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Isolation and characterization of *Vibrio harveyi* causing blackening disease among captive reared seahorses, *Hippocampus kuda*

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Abstract

A high mortality with symptoms such as external black patches on the body and anorexic conditions were noted among laboratory-cultured seahorse, *Hippocampus kuda* in Vizhinjam research unit in south west coast of India. Bacteria isolated from internal organs of infected animals were biochemically homogenized and identified as *Vibrio harveyi.* When infectivity of the isolated bacteria was studied in tilapia *Oreochromis mossambicus,* symptoms such as tail rot and erythema were noted apart from the blackening of skin. The lethal dose for tilapia with average body weight of 6.6g was 2.5 x 10^6 cells/fish, while for the seahorse with an average body weight of 4.41g, it was 4 x 10^4 cells/fish.

Keywords : blackening disease, *Hippocampus kuda,* infectivity of bacterial isolates, pathogenic bacteria, Seahorse, *Vibrio harveyi*

INTRODUCTION

Seahorse belongs to a single genus *Hippocampus* of the family *Syngnathidae.* This family also include pipe fishes and pipe horses under the genus *Hippocampus* (Anil *et al.,* 1999). In nature, the seahorses living in sea beds tend to be highly fragmented into small, isolated groups (Johannes, 1978). They are classified as threatened species. Seahorse culturing has proven technically challenging primarily because of problems with diet and disease, as the sea horses 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 health ailments caused by fungi, bacteria and parasites (*e.g. Glugea* heraldi) as reported by Vincent and Clifton-Hardley (1989). Captive breeding of sea horses had failed in many instances due their susceptibility to disease. Among the bacterial pathogens, different species of *Vibrio* are considered to be important (Alcaide *et al*., 2001). In general, strains of *V. harveyi* are implicated in luminous vibriosis (Zhang and Austin, 1999) and thus constitute an important pathogen of the penaeid shrimps in farming system (Lavilla-Pitago *et al.,* 1990; Karunasagar *et al.,* 1994; Montero and Austin, 1999). It was also reported as an opportunistic pathogen of the common snook (Kraxberger-Beauty *et al.,* 1990) and has beeen isolated from diseased marine fish such as *Acanthopagrus cuvieri* (Saeed, 1995), Sea bream, *Sparus aurata* (Balebona *et al.,* 1995), and dentex, *Dentex dentex,* cultured on the Mediterranean coast of Spain (Company *et al.,* 1999). This work was conducted when the seahorse *Hippocampus kuda* cultured in Vizhinjam Marine Biotechnology Laboratory, South India, turned black and exhibited abnormal breathing. Mortality was high and all fishes in the affected tanks died within 7 days. This paper describes the isolation and characterisation of the bacteria that transmits the disease to apparently healthy seahorse *H. kuda* and tilapia *O. mossambicus.*

MATERIALS AND METHODS

Isolation of the bacterial pathogen (bacterial isolates) from seahorse

The infected seahorses were segregated and bacteria were isolated. The primary isolations were performed on nutrient agar and stock cultures were kept in nutrient agar slant (Lavilla-Pitago *et al*., 1990). The infected seahorse, showing symptoms of disease during the moribund stage was cut open. The intestine, trunk and operculum were aseptically removed and incubated in nutrient broth in shaker (Remi, India) at 50 rpm at room temperature.

After 24 hrs. of incubation, the broth was streaked on nutrient agar plates to isolate the colonies by their form, color and their distinctive characters. Plates were incubated at room temperature (28°C) for 24 hrs. in nutrient agar. Different colonies obtained 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 isolates were studied based on their size, pigmentation, form, margin, elevation, motility and gram staining. Physiological characterisation of bacterial isolates was carried out with standard procedures

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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	+
VP test	-
Indole production text	-
Urease	-
Hydrogen Sulphide	-
ONPG text	-
Novobiocin (Sensitivity)	+
0/129 (Sensitivity)	+
Luminescence	+
Arabinose (Acid)	-
Ribose (Acid)	-
Growth at	
20^0 C	+
28^0 C	+
40^0 C	+
H_2S production	-
Growth percentage of NaCl	
0 %	-
3.0%	+
6.0%	+
8.0%	-

(+): Positive, (-): negative

(Collins *et al*., 2001). The production of exo-cellular and endo-cellular enzymes, utilisation of sugars and fermentation were studied by the methods such as carbohydrate fermentation, gelatin hydrolysis, casein hydrolysis, starch hydrolysis, glucose, arabinose, sucrose, sorbitol, maltose, lactose, mannitol, iIndole 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.

Determaination of LD_{50} of pathogenic bacteria to seahorse

The LD_{50} evaluation of the isolate was determined by following the method of Hodgson (2004). The 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. The resuspended pellet was serially diluted to get the desired bacterial concentration to be administered. Known number of bacterial cells was administered by intraperitoneal injection using 1.0 ml syringe needle (insulin syringe). Apparently healthy laboratory acclimated seahorse, *Hippocampus kuda,* were used for the experiments. The average size of seahorses used in the experiments ranged from 88.6 to 98.2 mm and 4.41 to 6.0 g. Initially, tilapia (*Oreochromis mossambicus*), acclimated in 35 % seawater was used as healthy experimental fish for the LD_{50} studies. The average size of tilapia used was 7.16 cm in length and 7.0 g in weight.

RESULTS

Morphological, biochemical and physiological characterisation of the isolates

Results of the morphological, biochemical and physiological characterisation of the bacterial isolate are presented in Table 1. The isolate was swarming, gram negative, motile, short rod and grew on TCBS agar, sodium chloride was required for growth and the isolate was sensitive to the vibriostatic agent O/129. Fermentation and acid production were noted from glucose, arabinose, lactose, mannitol and maltose. Growth occurred at 40^{0}C. Decarboxylase of lysine and ornithine was positive while arginine was negative. The isolate produced indole and negative for VP 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. The isolate grew in 6.0 % NaCl and tolerated 40°C. Based on the morphological, biochemical and physiological characteristics of the isolate and by comparison with the earlier reports, it was characterised as *Vibrio 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). The similarity of biochemical characteristics was further reduced to among 7 in the ornithine decarboxylase test followed by 4 in the sucrose utilisation test. In these, the probability of *V.cholerae* was rejected as for the VP test (Holt, 1994). Possibility of *V.charcariae* and *V. mediterraneii* was rejected in the trehalose and gelatinase test, respectively, as suggested by Holt *et al*. (1994). Therefore the present isolate was confirmed as *V. harveyi.*

Infectivity evaluation

In the case of seahorse, the normal body colour was changed into white due to infection. The fishes were anorexic, moved in vertical position with violent breathing. These symptoms were observed from the 10^4 cells/fish onwards. These symptoms were also observed from those fishes (*Oreochromis mossambicus*) administered with 10^5 cells/fish.

Among the group of fishes injected with 10^8 cells/fish, 100% mortality was observed within 12 days, whereas in fishes injected with 10^7, 10^6 and 10^5 cells, the mortality was 62.5%, 37.5% and 12.5%, respectively in 12 days. For the seahorse, 83.3 % mortality was noted within 7 days at 10^6 cells/fish. Fishes injected with 10^4 cells showed 100 % survival.

The LD_{50} was derived as 2.5 x 10^6 cells/fish for an average 6.6g body weight for tilapia *Oreochromis mossambicus* and for the seahorse *Hippocampus kuda*, the LD_{50} value was 2.3 x 10^4 cells/fish (Figs. 1 & 2).

DISCUSSION

The colony morphology of seahorse isolate, characterised as *V. harveyi* strain was reported earlier as cream colored, occasionally transleucent, luminescent raised and shiny colonies and the characteristic feature of negative VP reaction was observed for the *V.harveyi* seahorse strain (Alvarez *et al.,* 1999).

The bacterial isolates administered to the apparently healthy *O.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, and white colouration at 10^5 cells/fish. Earlier Soltani and Bruke (1994) determined the pathogenicity of *Cytophaga johnsonae* isolated from a number of diseased fresh water fish by administering different cell densities. Injection of *Piscrickettsia salmonis* was reported to cause the disease resulting in mortality (Smith *et al.,* 1996). Cvitanich *et al.* (1991) succeeded in reproducing the disease in Coho salmon by using 6 x 10^6 and 6 x 10^5 infective rickettsial units. Nelson *et al.* 1985) injected *V.angullarum* at 2.5 x 10^6 CFU/fish to produce this in Rainbow trout (*Salmo gaidneri)*. In their study Bruno *et al.* (1986) achieved successful infection by inoculating 1.04 x 10^5 bacteria intraperitoneally in to Atlantic salmon in which similar pathological results were obtained.

In the present set of experiments, the LD_{50} of seahorse isolate was determined as 2.3 x 10^6 cells/fish. Besides, 100 % mortality was noted in fishes with in 10^8 cells/fish within 12 days and 100 % mortality was observed in *O.mossambicus* injected with 10^8 cells within 10 days. However, the same pathogens were found to be more virulent for the seahorse, resulting 83.33 and 66.7 % mortality at 10^6 and 10^4 cells/fish within 7 days after injection for seahorse *Vibrio* isolates, respectively. This is comparable to Alcaide *et al.* (2001) who reported the LD_{50} of *V.harveyi* for 6 seahorses.

In the present set of experiments, the infection or mortality started after 1 to 4 days of the post challenge with *V.harveyi*. Alcaide *et al.* (2001) also reported that mortality began from 1 to 7 days. In general, *V. harveyi* infections were mostly reported from shrimp hatcheries and grow out ponds. Jiravanichpaisal *et al.* (1994) isolated *V. harveyi* as a minor component from the exoskeleton of female black tiger shrimp in Thailand and the LD_{50} value of *V. harveyi* isolated from shell diseased shrimp was 106 CFU/shrimp. The symptoms in *P. monodon* such as red disease syndrome could be reproduced by injection with 10^7 CFU/shrimp as

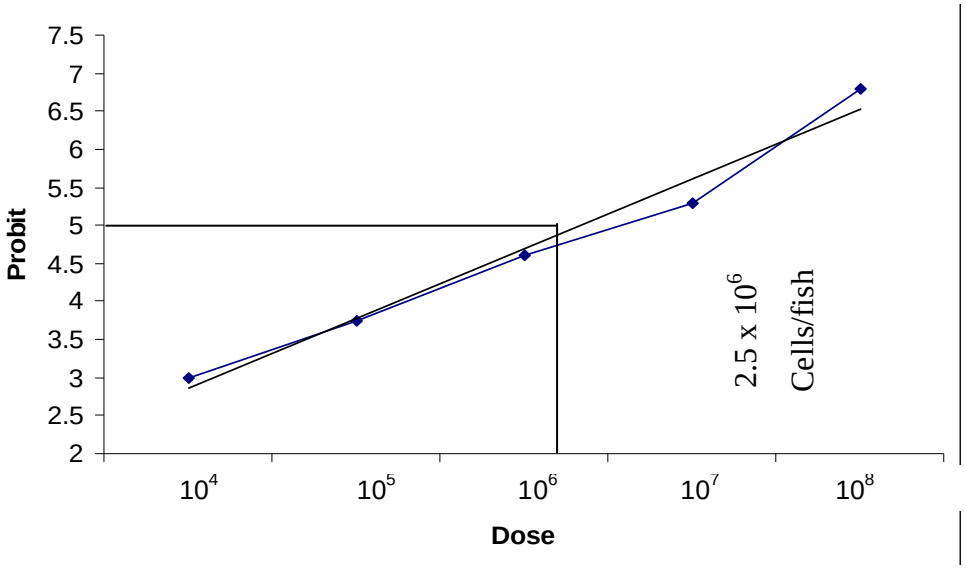

Figure 1. LD_{50} of seahorse isolated bacteria (*Vibrio* sp.) for *Oreochromis mossambicus*

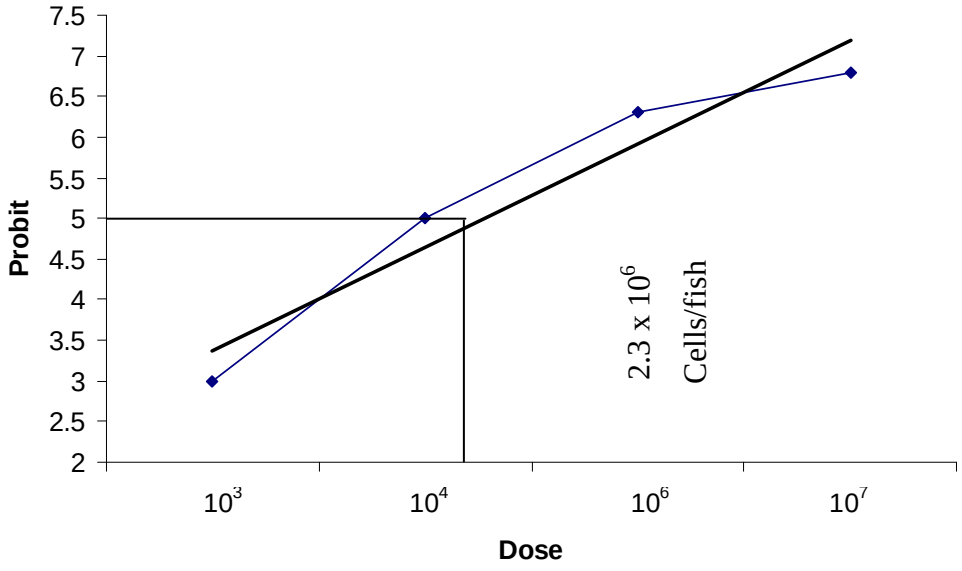

Figure 2. LD_{50} of seahorse isolated bacteria(*Vibrio* sp.) for *Hippocampus kuda*

Table 2. Key biochemical reactions of probable *Vibrio* species

Species	Indole production	Lysine decarboxylase	Ornithine Decarboxylase	Sucrose utilisation	VP	Gelatinase	Trehalose
V. alginolyticus	+	+	(+)	+	-	-	-
V. cholerae	+	+	+	+	-	d	-
V. charcariae	+	+	+	+	+	+	-
V. hollisae	+	-	-	-	-	-	-
V. arahaemolyticus	+	+	+	-	-	+	+
V. vulnificus	+	+	+	(-)	-	+	+
V. aesturiansus	+	v	-	+	-	+	
V. mediterarnei	+	v	v	+	-	-	+
V. nigripulchritudo	+	-	-	-	-	+	-
V. orientalis	+	+	-	+	-	+	+
V. pelagicus 2	+	-	-	v	-	+	+
V. proteolyticus	+	+	-	-	-	+	+
V. splendidus 1	+	-	-	(+)	-	+	+
V. splendidus 2	+	-	-	-	-	+	+
V. tubaishi	+	-	-	+	-	+	+
V. mimicus	+	+	+	-	-	nd	+
V. diazortopicus	+	-	-	+	-	-	+

+ = 90 to 100% positive; (+) = 75 to 89.9% positive; v =25.1 to 74.9% positive; (-) positive control, 0 to 10.5% positive
d - dehydrolysis; nd - no dehydrolysis

reported by Tendencia and Dureza (1997). According to Otta *et al*. (1999) the LD_{50} values of *V. harveyi* ranged from 1.4 x 10^6 to 2.8 x 10^7 CFU / shrimp which indicated their low virulence capability to shrimps.

Vibrio harveyi has also been reported previously as an opportunity pathogen of several marine fishes (Alcaide *et al.,* 2001). Austin and Austin (1993) reported that *V. splendidus* was pathogenic to rainbow trout, with an LD_{50} of 10^5 cells. In estuarine fish, Rajan (2001) documented that the LD_{50} dose of *Vibrio aeustrurianus* for *Etroplus maculatus* was 1 x 10^7 cells (1.25x10^4 CFU / fish or 1.25 x 10^3 / g of fish). Total mortality was noted in 1.25x10^5 CFU / fish or 1.25x10^4 CFU / g of fish within 8 hours after injection. Considering these, the present luminescent *V. harveyi* isolates from seahorse could be ranked as a highly virulent (10^4 CFU / seahorse) one.

Apart from *Vibrio,* Sirirat *et al*.(1999) 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 at high doses such as 10^8 CFU / fish (Esteve, 1995). Other pathogenic bacterial isolates of fish such as *Pseudomonas aeruginosa* and *A.hydrophila* were also tested for their pathogenicity (Lipton, 1987). It was observed that the fish isolate of *P.aeruginosa* had a lethal dose of 1.5x10^5 cells / fish for *Cyprinus carpio* and 4.2x10^5 cells for *O. mossambicus.* The fish pathogen *A.hydrophila* had lethal doses of 2.1 x 10^6, 6.8 x 10^5 and 3.2 x 10^6, cells / fish, respectively, for *C. carpio*, *L. rohita* and *O. mossambicus,* respectively (Lipton, 1987).

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REFERENCES

Alcaide, E., Gill-Sanz, C., Sanjuan, E., Esteve, D., Amaro, C. and Silveria, L. 2001. *Vibrio harveyi* causes disease in seahorse, *Hippocampus* sp. *J. Fish* disease, 24: 211-313.

Alsina, M. and Blanch, A.R. 1994. A set of kesy for biochemical identification of environmental *Vibrio* sp. *Appl. Bacteriol.* 76: 313-316.

Anil, M.K., Kakati, V.S., Ganga, V. and Zachoria, S. 1999. Larval Rearing of Seahorse *Hippocampus kuda* under laboratory conditions. *Marine Fisheries information Service,* 162: 23-25.

Austin, B. and Austin, D.A. 1993. Control of bacterial fish diseases *In*: Bacterial fish pathogens, Diseases in farmed and Wild Fish, II edition, Ellis Horwood Ltd. Publisher, Chichester Halsted Press, Chichester. P. 350-373.

Balebona, M.C., Morinigo, M.A., Fairs, A., Krovacek, K., Mansson, I., Bordas, M.A. and Borrego, J.J. 1995. Influence of salinity and pH on the adhesion of pathogenic *Vibrio* strains to *Sparus aurata* skin mucus. *Aquaculture,* 132: 113-120.

Bruno, D.W., Hastings, T.S and Ellis, A.E 1986. Histopathology, bacteriology and experimental transmission of cold-water vibriosis in Atlantic salmon. *Salmo salar*. *Dis. Aquat. Org.,* 1: 163-168.

Collins, C.H., Lyne. P.M. and Grange, J.M. 1995. Collins and Lyne's Microbiological Methods. 7th edition Butterworth, Heine Anann. P. 347.

Company, R., Sitija-Bbadila, A., Pujalte, M.J.Garay, E., Alvarez-Pellitero, P. and Perez Sanchez, J. 1999. Bacterial and parasitic pathogens in cultured common *Dentex dentex* L. *J. Fish Dis.,* 22: 299-309.

Cvitanich, J.D., Garate, N.O. and Smith, C.E 1991. The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. *J. Fish Dis.,* 12: 121-145.

Esteve, C., Amaro, C., Garay, E., Santos, Y. and Toranzo, A.E 1995. Pathogenicity of live bacteria and extracellular products of motile *Aeromonas* isolated from eel. *J. Appl. Bacterial.,* 78: 555-562.

Hodgson, E. 2004. *A Textbook of Modern Toxicology*. Wiley-Interscience, P. 35-73.

Holt, J., Nordmo, R and Gabrielsen, B.O 1994. Immune stimulation in Atlantic fed alginate-containing feed. *In:* 3rd International Marine Biotechnology Conference. Program, Abstracts and List of Participants. Tromsoe University, Tromsoe (Norway). P. 80.

Jiravanichpaisal. P., Myasaki, T. and Lisuwan, C 1994. Histopathology, biochemistry and pathogenicity of Vibrio *harveyi* infecting black tiger prawn *Penaeus monodon. J. Aqua. Anim. Health,* 6: 27-35.

Johannes, R.E. 1978. Reproductive strategies of coastal marine fishes in the tropics. *Env. Biol. Fish,* 3: 65-84.

Karunasagar, I., Pai, R., Malathi, G.R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture,* 128: 203-209.

Kraxberger Beauty, T. MeGary, D.J., Grier, H.J. and Lim, D.V. 1990. *Vibrio hareyi* an opportunistic pathogen of common snook, *Centropomus unidecimalis* (Bloch), held in captivity. *J. Fish Dis.,* 3: 557-560.

Lavilla-Pitago, C.R., Baticodes, M.C.L., Cruz-Lacierda, E.R. and de la Pena, L.D. 1990. Occurance of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines Aquaculture. *J. World Aquaculture Soc.,* 37: 1749-1760.

Lipton, A.P 1987. Studies on microbial diseases of some commercially important freshwater fishes with special reference to *Aeromonas* sp. and *Pseudomonas* sp. Ph. D. Dissertation, Madurai Kamaraj University, Madurai, India. P. 286.

Liu. Priya, C., Lee, K.K., Kou, G.H. and Chen, S.N. 1996. Isolation of *Vibrio harveyi* from disease Kuruma prawns *Penaeus japonicus. Curr. Microbiol.,* 33: 129-132.

Montero, A.B. and Aurtin, B. 1999. Charecterization of extracelllar products from an isolate to *vibrio harveyi* recovered from diseased post-larval *Penaeus vannamei* (Bonne). *J. Fish Dis.,* 22: 377-386.

Nelson, J.S., Rohovec, J.S. and Fryer, J.L. 1985. Location of *Vibrio angullarum* in tissues of infected rainbow trout (*Salmo gairdneri*) using the fluorescent antibody technique. *Fish Pathol.,* 20: 29-235.

Otta, S.K., Karunasagar, I. and Karunasagar, I. 1999. Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India. *J. Aqu. Trop.,* 14: 309-318.

Rajan, A.N. 2001. Studies on ulcerative bacterial diseases in *Etroplus* maculates caused by *Vibrio* sp. and its management using Bacterin and marine natural products. Ph.D., dissertation, Manonmaniam Sundaranar University, Tirunelveli, India. P.69.

Saeed, M.O. 1995. Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. *Aquaculture,*136: 21-29.

Sirirat, T., Intuseth, I., Chanphong, J., Thompson, K., Chinabut, S. and Adams, A. 1999 Characterisation of *Aeromonas hydrophila* extracellular products with reference to toxicity, virulence, protein and antigenicity. *Asian Fish Sci.,* 12: 371-379.

Smith, P.A. Contreras, J.R., Garees, L.H. and Larenas, J.J. 1996. Experimental challenge of coho salmon and rainbow trout with *Piscirickettsia salmonis*. *J. Aqua. Anim. Health,* 8:130-134.

Soltani, M. and Burke, C.M. 1994. Responses of fish pathogenic *Cytophaga flexibacter* like bacteria(CFLB) to environment conditions. *Bull. Eur. Ass. Fish. Pathol.,* 14: 185-187.

Tendencia, E.V.A. and Dureza, H.A. 1997. Isolation of *Vibrio* sp. From *Penaeus monodon* (Fabricins) with red disease syndrome. *Aquaculture,* 154: 107-114.

Vincent, A.C.J. and Clifton-Hardley, R.S. 1989. Parasitic infection of the seahorse (*Hippocampus erectus* – a case report. *J. Wildl. Dis.,* 25: 404-406.

Zhang, X.H. and Austin, B. 1999. Pathogenicity of *Vibrio harveyi* to salmonids. *J. Fish Dis.,* 23: 93-102.