
J. Mar. Biol. Ass. India, **50** (1) : 1 - 6, January - June 2008

Phenotypic characteristics and antibiotic sensitivity of *Vibrio parahaemolyticus* strains isolated from diseased groupers (*Epinephelus* spp.)

*P. S. Shyne Anand, K. S. Sobhana, K. C. George and R. Paul Raj

*Physiology, Nutrition and Pathology Division, Central Marine Fisheries Research Institute, Kochi, India – 682 018, *E mail: shyne.anand@gmail.com*

Abstract

Bacterial isolations were made from the kidney/blood of diseased groupers (*Epinephelus malabaricus* and *E. tauvina*) on thiosulfate citrate bile salt sucrose agar (TCBS) supplemented with 2% NaCl for selective isolation of vibrios. The sucrose non-fermenting isolates obtained on TCBS agar were screened for sensitivity to 10 μg as well as 150 μg 0/129 vibriostatic discs. The presumptive *Vibrio parahaemolyticus* isolates which were found resistant to 10 μg and sensitive to 150 μg discs were further tested for the detailed phenotypic characteristics. Out of the 10 suspected isolates tested, 9 strains showed typical reactions of *V. parahaemolyticus*. The phenotypic and biochemical reactions of these *V. parahaemolyticus* strains were compared with that of a type strain of *V. parahaemolyticus* (MTCC 451). These strains were also subjected to antibiotic sensitivity studies using 10 different antibiotics. There was wide variation in the antibiotic sensitivity of the strains. Among the various antibiotics tested, chloramphenicol was found to be sensitive to 89% of the *V. parahaemolyticus* strains used in the study. Streptomycin and nalidixic acid were found sensitive to 67% while neomycin was sensitive to 44% of the strains. All the other antibiotics tested were resistant to most of the strains.

Keywords: *Vibrio parahaemolyticus,* antibiotic sensitivity, grouper, vibriosis

Introduction

Fish diseases of bacterial origin have been one of the most important factors of economic loss since the beginning of marine fish culture (Balebona *et al*., 1998). Bacteria of the genus *Vibrio* are ubiquitous in marine and estuarine aquatic ecosystems in which finfishes and shellfishes occur naturally and are farmed (Denner *et al*., 2002; Heidelberg *et al*., 2002). Several *Vibrio* spp. form part of the natural biota of fish and shellfish (Ruangpan and Kitao, 1991; Otta *et al*., 1999) and these bacteria behave as opportunistic fish pathogens in marine environments. The onset of vibriosis is generally associated with deteriorating culture conditions or physical damage to the cultured fish (Colorni *et al*., 1981). Susceptibility to *Vibrio* infections can be caused by a number of environmental and host factors causing fish to be stressed (Anderson, 1990). Environmental factors such as rapid changes in water characteristics including temperature, salinity, organic content and low oxygen levels (Anderson, 1990; Newman, 1993) are associated with vibriosis outbreaks in fish farms. The principal bacteria identified in epidemic diseases of marine finfishes and shellfishes are *Vibrio anguillarum, V. alginolyticus, V. fluvialis, V. furnissii, V. harveyi, V. parahaemolyticus* and *V. vulnificus* (Buck, 1990; Lightner *et al*.,1992).

Vibrio parahaemolyticus is one of the major *Vibrio* species associated with vibriosis in marine shellfish (Lee *et al*., 2003). To date, there are several reports on the pathogenecity of *V. parahaemolyticus* in marine finfish. *V. parahaemolyticus* has been isolated from skin ulcers in red seabream and other marine fishes in

Japan (Kusuda *et al*., 1979; Hatai *et al*., 1981). Wong and Leong (1986) reported *V. parahaemolyticus* as a predominant species associated with vibriosis in the kidney or spleen of *Lates calcarifer*. Liao *et al*. (2004) reported *V. parahaemolyticus* as one of the secondary invaders along with other vibrios in cobia culture in Taiwan. These bacteria are also important in public health and many of them are capable of producing gastrointestinal disorders in humans who have ingested contaminated fish and shellfish (Nolan *et al*., 1984).

According to Bergey's manual of systematic bacteriology (Brenner *et al*., 2005), members of the genus *Vibrio* (Family:Vibrionaceae) are Gram negative, usually motile rods having a facultative fermentative metabolism. They are generally able to grow on marine agar and on the selective medium thiosulfate-citrate bile salt-sucrose agar (TCBS), and are mostly oxidase positive. We made an attempt for phenotypic characterisation and to study the antibiotic sensitivity of *V. parahaemolyticus* strains isolated from diseased groupers (*Epinephelus* spp.). The study formed part of the research work on identification of specific cell wall antigens of *V. parahaemolyticus* so as to develop an indirect enzyme linked immunosorbant assay (ELISA) technique for rapid detection of *V. parahaemolyticus.*

Materials and methods

Test strains: Diseased groupers (*Epinephelus malabaricus* and *E. tauvina*) from different disease incidences were screened for vibrios using thiosulfate citrate bile salt sucrose (TCBS) agar, (Difco) agar supplemented with 2% NaCl. The isolations were done from kidney and blood of the diseased fish. Out of the presumptive vibrio isolates obtained on TCBS agar, the sucrose non-fermenting isolates (16 numbers) were tested for sensitivity to 10 ì g as well as 150 μg 0/129 vibriostatic discs. The isolates which were resistant to 10 μg and sensitive to 150 μg discs were further tested for detailed phenotypic characteristics by performing a series of biochemical tests for identification up to species level as per Alsina and Blanch (1994) and Brenner *et al*. (2005). The phenotypic and biochemical reactions were compared with that of a type strain of *V. parahaemolyticus* (MTCC 451) procured from the Microbial Type Culture Collections of the Institute of Microbial Technology (IMTECH), Chandigarh.

***Phenotypic characterisation of the test isolates* :** The strains were characterized based on their growth on selective media (TCBS agar, Difco), colony characteristics, Gram staining, motility and a series of biochemical tests, such as amino acid decarboxylase test, sugar fermentation test, growth in salt tryptone broth to study halophilism, growth at 42°C, cytochrome oxidase test, nitrate reduction test, catalase test, methyl red and Voges Proskaeur test, indole production, triple sugar iron agar (TSI) and Kligler iron agar (KIA) reactions, oxidation/ fermentation (O/F) test, urease test, citrate utilization test, O-nitrophenyl β-D galactopyranosidase ONPG (â-galactosidase) test and gelatinase test (Holding and Collee,1971; West and Colwell, 1984). All media used for the test were supplemented with 2% NaCl (W/V). The isolates were then identified to species level according to Alsina and Blanch (1994) and Brenner *et al*. (2005).

***Kanagawa reaction to test haemolytic activity (β – haemolysis*):** The Kanagawa reaction was performed on Wagatsuma agar (Wagatsuma, 1967). Haemolytic activity of the bacterial strains were tested by spot inoculating the strains onto each freshly prepared and dried Wagatsuma agar (Hi-Media, India) containing a suspension of mammalian/fish red blood cells. The plates were incubated for 18 $\pm$ 2 h at 37°C and observed for presence of zone of haemolysis around the colony.

***Antibiotic susceptibility testing*:** The nine *V. parahaemolyticus* strains giving typical reactions were tested for antibiotic susceptibility on Mueller Hinton agar (Hi-Media, India) supplemented with 2% NaCl, as per Kirby-Bauer disk diffusion method (Bauer *et al*., 1996). The isolates were tested using the antibiotic discs (Hi-Media, Mumbai) for their susceptibility to a set of ten antibiotics *viz*., streptomycin, sulphadiazine, gentamycin, nitrofurantoin, kanamycin, chloramphenicol, nalidixic acid, neomycin, amoxycillin and oxytetracycline. The results were

recorded on the basis of the diameter of the inhibition zone from the zone size interpretative chart supplied by the manufacturer. Organisms with "intermediate" levels of resistance were included in the percentage of resistant organisms for final analysis.

Results

***Phenotypic characteristics of the test strains*:** Table 1 summarises the phenotypic characteristics of the vibrio isolates used in the study. Out of the suspected 10 isolates tested, 9 strains gave typical reactions of *V. parahaemolyticus* as per Alsina and Blanch (1994) and Brenner *et al*. (2005). The type strain of *V. parahaemolyticus* (MTCC 451) also gave identical reactions. One strain (V18) did not grow at 8% salt level and also gave negative reaction for fermentation of arabinose and positive reaction for cellobiose.

***Kanagawa reaction (β – haemolysis)*:** All the test strains and the MTCC reference strain of *V. parahaemolyticus* gave negative reaction for haemolytic activity on Wagatsuma agar indicating that they were all Kanagawa negative.

***Antibiotic sensitivity*:** The antibiotic sensitivity pattern to individual antibiotics are summarised in Table 2. All the strains tested were resistant to sulphadiazine and amoxycillin. About 78% of the *V. parahaemolyticus* strains from diseased groupers were found resistant to gentamycin, while 89% of the strains were resistant to kanamycin and oxytetracycline. Among the various antibiotics tested, chloramphenicol was found to be sensitive to 89% of the strains. Streptomycin and nalidixic acid were found sensitive to 67% while neomycin was sensitive to 44% of the strains.

Discussion

Bacterial disease is one of the major problems affecting production, development and expansion of aquaculture. The control of disease is particularly difficult because fish are often farmed in systems where production is dependent on natural environmental conditions. Most of the bacterial diseases are associated with changes or deterioration of the aquatic environment. Identification of the causative agents is important to ensure successful control measures. Traditionally, the diagnosis of infectious diseases has been accomplished by the isolation of the infecting microorganism in pure culture. Classical methods of microbial isolation and identification have been invaluable in the study of bacterial, viral and fungal infections, even though these methods offer disadvantages for the rapid diagnosis of infectious diseases.

All the test strains used in the present study were originally isolated from diseased groupers (*Epinephelus* spp.). Out of the sixteen non-sucrose fermenting vibrio isolates, 10 presumptive *V. parahaemolyticus* strains which were found resistant to 10 μg and sensitive to 150 μg 0/129 vibriostatic discs were further tested for detailed phenotypic characteristics. The phenotypic variability of isolates from the environment and from fish makes it difficult to distinguish accurately between *V. parahaemolyticus* and other members of the genus, particularly *V. alginolyticus*, *V. harveyi* and *V. mimicus* by means of biochemical tests. *V. parahaemolyticus* and *V. alginolyticus* are very closely related (Wachsmuth *et al*., 1980). One of the major difficulties in biochemical identification of *V. parahaemolyticus* is the variability in some of the activities such as fermentation of sugars like sucrose, arabinose and cellobiose (Karunasagar *et al*., 1997). However in the present study, except V18 all other strains gave typical reactions as per Brenner *et al*. (2005). The type strain of *V. parahaemolyticus* (MTCC 451) used in the present study also gave similar biochemical and phenotypic characteristics.

All the test strains and the MTCC reference strain of *V. parahaemolyticus* gave negative reaction for haemolytic activity on Wagatsuma agar indicating that they were Kanagawa negative. However, all the strains used tested positive for urease production. In the Kanagawa reaction test for the presence of specific β- haemolysis on Wagatsuma agar, a positive reaction correlates with the human pathogenecity of the organism. Even though most of the environmental strains of *V. parahaemolyticus* are typically not human pathogens, they cause disease in fish and shellfish (Chen and Hanna, 1992; Montilla *et al*., 1994).

Table 1. Phenotypic characteristics of *Vibrio parahaemolyticus* strains

Biochemical tests	Test strains										
	V8	V9	V10	V21	V23	V24	V97	V98	E45	V18	MTCC 451
Gram reaction	-	-	-	-	-	-	-	-	-	-	-
Cell morphology	short rod	short rod	short rod	short rod	short rod	short rod	short rod	short rod	short rod	short rod	short rod
Growth on TCBS	G	G	G	G	G	G	G	G	G	G	G
Luminescence	-	-	-	-	-	-	-	-	-	-	-
Swarming on solid media	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+
0/129 sensitivity :											
10μg	R	R	R	R	R	R	R	R	R	R	R
150μg	S	S	S	S	S	S	S	S	S	S	S
Decarboxylase tests:											
Arginine	-	-	-	-	-	-	-	-	-	-	-
Lysine	+	+	+	+	+	+	+	+	+	+	+
Ornithine	+	+	+	+	+	+	+	+	+	+	+
Growth in % NaCl:											
0	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	-	+
10	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-
Methyl Red	+	+	+	+	+	+	+	+	+	+	+
O/F test	F	F	F	F	F	F	F	F	F	F	F
Growth at 42°C	+	+	+	+	+	+	+	+	+	+	+
Acid from :											
Sucrose	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+	+	+	-	+
Cellobiose	-	-	-	-	-	-	-	-	-	+	-
Inositol	-	-	-	-	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Enzyme production:											
Gelatinase	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+
Simmons Citrate	+	+	+	+	+	+	+	+	+	+	+
KIA	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
TSI	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A	K/A
Gas production	-	-	-	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-

+ : Positive reaction; - : negative reaction; G : green colony; R : resistant; S : sensitive; F : fermentative; K : alkaline; A : acidic

Table 2. Antibiotic sensitivity of *Vibrio parahaemolyticus* strains

Antibiotics	Conc. μg/disc	V8	V9	V10	V21	V23	V24	V97	V98	E45
Streptomycin (S^{10})	10	S	S	S	R	R	S	S	S	R
Sulphadiazine (Sz^{300})	300	R	R	R	R	R	R	R	R	R
Gentamicin (G^{10})	10	R	S	R	R	S	I	R	R	R
Nitrofurantoin (Nf^{300})	300	R	I	R	I	S	I	R	R	R
Kanamycin (K^{30})	30	R	I	S	R	R	R	R	I	R
Chloramphenicol (C^{10})	10	S	S	S	I	S	S	S	S	S
Nalidixic acid (N^{30})	30	S	S	S	S	I	I	I	S	S
Neomycin (N^{30})	30	I	S	S	R	S	R	S	I	R
Amoxycillin (Am^{10})	10	R	R	R	R	R	R	R	R	R
Oxytetracycline (O^{30})	30	I	R	R	I	I	I	I	I	S

R – Resistant; I – Intermediate; S - Sensitive

The antibiogram of the isolates indicated the sensitivity pattern of the strains to commonly used/ conventional antibiotics which in turn, may be useful in determining the therapeutants. All the strains tested were resistant to sulphadiazine and amoxycillin. Only streptomycin, chloramphenicol and nalidixic acid were sensitive to more than 50% of the *V. parahaemolyticus* strains tested. Because of the widespread use of antibiotics, the resistance profile of the microorganisms are changing, as evidenced by the increasing resistance among bacterial population from aquatic and other environments (Shahul Hameed *et al*., 2003). Environmental contamination with antibiotics contributes to the maintenance and spread of antibiotic resistance genes (Goni-Urriza *et al*., 2000). One mechanism that allows the perpetuation of such genes is the spread of resistance plasmids between unrelated bacteria in natural environments (Kruse and Sorum, 1994). Although vibrios are widespread in aquatic ecosystems and have been designated as an emerging threat to human health, little is known about their antibiotic resistance profiles.

Acknowledgements

The authors wish to express their gratitude to Dr. Mohan Joseph Modayil, Director, Central Marine Fisheries Research Institute for providing necessary facilities for carrying out this research work. The financial support in the form of a junior research fellowship awarded to the first author by the Indian Council of Agricultural Research (ICAR), New Delhi during the period of study is gratefully acknowledged.

References

Alsina, M. and A. Blanch. 1994. Improvement and update of a set of keys for biochemical identification of *Vibrio* species. *J. Appl. Bacteriol*., 77: 719-721.

Anderson, D. P. 1990. Immunological indicators: Effects of environmental stress on immune protection and disease outbreaks. *Am. Fish. Sci. Soc. Symp*., 8: 38-50.

Balebona, M. C., I. Zorrilla, M. A. Morinigo and J. J. Borrego. 1998. Survey of bacterial pathologies affecting farmed gilt-head sea bream *Sparus aurata* L. in southwestern Spain from 1990 to 1996. *Aquaculture*, 166: 19–35.

Bauer, A. W., W. M. M. Kirby, J. C. Shenis and M. Turk. 1996. Antibiotic susceptibility testing by a standardized single disc method. *A. J. Clin. Pathol*., 45: 493 – 498.

Brenner, D. J., N. R. Krieg and J. T. Staley. 2005. *Bergey's Manual of Systematic Bacteriology*,Vol. 2, Springer, New York, II. 491 – 555.

Buck, J. D. 1990. Potentially pathogenic marine *Vibrio* species in sea water and marine animals in the Sarasota, Florida area. *J. Coast. Rev*., 6: 943-948.

Chen, D. and P. J. Hanna. 1992. Attachment of *Vibrio* pathogens to cells of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis*., 15: 331–337.

Colorni, A., I. Paperna and H. Gordin. 1981. Bacterial infections in gilt-head sea bream *Sparus aurata* cultured at Eilat. *Aquaculture,* 23: 257–267.

Denner, E. B., M. D. Vybiral, U. R. Fischer, B. Velimirov and H. J. Busse. 2002. *Vibrio calviensis* sp. *nov*., a halophilic, facultatively oligotrophic 0.2 micron-filterable marine bacterium. *Int. J. Syst. Evol. Microbiol*., 52: 549 - 553.

Goñi-Urriza, M., M. Capdepuy, C. Arpin, N. Raymond, P. Caumette and C. Quentin. 2000. Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas* spp. *Appl. Environ. Microbiol.*, 66:125-132.

Hatai, K., S. Yasumoto and N. Yasunaga. 1981. On *Vibrio* strains isolated from cultured Japanese horse mackerel (*Trachurus japonicus*). *Fish Pathol.,* 16: 111-118.

Heidelberg, J. F., K. B. Heidelberg and R. R. Colwell. 2002. Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. *Appl. Environ. Microbiol.,* 68: 5498–5507.

Holding, A. J. and J. G. Collee. 1971. Routine biochemical tests. *In:* Norris, J. R. and D. W. Robbins (Eds.), *Methods in Microbiology,* 6A, Academic Press, London, p.1 – 32.

Karunasagar, I., B. B. Nayak and I. Karunasagar. 1997. Rapid detection of *Vibrio parahaemolyticus* from fish by polymerase chain reaction (PCR). *In:* Flegel, T. W. and I. H. MacRae (Eds.), *Diseases in Asian Aquaculture III*, Fish Health Section, Asian Fisheries Society, Manila, p. 119– 122.

Kruse, H. and H. Sørum. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Appl. Environ. Microbiol*., 60: 4015-4021.

Kusuda, R., H. Salco and K. Kawai. 1979. Classification of vibriosis isolated from diseased fishes. Part 1. On the morphological and biochemical properties. *Gyobyo Kenkya*, 13: 123-138.

Lee, K. K., P. S. Liu and C. Y. Huang. 2003. *Vibrio parahaemolyticus* infectious for both humans and edible mollusk abalone. *Microbes and Infection*, 5: 481–485.

Liao, I. C., T. S. Huang, W. S. Tsai, C. M. Hsueh, S. L. Chang and E. M. Leano. 2004. Cobia culture in Taiwan: Current status and problems. *Aquaculture*, 237: 155-165.

Lightner, D. V., T. A. Bell, R. M. Redman, L. L. Mohney, J. M. Natividad, A. Rukyani and A. Poernomo. 1992. A review of major diseases of economic significance in penaeid prawns/ shrimps of the Americas and Indo-Pacific, *In:* Shariff, M., R. P. Subasinghe and J. R. Arthur (Eds.), *Diseases in Asian aquaculture, I*. Fish Health Section of the Asian Fisheries Society, Manila, Philippines, p. 57-80.

Montilla, R., J. Palomar, M. Santmarti, C. Fuste and M. Vinas. 1994. Isolation and characterization of halophilic *Vibrio* from bivalves bred in nurseries at the Ebro Delta. *J. Invertebr. Pathol*., 63: 178 -181.

Newman, S. G. 1993. Bacterial vaccines for fish. *Annu. Rev. Fish. Dis*., p. 145-185.

Nolan, C. M., J. Ballard, C. A. Kaysner, J. L. Lilja, L. P. J. Williams and F. C. Tenover. 1984. *Vibrio parahaemolyticus* gastroenteritis. An outbreak associated with raw oysters in the Pacific Northwest. *Diagn. Microbial. Infect. Dis*., 2: 119-128.

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

Ruangpan, L. and T. Kitao. 1991. *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon* Fabricius. *J. Fish Dis.,* 14: 383-388.

Shahul Hameed, A. S., K. A. Rahman, A. Alagan and K. Yoganandan. 2003. Antibiotic resistance in bacteria isolated from hatchery reared larvae and postlarvae of *Macrobrachium rosenbergii*. *Aquaculture*, 217: 39 – 48.

Wachsmuth, I. K., G. K. Morris and J. C. Feely. 1980. *Manual of Clinical Microbiology*. American Society for Microbiology, Washington, DC.

Wagatsuma, S. 1967. On a medium to test the hemolytic reaction of *Vibrio parahaemolyticus*. *Media Circle,* 3: 159–162.

West, P. A. and R. R. Colwell. 1984. Identification and classification of Vibrionaceae – an overview. *In*: Colwell, R. R. (Ed.), *Vibriosis in the Environment*, John Willey and Sons, New York, p. 285 - 363.

Wong, S. Y. and T. S. Leong. 1986. Bacterial flora of sea bass, *Lates calcarifer* Bloch, imported from Thailand for cage culture in Penang, Malaysia. *In: Proceedings of the the First Asian Fisheries Forum*, *MacLean, J. L., L. B. Dizon and L. V. Hosillos (Eds.)*, Asian Fisheries Society, Manilla, Philippines, p. 243 - 246.

Received: 7 November 2007
Accepted: 14 February 2008