EXPERIMENTAL TRANSMISSION AND HISTOPATHOLOGY OF BROWN SPOT DISEASE IN SHRIMP (*PENAEUS INDICUS*) AND LOBSTER (*PANULIRUS HOMARUS*)

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ABSTRACT

The occurrence of shell disease in prawn and lobster is reported. Black lesion was observed on abdominal appendages and telson of larvae and adult *Penaeus indicus* and adult *Panulirus homarus*. Bacterial isolates belonging to the genus *Vibrio*, especially *V. alginolyticus*, were isolated. *Vibrio alginolyticus* caused black lesion on abdominal segments of larvae in experimental transmission. Histopathological changes were observed in hepatopancreas, gut, and muscle of infected animals.

Diseases of exoskeleton of decapod crustacea are common and are variously referred to as spot disease, brown spot, black spot, spotted disease, or shell disease. This disease is characterized by brown to black spots on the external carapace or cuticle of prawn. Ulceration and melanization of the uropods have been observed in tank-reared *Macrobrachium rosenbergii* (Burns *et al.*, 1979). A number of bacteria capable of chitinoclastic activity were isolated from shell lesions of *M. rosenbergii* and their morphological and biochemical properties were characterized. Chitinoclastic bacteria such as *Beneckea* and *Vibrio* have been isolated from the shell lesion of *M. rosenbergii*, the penaeid prawn *Penaeus setiferus*, and the blue crab (*Callinectes sapidus*) (Cook and Lofton, 1973; Delves-Broughton and Poupard, 1976). Alfaro *et al.* (1993) have observed the blackening of male reproductive system of captive *P. setiferus* and isolated *Vibrio alginolyticus* from the infected parts. In the present case, ulceration and melanization of uropod were observed in the tank-reared larvae and adult prawn *P. indicus* and adult lobster *Panulirus homarus*. Attempts were made to identify the causative organism and to find out its pathogenicity on the larvae of *P. indicus*.

J. Aqua. Trop., **9** (1994) 311–322.
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MATERIALS AND METHODS

Collection of specimens

The infected specimens were collected from prawn hatchery of Central Marine Fisheries Research Institute located at Kovalam near Madras. At the hatchery, the prawn seeds were produced following the modified Galveston system. The infected larvae of *P. indicus* were collected from the rearing tanks and the adult *P. indicus* and *P. homarus* were collected from breeding and rearing tanks respectively.

The stage, percentage of mortality, number of specimens observed, number of samples used for isolation, and number of isolates characterized are presented in Table 1.

Bacteriological analysis

Ten larvae were selected at random in each sampling. The infected portions of specimens were cut with sterile blade, placed in a test-tube along with 1 ml of sterile sea water, and macerated with a glass rod. The same procedure was followed for adults. Ten-fold serial dilutions were done to avoid overgrowth of bacteria as described by Bullock (1971). The diluted samples were inoculated on TCBS agar and seawater nutrient agar by spread plate technique.

After inoculation, plates were incubated at 28°C for 24 to 48 hrs. Morphologically similar and dominant bacterial colonies were selected and streaked on nutrient agar plates to obtain pure culture. After obtaining the pure culture, isolates were maintained on nutrient agar for further study. Bacterial isolates were identified according to the taxonomic schemes of Buchanan and Gibbons (1984) and West and Colwell (1984).

Experimental transmission

Pathogenicity experiment was carried out on the mysis larvae of *P. indicus*. Immersion method was followed to infect the larvae. Active and healthy larvae were collected and washed with sterile sea water to remove the food and other adsorbed detritus adhering to the body and maintained in a clean 5 litre beaker

Table 1. Incidence of brown spot disease in larvae and adult *Penaeus indicus* and adult *Panulirus homarus*

Date of collection	Host	% of mortality	No. of specimens observed	No. of specimens with brown spot	No. of samples used for isolation	No. of isolates characterized
14.08.86	Mysis	28	100	52	10	14
16.08.86	Mysis	30	100	47	10	16
16.08.86	Adult shrimp	0	12	8	3	14
16.08.86	Lobster	0	16	12	3	13

containing sterilized sea water for nearly 3–5 hrs for acclimatization before experiments.

Natural sea water was used in the experiment. Sea water pumped from the adjacent sea was initially stored for some time to allow sand and particulate matter to settle. In the laboratory, sea water was filtered through Sartorius filter paper (0.2 μm pore size) and exposed to ultraviolet light for sterilization. Salinity varied between 30 and 34 ppt and the temperature was maintained at 28°C.

Vibrio alginolyticus was grown on TCBS agar plates prepared with sea water. After incubation at 28°C for 24 hrs, the culture was harvested in sterile sea water and diluted to a standard concentration equal to a particulate suspension that had an optical density of 1.0 at a wavelength of 530 nm. This standard suspension of bacteria contained approximately 1.12×10^{11} cells/ml as determined by standard dilution and plating methods. It was either introduced directly to the larval rearing water (400 ml) or diluted by ten-fold serial dilutions (1.12×10^{9}) prior to being introduced to the water.

Mysis of *P. indicus* at the rate of 10 were reared in sterilized beaker (500 ml) containing 400 ml of sterile sea water. The water was provided with mild aeration throughout the experiment without harming the larvae. Air stones and air tubes were sterilized by immersion in 2.6% sodium hypochloride and then washed thoroughly with sterilized tap water. The beakers were covered in order to prevent contamination. Aseptic techniques were observed throughout the experiments.

The larvae were fed with *Chaetoceros* sp. and *Skeletonema* sp., which were cultured aseptically in conical flask (2 litre). To ensure the experimental level of concentration of bacterial cells in larval rearing water, the phytoplankton culture was sieved through filter paper (Whatman no. 41) and plankton thus collected was added to water for feeding the larvae. One ml of bacterial suspension from each of two concentrations (1.12×10^{11} and 1.12×10^{9}) mentioned earlier was added to the rearing water of larvae. One ml of sterilized sea water was added to the control. Three replicates in each concentration and control were carried out. The larvae were examined twice daily for clinical signs of disease and mortality. Dead animals were removed.

The specific action of *V. alginolyticus* as a pathogen was confirmed by re-isolating *V. alginolyticus* from moribund larvae to satisfy Koch's Postulate.

Histopathology

Representative specimens were fixed in Davidson's fixative. Fixed materials were processed and embedded in wax, cut at 5–7 μm thickness and stained with haematoxylin and eosin (Preece, 1972).

RESULTS

Typical necrotic lesions observed on the larvae and adult shrimp and adult lobster are shown in Figs. 1 to 4. In the larvae, the furcal setae were degenerated and telson became necrotic and blackened (Fig. 1). Some larvae had ulcerated uropod

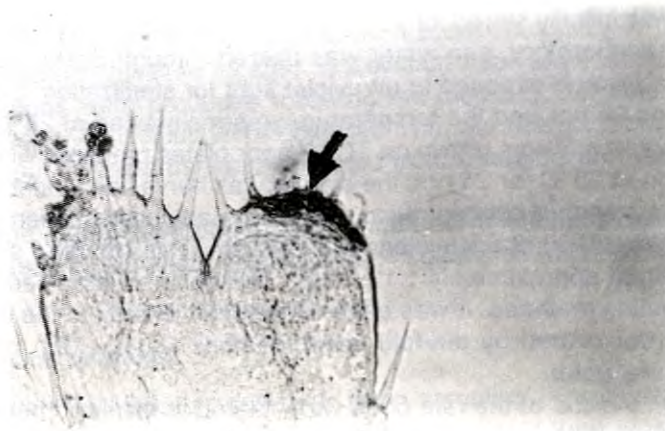

Fig. 1. Telson of infected mysis of *Penaeus indicus* showing the absence of furcal setae and blackening of telson (arrow)

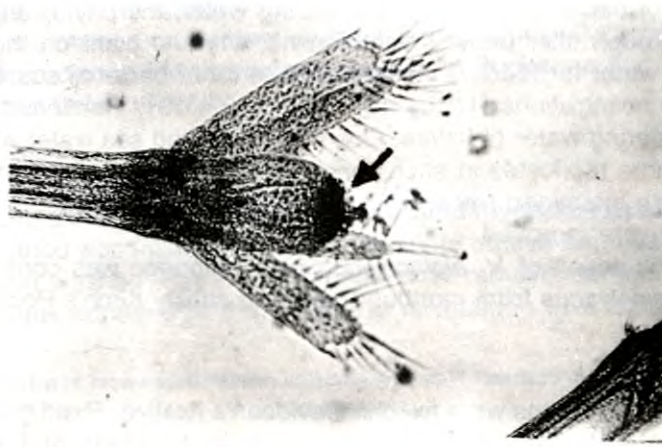

Fig. 2. Uropod of infected mysis showing the degenerating of setae and blackening of necrosed area (arrow)

with broken setae (Fig. 2). Mortality of larvae up to 30% was observed in this incident. These signs were also observed in the adult shrimp and lobster that were being reared in the laboratory (Figs. 3 and 4). However, it was seen that adults usually recovered from this condition after the affected shrimp or lobster moulted.

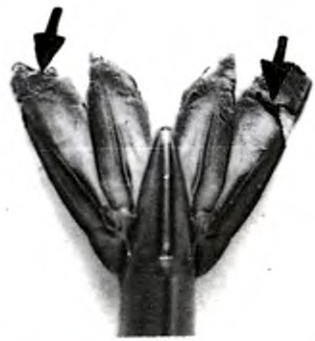

Fig. 3. Necrosis and blackening of adult *Penaeus indicus* (arrow)

Fig. 4. Tail region of *Panulirus homarus* with necrosed uropods and telson

Microscopic examination of materials from these lesions indicated the presence of numerous motile, rod-shaped bacteria. Fungi were not observed in any of the samples. The various characteristics of the isolates studied are summarized in Table 2. Isolates were identified as predominantly *Vibrio* spp., especially *V. alginolyticus* (Fig. 5).

Table 2. Characteristics of bacterial isolates isolated from larvae and adult *Penaeus indicus* and adult *Panulirus homarus*

Characteristics	Mysis				Adult shrimp			Lobster		
	I	II	III	IV	I	II	III	I	II	III
	(30)				(14)			(13)		
Swarming	–	–	+	–	–	+	–	–	+	–
Gram stain	–	–	–	–	–	–	–	–	–	–
Motility	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+
NaCl tolerance										
0%	–	–	–	–	–	–	–	–	–	+
3%	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	–	+	+	–	+	+	–
8%	–	–	+	–	–	+	–	–	+	–
10%	–	–	+	–	–	+	–	–	+	–
Growth on MacConkey agar	+	+	+	–	+	+	–	+	+	–
Growth on TCBS agar	G	G	Y	–	G	Y	–	G	Y	–
Sensitivity to 0/129	+	+	+	–	+	+	–	+	+	–
Arginine dihydrolase	–	–	–	+	–	–	+	–	–	*
Lysine decarboxylase	+	+	+	–	+	+	–	+	+	*
Ornithine decarboxylase	–	+	+	–	+	+	–	–	+	*
Nitrate reduction	+	+	+	–	+	+	–	+	+	+
Voges-Proskauer reaction	–	–	+	–	–	+	–	–	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
H_2S production	+	+	+	–	+	+	–	+	+	+
Indole production	+	+	+	–	+	+	–	+	+	+
O/F test	F	F	F	–	F	F	–	F	F	F
Gas from glucose	–	–	–	–	–	–	–	–	–	+
Amylase	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+
Lipase	+	+	+	+	+	+	+	+	+	+
Chitinase	+	+	+	+	+	+	+	+	+	+
Casein digestion	+	+	+	+	+	+	+	+	+	+
Acid from										
Arabinose	–	+	–	–	+	–	–	–	–	–
Fructose	+	–	+	–	–	+	–	+	+	+
Glucose	+	+	+	–	+	+	–	+	+	+
Lactose	–	–	–	–	–	–	–	–	–	–
Maltose	+	+	+	–	+	+	–	+	+	+
Mannitol	+	+	+	–	+	+	–	+	+	+
Sucrose	–	–	+	–	–	+	–	–	+	+
Identification	V1	V2	V3	P	V2	V3	P	V1	V3	A

( ) = number of isolates characterized; + = positive; – = negative; G = green colony; Y = yellow colony; F = fermentative; V1 = *Vibrio* sp.; V2 = *Vibrio* sp.; V3 = *V. alginolyticus*; P = *Pseudomonas*; A = *Aeromonas*.

The accumulated percentage mortality of larvae at different time intervals is given in Table 3. At concentrations of 2.8×10^6 and 2.8×10^8 cells/ml, *V. alginolyticus* caused 20 and 36.6% mortality respectively in the larvae after 48 hrs of exposure. This bacterium was not able to cause lesion on the uropod and telson

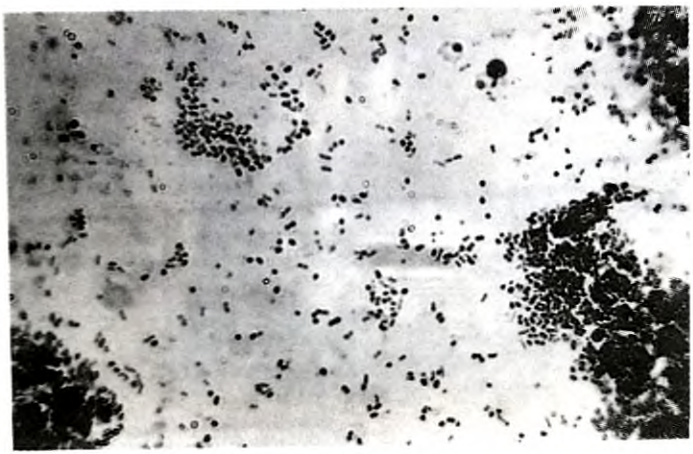

Fig. 5. Smear from the 24 hr culture of *Vibrio alginolyticus* cultured on seawater nutrient agar showing small rod-shaped bacterial cells (Gram staining) 20 μm

Table 3. Pathogenicity experiments of *Vibrio alginolyticus* on mysis of *Peneaus indicus*

Larval stage used	No. of larvae used	No. of bacterial cells/ml of rearing water	Accumulated percentage mortality of larvae at the end of		Percentage of larvae with black lesion on abdominal segment
			24 hrs	48 hrs	
Mysis I	10 × 3	0	0	0	0
Mysis I	10 × 3	28×10^5	13.3	20	37.5
Mysis I	10 × 3	28×10^7	23.2	36.6	78.9

as observed in nature, but black lesion was observed on the abdominal segments of experimental animals (Fig. 6). Such lesions were observed in 78.9% of the experimental animals after 48 hrs of inoculation. *Vibrio alginolyticus* was reisolated from experimentally infected larvae of *P. indicus*.

Histopathological studies showed certain structural changes in hepatopancreas, muscle, and gut of infected larvae. Extensive vacuolation was observed in hepatopancreatic epithelial cells and vacuoles were filled with eosinophilic materials (hyaline degeneration) (Fig. 7). The muscle fibres of the abdominal region were separated and haemocytic infiltration was observed between the muscle bundles. The sections of blackened area of host revealed melanization of exoskeleton and the muscle underneath (Fig. 8). The rod-shaped bacteria were observed in the blackened area (Fig. 9). The epithelium of gut was edematous. The epithelial cells were highly vacuolated and vacuoles, as in the hepatopancreas, contained

Fig. 6. Abdominal region of mysis of *Penaeus indicus* showing experimentally induced black lesion by *V. alginolyticus* (arrow). 0.1 mm

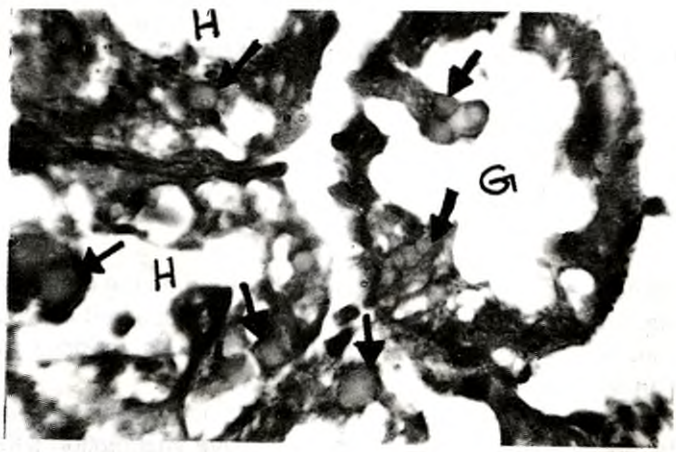

Fig. 7. Cross-section of cephalothoracic region of mysis of *Penaeus indicus* infected by *V. alginolyticus* showing hyaline droplets in the epithelial cells of gut and hepatopancreas (arrow). H—hepatopancreatic tubules, G—gut (H & E). 20 µm

eosinophilic materials (Fig. 7). The above histopathological changes were not observed in the control.

Fig. 8. Cross-section of melanized area of abdominal region of mysis showing the melanization of muscle fibres (arrow). M—normal muscle (H & E). 50 µm

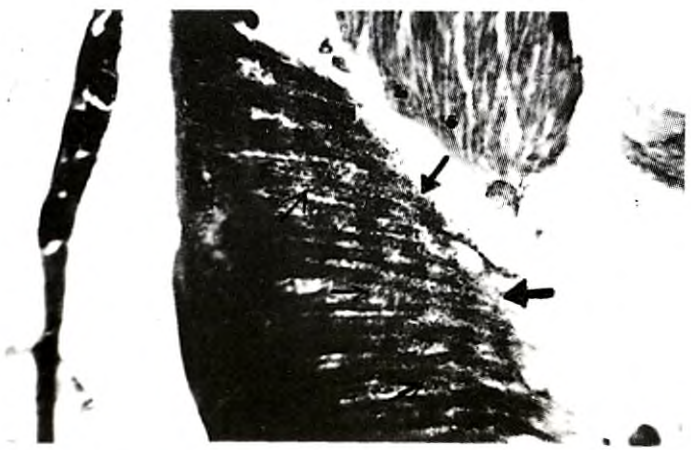

Fig. 9. Enlarged view of Fig. 8 showing small rod-shaped bacteria in the melanized area (arrow) (H & E) 20 µm

DISCUSSION

Several factors have been suggested for the manifestation of brown spot disease. These include bacterial species that produce extracellular lipases, proteases (Cipriani *et al.*, 1980), and chitinase (Delves-Broughton and Poupard,

1976; Sindermann, 1977; Cipriani *et al.*, 1980), fungi (Burns *et al.*, 1979; Johnson 1978), mechanical trauma (Delves-Broughton and Poupard, 1976; Sindermann, 1977), precipitating chemicals (Nimmo *et al.*, 1970; Johnson, 1978), nitrogenous waste products (Johnson, 1978), and nutritional deficiencies and developmental abnormalities that result in damage to the epicuticular layer of exoskeleton (Fisher *et al.*, 1976). *Vibrio* spp., especially *V. alginolyticus*, were predominantly isolated from the necrotic lesions on the appendages of larvae and adult shrimp and adult lobster. The biochemical tests carried out on *V. alginolyticus* showed its ability to produce lipase, protease, and chitinase. The isolation of *V. alginolyticus* from all the samples and its chitinoclastic nature make this organism a possible causative agent as observed by Cook and Lofton (1973), Delves-Broughton and Poupard (1976), and Sindermann (1977).

In the experimental transmission, black lesion was produced at the abdominal segments as observed by Lightner (1975). Pylant (1980) initiated the infection process of brown spot disease with *V. alginolyticus* through an injured integument in *P. setiferus*, but in the present case, black lesion was produced after two days without any injury by *V. alginolyticus*. It was possible because of the tenderness of the larval exoskeleton.

The epithelial cells of hepatopancreatic tubules were vacuolated and vacuoles contained eosinophilic materials. This reaction has been described as hyaline degeneration (Runnells *et al.*, 1960). Microscopically it appeared smooth, homogeneous, and deep pink in eosin-stained materials. The cause and pathological significance of hyaline degeneration are not known. Boyd (1970) believed that hyalinization was an end stage of many degenerative processes. Similarly, Bowser *et al.* (1981) and Elston *et al.* (1981) have observed high vacuolation in the hepatopancreas of *Vibrio*-infected *Homarus americanus* and larvae of *Crassostrea virginica* respectively.

The black colouration observed in the affected parts of the exoskeleton is due to melanin formation, which generally indicates host response to injuries, pathogens, or parasites. The presence of small rod-shaped bacteria in the melanized body parts of larvae of *P. indicus* and successful isolation of *V. alginolyticus* from the black lesion suggest the bacterium's ability to invade and induce melanization in the host.

ACKNOWLEDGEMENT

The author is grateful to Dr. P. Vedavyasa Rao, Principal Scientist, Central Marine Fisheries Research Insitute, Cochin, for his valuable guidance and ICAR, New Delhi, for the award of Senior Research Fellowship.

REFERENCES

Alfaro, J., Lawrence, A.L., and Lewis, D. (1993). Interaction of bacteria and male reproductive system blackening disease of captive *Penaeus setiferus*. *Aquaculture*, **117**: 1–8.

Bowser, P.R., Rosenar, R., and Reiner, C.R. (1981). A preliminary report of vibriosis in cultured American lobsters, *Homarus americanus*. *J. Invertebr. Pathol.*, **37**: 80–85.

Boyd, W. (1970). A Text Book of Pathology. Lea & Feibger, Philadelphia, 1464 pp.

Buchanan, R.E., and Gibbons, N.E. (1984). Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins Company, Baltimore, Maryland, 1268 pp.

Bullock, G.L. (1971). Identification of fish pathogenic bacteria. *In* S.F. Snieszko and H.R. Axelrod (eds.). Diseases of Fishes, TFH Publications, New York, p. 41.

Burns, C.D., Berrigan, M.E., and Hendrson, G.E. (1979). *Fusarium* sp. infections in the freshwater prawn *Macrobrachium rosenbergii* (De Man). *Aquaculture*, **16**: 193–198.

Cipriani, G.R., Wheeler, R.S., and Sizemore, R.K. (1980). Characterisation of brown spot disease of Gulf Coast shrimp. *J. Invertebr. Pathol.*, **36**: 255–263.

Cook, D.W., and Lofton, S.R. (1973). Chitinoclastic bacteria associated with shell disease in *Penaeus* shrimp and the blue crab (*Callinectes sapidus*). *J. Wildlife Dis.*, **19**: 154–159.

Delves-Broughton, J., and Poupard, C.W. (1976). Disease problems of prawns in recirculation systems in U.K. *Aquaculture*, **7**(3): 201–217.

Elston, R., Leibovitz, L., Relyea, D., and Zaltia, I. (1981). Diagnosis of vibriosis in a commercial oyster hatchery epizootic. Diagnostic tools and management features. *Aquaculture*, **20**: 53–62.

Fisher, W.S., Rosemark, T.R., and Shleser, R.A. (1976). Toxicity of malachite green to cultured American lobsters (*Homarus americanus*) larvae. *Aquaculture*, **8**: 151–176.

Johnson, S.K. (1978). Handbook of Shrimp Diseases. Sea Grant College Program Publ. TAMU-SG-75-603, 23 pp.

Lightner, D.V. (1975). Some potentially serious disease problems in the culture of penaeid shrimp in North America. Proc. U.S.-Japan Natural Resources Program. Symposium on Aquaculture Diseases, Tokyo, pp. 75–97.

Nimmo, D.R., Wilson, A.J., Jr., and Blackman, R.R. (1970). Localisation of DDT in body organs of pink and white shrimp. *Bull. Environ. Contam. Toxicol.*, **39**(5), 333–341.

Preece, A. (1972). A Manual for Histological Technician, 3rd ed. Little Brown & Co., Boston, 428 pp.

Pylant, R. (1980). A study of brown-spot disease and the histology of the integument in the white shrimp *Penaeus setiferus*. Master's thesis, University of Houston, Houston, Texas.

Runnells, R.A., Monulux, W.S., and Monlux, A.W. (1960). Principles of Veterinary Pathology. The Iowa State University Press, Ames, Iowa, 732 pp.

Sindermann, C.J. (1977). Disease diagnosis and control in North America marine aquaculture development. *In* Aquaculture and Fisheries Science, 6. Elsevier, New York, 329 pp.

West, P.A., and Colwell, R.R. (1984). Identification and classification of Vibrionaceae overview. *In* R.R. Colwell (ed.). Vibrios in the Environment. John Wiley, New York, pp. 285–363.