bacteria were isolated. Primary isolations were performed on Nutrient agar and stock cultures were kept in Nutrient agar slant[13]. Infected seahorse, showing symptoms of disease during the moribund stage, was cut open. Intestine, trunk and operculum were aseptically removed and incubated in nutrient broth in shaker (Remi-India) at 50 rpm at room temperature.

After 24 hours of incubation, the broth was streaked on nutrient agar plates to isolate the colonies by their form, color and their distinct characters. Plates were incubated at room temperature 28°C for 24 hours in nutrient agar. Different colonies were isolated and streaked on nutrient slants and they were maintained as axenic retrains.

Morphological, physiological and biochemical characteristics of bacterial isolates

Morphological and cultural characteristics of the bacterial isolate were studied based on their size,

pigmentation, form, margin, elevation, motility and Gram staining. Physiological characterization of bacterial isolates was carried out with standard procedures[14]. The production of exo-cellular and endo-cellular enzymes, utilization of sugars and fermentation were studied by the methods such as Carbohydrate fermentation, Gelatin hydrolysis, Caesin hydrolysis, Starch hydrolysis, Glucose, Arabinose, Sucrose, Sorbitol, Maltose, Lactose, Mannitol, Indole production test, VP test, Hydrogen Sulphide production test, ONPG test, TCBS agar test, O/129 (2:4 Diamino 6.7 Di iso-propyl pteridine phosphate), Penicillin sensitivity, Urease, Growth at different concentrations of NaCl(%) and growth at different temperatures.

Determination of LD_{50} of seahorse in response to pathogenic bacteria

Pathogenic isolate was sub-cultured in nutrient broth and after 18 hrs centrifuged at 3000 rpm for 15 minutes and washed in 0.85% sterile saline. Re-suspended pellet was serially diluted to get the desired bacterial concentration to be administered. Known numbers of bacterial cells were administered by intraperitoneal injection using 1.0 ml syringe needle (insulin syringe). Apparently healthy laboratory acclimated seahorse, *Hippocampus kuda* was used for the experiments. Average size of seahorse ranged from 88.6 to 98.2 mm and 4.41 to 6.0g. Initially, Tilapia (*Oreochromis mossambicus*), acclimated in 35 ppm seawater was used as healthy experimental fish for the LD_{50} studies. The average size of tilapia was 7.16 cm in length and 7.0 g in weight.

Results

Morphological, Biochemical and Physiological characterization of the isolates

Results of the morphological, biochemical and physiological characterization of the bacterial isolate was presented in (Table 1). The isolate was swarming, Gram negative, motile, short rod and grew on TCBS agar. NaCl was required for growth and the isolate was sensitive to the Vibriostatic agent O/129. It produced cytochrome oxidase, catalase and nitrate was reduced. Fermentation and acid production were noted from glucose, arabinose, lactose, mannitol and maltose except sucrose, Glucose and Ribose. Growth occurred at 40°C. Decarboxylase of lysine and ornithine were positive while arginine was negative. The isolate was produced indole. The negative for Vigasproscar test, as well as for H_2S production. Positive results for production of exo-cellular enzymes such as gelatinase, caesinase, amylase and chitinase was obtained. Isolate grew in 6.0% NaCl and tolerated 40°C based on the morphological, biochemical and physiological characteristics of the isolate and comparison with the earlier report, it was physiological characterized of the isolate and comparison with the earlier report, it was characterized *V. harveyi*.

Among the 17 probable species, which showed positive reaction in the indole production, the probability was reduced to 10 in the lysine decarboxylase test (Table 2). Similarity of biochemical characteristics was further reduced to among 7 in the ornithine decarboxylase test followed by 4 in the sucrose utilization test. In these, the probability of *V. cholerae* was rejected as for the VP test[15] Possibility of *V. charcariae* and *V. mediterraneii* was rejected in the trehalose and

Table 1—Characteristics of the predominant seahorse bacterial isolate

Tests	Isolate
Gram's stain	-
Gelatin hydrolysis	-
Casein hydrolysis	-
Starch hydrolysis	+
Glucose (Acid) formation	-
Sucrose (Acid) formation	-
Sorbitol (Acid) formation	+
Maltose (Acid) formation	+
Lactose (Acid) formation	+
Mannitol (Acid) formation	+
Vigasproscar test	-
Indole production test	+
Urease	-
Hydrogen Sulphide	-
ONPG test	+
Novobiocin (Sensitivity)	+
O/129 (Sensitivity)	+
Luminescence	-
Arabinose (Acid)	+
Ribose (Acid)	-
Growth at	
20°C	+
28°C	+
40°C	+
H_2S production	-
Growth percentage of NaCl	
0 %	-
3.0 %	+
6.0 %	+
8.0 %	-
(+) : Positive, (-) : Negative	

Table 2—Key of biochemical reactions to differentiate *V. harveyi* from other probable *Vibrio* species

Species	Indole Production	Lysine decarboxylase	Ornithine decarboxylase	Sucrose	VP	Gelatinase	Trehalose
V. alginolyticus	+	+	(+)	+	-	-	-
V. cholerae	+	+	+	+	-	d	-
V. cholerae	+	+	+	+	+	+	(+)
V. hollisae	+	-	-	-	-	-	-
V. arahaemolyticus	+	+	+	-	-	+	+
V. vulnificus	+	+	+	(-)	-	+	+
V. aesturianus	+	v	-	+	-	+	+
V. mediterarnei	+	v	V	+	-	-	+
V. nigripulchritudo	+	-	-	-	-	+	-
V. orientalis	+	+	-	+	-	+	+
V. pelagicus 2	+	-	-	v	-	+	+
V. proteolyticus	+	+	-	-	-	+	+
V. splendidus 1	+	-	-	(+)	-	+	+
V. splendidus 2	+	-	-	-	-	+	+
V. tubiashi	+	-	-	+	-	+	+
V. mimicus	+	+	+	-	-	nd	+
V. diazotropicus	+	-	-	+	-	-	+

+ = 90 to 100% positive; (+) = 75 to 89.9% positive; v = 25.1 to 74.9% positive; – = 0 to 10% positive, (d) – defined, (nd) not defined

gelatinase test respectively as suggested by Holt *et al*[16]. Therefore, the present isolate was confirmed as *V. harveyi*.

Infectivity evaluation

In the case of Seahorse, the normal body colour changed into white. Fishes were anerxic, moved vertical position with violent breathing. These symptoms were observed from the 10^4 cells/fish onwards. These same symptoms were also observed from those fishes (*Oreochromis mossambicus*) administered with 10^5 cells/fish onwards.

Fishes, which received 10^8 cells/fish, totally succumbed within 10 days. In those fishes injected with 10^7 and 10^6 cells/fish, the mortality was 50% and 12.5% respectively within 10 days. Fishes were injected with 10^5 cells did not die (100% survival). In seahorse, 66.6% mortality was observed in fishes which received 10^6 cells/fish within 10 days. Besides, 50% mortality was observed in 10^4 cells/fish.

The LD_{50} value of *Oreochromis mossambicus* (average body weigh: 8.8 g) was 8×10^6 cells/fish. For the seahorse, *Hippocampus kuda* the LD_{50} was 4×10^4 cells/fish (average body weight: 6.2 g). The results are depicted in Figs 1 and 2 respectively.

Discussion

Colony morphology of seahorse isolate, which characterized as *V. harveyi* strain was reported earlier as cream colored, occasionally translucent, luminescent raised and shiny colonies[17]. Biochemical characteristics of *V. harveyi* were also compared with the type strain of the species. The characteristic feature of negative VP reaction was observed for the *V. harveyi* seahorse strain. According to Lightner *et al*[18]. identification of *Vibrio bacteria* required complete biochemical characterization otherwise, *V. harveyi* would be misidentified as *V. parahaemolyticus* or *V. alginolyticus*.

Bacterial isolates administered to the apparently healthy *Oreochromis mossambicus* and *Hippocampus kuda* produced similar disease symptoms as compared to the earlier naturally infected fish. In addition, the experimental group of fishes responded according to the different densities of cells indicating the dose dependence phenomenon.

In the infectivity studies, the fishes administered with Seahorse isolate succumbed with disease symptoms like tail rot, red patches on the body white colouration at 10^5 cells/fish. Soltani and Bruke[19] determined the pathogenicity of *Cytophaga johnsonae* isolated form a number of diseased fresh water fish by administering different cell densities. Injection of *Piscrickettsia salmonis* established the disease resulting in mortality[20]. Cvitanich *et al*.[21] succeeded in reproducing the disease in Coho salmon by using 6×10^6 and 6×10^5 infective rickettsial units. Nelson *et al*.[22] injected *V. angullarum* @ 2.5×10^6 CFU/fish to

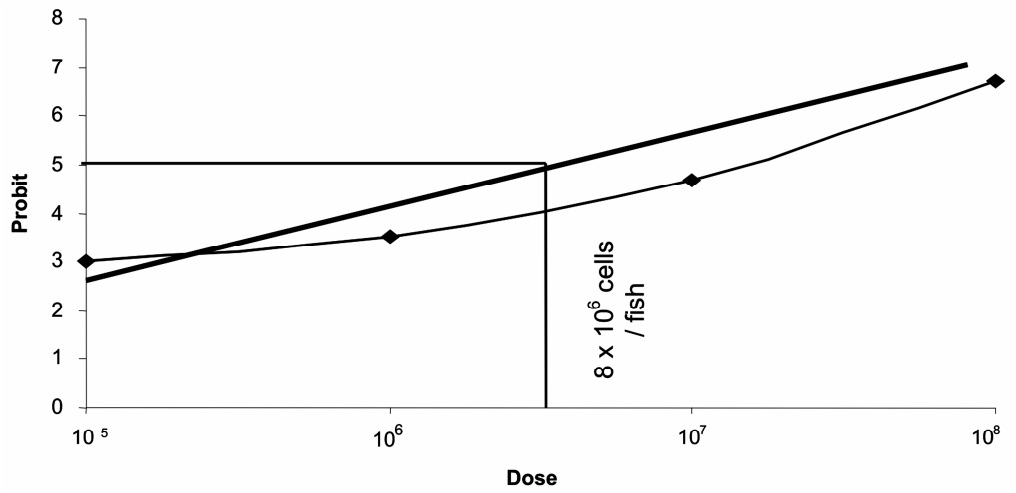

Fig. 1—LD_{50} of seahorse isolated bacteria for *Oreochromis mossambicus*

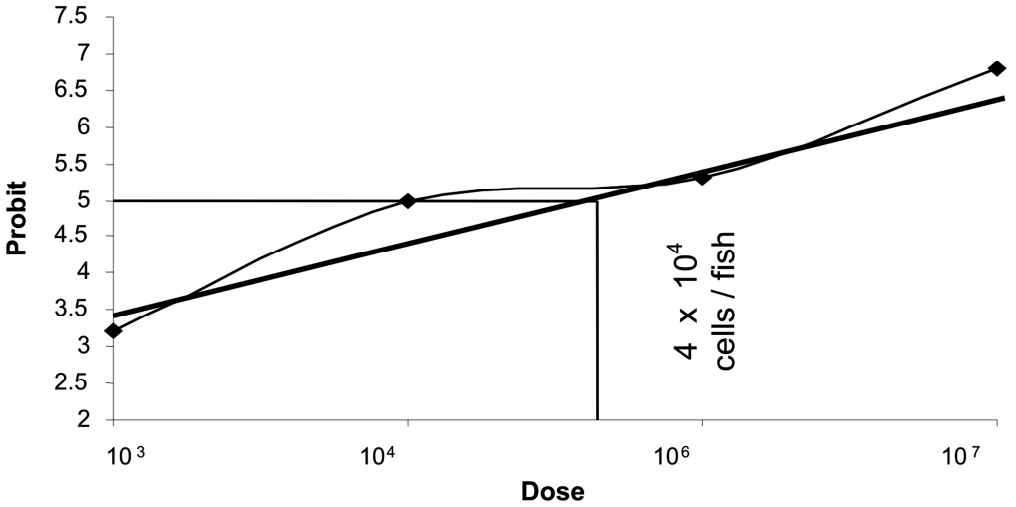

Fig. 2—LD_{50} of seahorse isolated bacteria for *Hippocampus kuda*

produce this in Rainbow trout (*Salmo gaidneri*). In their study Bruno *et al.*[23] achieved successful infection by inoculating 1.04×10^5 bacteria intraperitoneally into Atlantic salmon in which similar pathological results were obtained.

In the present set of experiments, the LD_{50} of Seahorse isolate was determined as 8×10^6 cells/fish. Besides, 100% mortality was noted in fishes injected with 10^8 cells/fish within 12 days. And 100% mortality was observed in *Oreochromis mossambicus* injected with 10^8 cells within 10 days. However, the same pathogens were found to be more virulent of the seahorse, resulting 83.33 and 66.7% mortality at 10^4 cells/fish within 3 days after injection for Seahorse *Vibrio* isolates respectively. Alcaide *et al.*[8] there is no other specific information on *V. harveyi* as a pathogen for *Hippocampus sp.* Alcaide *et al.*[8] reported the LD_{50} of *V. harveyi* with batches of 6 seahorses per dose by intraperitoneal injection.

In the present set of experiments, the infection or mortality started after 1 to 4 days of the post challenge with *V. harveyi* and the LD_{50} value was 4×10^4 cells/fish seahorse isolate respectively. Alcaide *et al.*[8] reported that mortality began from 1 to 7 days. In general, *V. harveyi* infection were mostly reported form shrimp hatcheries and grow out ponds. Jiravanichpaisal *et al.*[24] isolated *V. harveyi* as a minor component from the exoskeleton of female black tiger shrimp in Thailand. The LD_{50} value of *V. harveyi* isolated from shell diseased shrimp was 10^6 CFU/shrimp. The symptoms in *P. monodon* such as

red disease syndrome could be reproduced by injection with 10^7 CFU/shrimp as reported by Tendencia and Dureza[25]. According to Otta *et al.*[26] the LD_{50} values of *V. harveyi* ranged form 1.4×10^6 to 2.8×10^7 CFU/shrimp which indicated their low virulence capability.

Vibrio harveyi has also been reported previously as an opportunity pathogen of several marine fishes. Austin and Austin[27] reported that *V. splendidus* was pathogenic to rainbow trout, with and LD_{50} of 10^5 cells. In estuarine fish, Rajan[28] documented, that the LD_{50} dose of *Vibrio aeusturianus* for *Etroplus maculatus* was 1×10^7 cells (1.25×10^4 CFU/fish or 1.25×10^3/g of fish). Total mortality was noted in 1.25×10^4 CFU/fish or 1.25×10^4 CFU/g of fish within 8 hr after injection. Considering these, the present luminescent *V. harveyi* Seahorse and seahorse isolates could be ranked as a highly virulent (10^4 CFU/seahorse) one.

Apart form *Vibrio*, Sirirat[29] reported the virulence pattern of *Aeromonas hydrophila* isolates to catfish. Isolates of *A. sobria, A. caviae* and *A. allosaccharophila* were avirulent for European eels since no moralities were recorded in challenged elvers at high doses such as $10^{8.4}$ CFU/fish Esteve[30]. Other pathogenic bacterial isolates of fish such as *Pseudomonas aeruginosa* and *A.hydrophila* were tested for their pathogenicity[31]. It was observed that the fish isolate of *P. aeruginosa* had a lethal dose of 1.5×10^5 cells/fish for *Cyprinus carpio* and 4.2×10^5 cells for *Oreochromis mossambicus*. The fish pathogen *A. hydrophila* had lethal doses of 2.1×10^6, 6.8×10^5 and 3.2×10^6 respectively for *Cyprinus carpio*, *Labeo rohita* and *Oreochromis mossambicus*[31].

Conclusion

Pathogenicity the Seahorse isolate was characterized as *V. harveyi* through the colony morphology. Biochemical characteristics were also compared with the early literature and the type strain of the species. It was absorbed that seahorse developed symptoms such as tail rot, erythemia and as white patches on the body. The LD_{50} value was depends upon the body weight of the animals.

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