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Production of Extracellular Enzymes by Pathogenic *Vibrio* spp. in Fish

K. Dhevendaran and M.I. Georgekutty
Department of Aquatic Biology and Fisheries,
University of Kerala, Trivandrum - 695 007, India

Production of extracellular enzymes and haemolysin in a few species of *Vibrio* was examined. Majority of *Vibrio* spp. produced lipase, protease and arylsulfatase. More than 90% of the strains released haemolysin on fish blood agar and a few on calf blood and sheep blood agar. Representative strains isolated from fish showed lethality against white mice. Infected parts of fish harboured arylsulfatase producing bacteria. The pH activity curve of arylsulfatase showed a primary peak at pH 5.2 and secondary peak at pH 6.2.

Key words: ***Vibrio*** **spp., extracellular enzymes, haemolysin, arylsulfatase.**

The members of vibrionaceae are pathogenic to humans, fish, crustacea and bivalves (Colwell, 1984). Tareen (1984) observed pathological changes in *Oreochromis aureus* due to *Vibrio alginolyticus* infection. Albuminase, elastase, sulfatase and hyaluronidase are thought to be active in the breakdown of mucopolysaccharides of tissue. These enzymes may be involved in the potentially fatal wound infections which *Vibrio vulnificus* is capable of producing. *Vibrio salmonicida* and *Vibrio damsela* have been implicated in epizootics of cultured and wild fish (Egidius *et al.* 1986; Fujioka *et al.*, 1988). Georgekutty (1989) made a survey on the distribution of *Vibrio* spp. in water and infected parts of diseased fish in Trivandrum coast. Maya et al. (1995) observed the proliferation of *Vibrio* spp. in relation to weight of alimentary canal of *Etroplus suratensis* and *Etroplus maculatus*. A great variety of extracellular substances have been associated with the virulence of pathogenic bacteria. They include a variety of proteases (Kothary & Kreger, 1985), lipase and nuclease (Janda *et al.*, 1984). Similarly haemolysin (Miyamoto *et al.*, 1969) and arylsulfatase (Baum *et al.*, 1959) are also involved in the pathogenesis. Inamura *et al.* (1985) regarded protease produced by *Vibrio* spp. as one of the virulence determinants in infection of fish. The present study was undertaken to examine the production of extracellular enzymes and haemolysin by *Vibrio* spp. associated with diseased fish.

Materials and Methods

A total of 1589 fish were collected over a period of one year (from June 1990 to May 1991) from Veli, Akkulam lake, Valiathura and Poonthura backwaters near Trivandrum, Kerala. Of these, 379 fish showing abnormalities such as skin lesions, fin and tail rot, haemorrhagic syndrome etc. were secluded and transported alive to the laboratory within the shortest period of time. The infected external parts and damaged internal organs of moribund fish were aseptically removed and homogenized with 99 ml

of 50% sea water for the isolation of pathogenic microorganisms.

Isolation of bacterial pathogens from the infected site was carried out following the method of Tareen (1984) and Georgekutty (1989) using nutrient agar, ZooBells 2216E agar, Vibrio agar and TCBS media. Haemolysin production was studied using Wagatsuma agar medium (Miyamoto *et al.*, 1969). The medium was melted in a water bath and cooled to 50°C. Citrated, non-coagulated blood from sheep, calf and fish were added to the above medium and immediately poured into the sterile petri dish. 18 h old tryplic say broth was spotted on well-dried wagatsuma agar plates and the plates were incubated at 37°C for 18 h. Plates showing haemolysin production with a zone of transparent clearing of blood around a colony was considered as positive.

Vibrios were identified upto species level based on Bergey's Manual of Systematic Bacteriology (Statey *et al.*, 1989).

Protease production was determined using casein and lipase, using Tween 80 (Harringan & Mc Cance, 1972). The arylsulfatase producing bacteria were estimated by the method of Dhevendaran *et al.* (1985) using chromogenic substrates, tripotassium phenolphthalein disulfate (PDS) and nitrocatechol sulfate (NCS). In order to know the nature of arylsulfatase enzyme in liver, *V. anguillarum* was injected intraperitoneally into *O. spiluris*. When the fish died, the damaged part of liver was collected and enzyme activity was determined at pH 4.0 to 8.0 using 0.0002 M NCS as the substrate. 1 gm of infected part of the flesh from the diseased fish was dissected and mixed with 5 ml of distilled water and ground well in tissue homogenizer. 1 ml of the sample was pipetted out into the Mc Cortney bottle and mixed with 3 ml of 0.2 M buffer of pH range of 4.8 to 10.6, along with 1 ml of substrate. Toluence (0.5) ml was added to arrest microbial activity (Dhevendaran *et al.*, 1980). It was incubated at room temperature for 12 h and the activity was stopped by addition of 1 ml of 1

Table 1. Identification of different *Vibrio* isolates

Names of fish	Total isolates	Number identified	*V. fischeri*	*V. vulnificus*	*V. costicola*	*V. cholerae*	*V. anguillarum*	*V. alginolyticus*	*V. parahaemolyticus*
Anabas testudineus	31	30	2	4	-	3	7	3	1
Etroplus suratensis	26	25	-	2	-	2	5	4	2
Etroplus maculatus	27	24	1	2	-	3	5	2	1
Oreochromis spiluris	32	29	2	6	-	3	12	6	-
Puntius amphibius	11	10	-	-	-	1	4	5	-
Clarias batrachus	9	6	-	-	-	1	1	2	2
Channa striatus	14	9	3	1	1	1	2	1	-
Liza tade	19	13	3	-	-	1	4	2	3
Total	169	146	11	15	1	15	40	25	9
Unidentified	29								

Table 2. Production of extracellular enzymes, haemolysin and lethality in mice by *Vibrio* spp

Sl. No.	Bacteria	Culture Number	Enzyme activity			Haemolysis			Lethality for mice
			Arylsulfatase	Protease	Lipase	Fish blood	Calf blood	Sheep blood	
1.	*Vibrio anguillarum*	AQB-ES-23	+	+	+	+	-	-	-
2.	-do-	AQB-OS-23	+	+	+	+	-	-	+
3.	-do-	AQB-OS-03	+	+	-	+	-	-	-
4.	-do-	AQB-MC-03	+	+	+	+	+	-	-
5.	-do-	AQB-OS-04	+	+	+	+	-	+	-
6.	*Vibrio alginolyticus*	AQB-ES-01	+	+	+	+	+	-	-
7.	-do-	AQB-OS-06	+	+	+	+	-	+	-
8.	-do-	AQB-A-04	+	+	+	-	-	+	-
9.	-do-	AQB-S-07	+	+	-	+	-	+	-
10.	-do-	AQB-EM-01	+	+	+	+	-	+	-
11.	-do-	AQB-VC-08	+	+	+	+	+	+	-
12.	-do-	AQB-RK-07	+	+	+	+	-	-	-
13.	*Vibrio vulnificus*	AQB-OS-09	+	+	+	+	-	+	+
14.	-do-	AQB-ES-08	+	+	+	-	+	+	+
15.	-do-	AQB-EM-24	+	+	+	+	-	-	+
16.	-do-	AQB-CS-08	+	+	+	+	-	+	+
17.	-do-	AQB-A-21	-	+	+	+	+	+	+
18.	-do-	AQB-P-16	+	+	+	+	-	+	+
19.	-do-	AQB-GF-11	+	+	-	+	+	+	+
20.	-do-	AQB-RK-10	-	+	+	+	-	-	+
21.	*Vibrio cholerae*	AQB-VC-12	+	+	+	+	+	-	+
22.	-do-	AQB-ES-11	+	+	-	-	-	-	-
23.	-do-	AQB-EM-13	-	+	+	+	-	+	+
24.	*Vibrio parahaemolyticus*	AQB-ES-07	+	+	+	-	+	+	+
25.	-do-	AQB-EM-11	+	+	+	-	-	-	-
26.	-do-	AQB-OS-14	-	+	+	+	-	+	+
27.	-do-	AQB-OS-17	+	+	+	+	+	-	+
28.	-do-	AQB-PI-16	-	+	+	+	-	-	+
29.	-do-	AQB-PI-21	+	+	-	-	+	+	-
30.	-do-	AQB-MC-19	-	+	+	+	-	+	+

+ positive, - negative, AQB - Aquatic Biology, ES - *E. suratensis*, EM - *E. maculatus*, P - *Puntius*, OS - *O. spiluris*, A - *Anabas*, VC - *V. cyprinoides*, MC - *M. cephalus*, CS - *C. striatus*.

N NaOH. The colour was measured in spectrophotometer at 515 nm. Lethality for laboratory mice was tested by intraperitoneal injection of cell suspension (0.5 ml) grown overnight in heart infusion broth at 25°C (Oliver *et al.*, 1986).

Results and Discussion

Since *Vibrio* spp. constituted an important group in brackish, marine and estuarine environments, they were identified to the species level (Table 1). The salinity of water in these areas ranged from 2 to 9‰. The species

Table 3. Arylsulfatase activity in selected bacterial pathogens using Nitrocatchol sulphate (NCS)

Source	Culture Number	Enzyme activity (µgNCS/mg/hr)
Mugil cephalus		
Skin	AQB-MCS1	260
	AQB-MCS2	280
	AQB-MCS3	260
Gut	AQB-MCG1	180
	AQB-MCG2	180
	AQB-MCG3	180
	AQB-MCG4	210
	AQB-MCG5	190
Gill	AQB-MCGL1	220
	AQB-MCG2	220
Liver	AQB-MCL1	280
	AQB-MCL2	270
	AQB-MCL3	300
	AQB-MCL4	300
	AQB-MCL5	290
	AQB-MCL6	280
	AQB-MCL7	270
	AQB-MCL8	250
	AQB-MCL9	260
Etroplus suratensis		
Mouth	AQB-ESM1	510
	AQB-ESM2	500
	AQB-ESM3	580
	AQB-ESM4	500
Skin	AQB-ESS1	500
	AQB-ESS2	510
	AQB-ESS3	500
	AQB-ESS4	510
	AQB-ESS5	410
Etroplus maculatus		
Kidney	AQB-EMK1	250
	AQB-EMK2	230
	AQB-EMK3	240
	AQB-EMK4	200

AQB - Aquatic Biology & Fisheries, MCS - *Mugil cephalus* skin, MCG - *M. cephalus* gut, MCL - *M. cephalus* liver, ESM - *Etroplus suratensis* mouth, ESS - *E. suratensis* skin, EMK - *E. maculatus* kidney.

encountered were *V. parahaemolyticus, V. alginolyticus, V. cholerae, V. costicola, V. anguillarum, V. vulnificus* and *V. fisheri. V. anguillarum* was most common species constituting 31-42% of total *Vibrio* spp. followed by *V. alginolyticus.*

Table 4. Screening of pathogens for arylsulfatase activity using Phenopthalein disulphate

Culture No.	Culture Source	Enzyme activity (µg phnolphthalein/mg/hr)
AQB-OS. 01	*O. spiluris*	90
02	"	80
03	"	90
04	"	75
05	"	70
06	"	68
07	"	60
08	"	60
09	"	58
10	"	80
AQB-C. 01	*Channa straitus*	63
02	"	64
03	"	58
04	"	64
05	"	78
06	"	62
07	"	85
08	"	50
09	"	64
10	"	70
ABC-C. 01	"	100
02	"	70
03	"	85
04	"	100
05	"	70
06	"	78
07	"	60
08	"	60
09	"	50

Mass development of *Vibrio* at the infected site of fish was observed by Sakasakai (1967). The earlier observations (Georgekutty & Dhevendran 1994, Maya *et al.*, 1995) revealed the predominance of *Vibrio* in the infected sites of fish and in the alimentary canal of *E. Suratensis* and *E. maculatus.* The results of the present study are in agreement with the above finding. Maya *et al.* (1995) found positive correlation between population of *Vibrio* spp. and weight of the alimentary tract.

Randomly selected *Vibrio* strains were tested for their ability to produce extracellular enzymes and haemolysin which are considered to be responsible for the virulence of the pathogen (Table 2). From the observation it could be found that majority of the strains exhibited lipase, protease and arylsufatase activities. The hydrolysis of Tween 80 by lipase took about 48 h. Oliver *et al.* (1986) observed similar results. More than 90% of *Vibrio* degraded serum albumin protein. Inamura *et al.* (1985), and Sandhya (1995) have reported correlation between virulence and protease activity.

The production of haemolysin was tested with fish, calf and sheep bloods. More than 90% of the strains released haemolysin (Table 2) with fish blood whereas 33.33% of strains haemolysed the calf blood. Oliver *et al.* (1986) observed the production of haemolysin using prepared blood agar plates containing 5% sheep erythrocytes (BBC) and they found that all strains of *V. vulnificus* examined were haemolytic on sheep blood agar. They also correlated extracellular enzymes and haemolysins with pathogenicity of *Vibrio* spp.

Infected parts of selected fish such as *Mugil cephalus, E. suratensis, E. maculatus, O. spiluris* and *Channa striatus* harboured arylsulfatase producing bacteria (Table 3 & 4). This is in agreement with the earlier observations of Dhevendaran *et al.* (1985) and Maya *et al.* (1990) in marine environment and in healthy fish. Experimentally infected *O. spiluris* exhibited arylsulfatase activity in liver. Primary and secondary peaks of the enzyme activity was noticed at pH 5.2 and 6.2 respectively (Fig. 1). Aryl

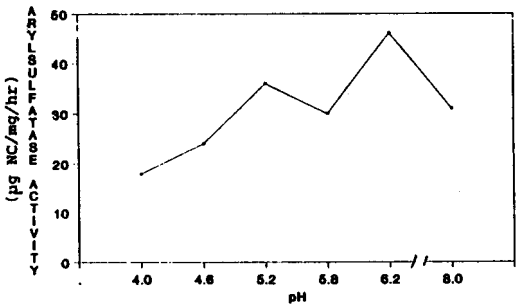

Fig. 1. pH activity curve of arylsulfatase from infected part of fish liver using NCS

sulfatase from healthy gastropod, *Telescopium telescopium* (Dhevendaran *et al.* 1980); polychaete, *Neries* (Dhevendaran, 1984); prawn, *Penaeus indicus*; fish, *Therapon jarbua* and bivalve, *Villorita cyprinoides* (Maya *et al.* 1990; 1995) exhibited the primary peak at pH 5.6 and secondary peak at pH 10.6. However, human pathogens like *Salmonella, Mycobacterium, Vibrio* and *Proteus vulgaris* showed the maximum activity at pH 6.2 (Dhevendaran *et al.*, 1985). It is stated that arylsulfatase activity in the gut of *T. jarbua* was partially contributed by indegenious bacteria. (Maya *et al.*, 1990). It had been observed that the maximum arylsulfatase activity in the mantle of *Balanus eburneus* had been shifted from pH 5.6 to 5.1 due to inhibition of endogenous phosphate. It is presumed that certain compounds secreted by bacteria might have shifted the pH peak from 5.6 to 5.2. However, occurrence of this type of arylsulfatase (pH 5.2) in the diseased part of fish is quite unique.

Lethality test using laboratory mice (Oliver *et al.*, 1986) to differentiate pathogenic and non-pathogenic vibrios had shown that *V. anguillarum, V. vulnificus, V. cholerae* and *V. parahaemolyticus* were lathal. Numerous studies have been reported indicating a

correlation between virulence and the production of certain extracellular enzymes in the members of the genus *Vibrio*. The present study indicated some correlation between production of protease and virulence in *Vibrio* spp.

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References

Baum, H., Dodgson, K.S. & Spencer, B. (1959) *Cli. Chem. Acta.* **4**, 453

Colwell, R.R. (1984) In: *Vibrios in the Environment* p. 623, John Wiley & Sons, New York, USA

Dhevendaran, K. (1984) *Fish Technol.* **21**, 57

Dhevendaran, K. Kannupandi, T. & Natarajan, R. (1980) *Mahasagar,* **13**, 173

Dhevendaran, K., Chandramohan, D. & Natarajan, R. (1985) *Fish. Technol.* **22**, 121

Egidius, E., Wilk, R., Anderson, K., Hobb, K.A. & Hjeltner, B. (1986) *Int. J. Syst. Bacteriol.*, **36**, 518

Fujioka, R.S., Greco, S.B., Cotes, M.B. & Schoroedor, J.P. (1988) *Dis. Aqu. Organism,* **4**, 1

Georgekutty, M.I. (1989) *Studies on Vibriosis in Fishes of Trivandrum Coast,* M. Phil Thesis, University of Kerala.

Georgekutty M.I. & Dhevendaran, K. (1994) *Proc. Sixth Kerala, Sci. Cong.* 371

Harringan, W.F. & McCance (1972) *Laboratory Methods in Microbiology* Academic Press, London

Inamura, H., Nakar, T.N., & Muroka, K. (1985) *Bull. Jap Soc. Sci. Fish.* **57**, 1951

Janda, J.M., Reitano, M. & Buttone, E.J. (1984) *J. Clin. Microbiol.* **19**, 44

Kothary, M.H. & Kreger, A.S. (1985) *Infection and Immunity* **50**, 534

Maya, K., Dhevendaran, K. & Natarajan, P. (1990) In: *Microbiology in Poikilotherms,* (Lessel, R., Ed.) p. 203, Elsavier Science, Publishers, B.V. Biomedical Division, Amsterdam

Maya, K., Dhevendaran, K. & Natarajan, P. (1995) *J. Mar. Biol. Ass. India,* **37**, 259

Maya, R., Dhevendaran, K., Annie Mathew, Georgekutty, M.I. & Natarajan P. (1995) *Indian J. Mar. Sci.* **24**, 225

Miyamoto, Y., Kato, T. Obara, Y., Akiyama, S., Takizawa, K. & Yamai, S. (1969) *J. Bacteriol.* **100**, 1147

Oliver, J.D., Ween, J.E., Thomas, M.B., Wanner, M. & Linder, K. (1986) *Diag. Microbiol. Infec. Dis.* **5**, 99

Sakasakai, R. (1967) *Isolation and Identification of Vibrio parahaemolyticus,* Nayashotan, Tokyo, Japan

Sandhya, S.D. (1995) *Studies on protein degradation by bacterial proteolytic activity and their growth in fish flesh extracts of certain fish.* M.Sc. Project, University of Kerala, Trivandrum

Statey, T.J., Bryant, P.M. & Pfenning, N. (Eds) (1989) *Bergey's Manaul of Systematic Bacteriology,* William and Willkins, Baltimore

Tareen, I.V. (1984) *Bull. Eun. Ass. Fish Pathol,* **4**, 47