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Assessment of Virulence and Antimicrobial Susceptibility of *Vibrio vulnificus* Isolated from *Etroplus suratensis* (Bloch)

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ABSTRACT

Pearl spot (discolourised skin, hemorrhagic patches, bloody blotches around fins and mouth of fish) *Etroplus suratensis*, Cichlidae family were identified at backwaters of Muttukadu, CIBA and Chennai. Diseased fishes were collected and the causative agent isolated from tissues of kidney. A control set of fishes were inoculated with *Vibrio vulnificus* and it produces the identical signs of vibriosis. Based on morphological and biochemical characters, the pathogen identified as *V. vulnificus.* Further, the virulence of *V. vulnificus* tested by Lethal Dosage (LD50) experiment and sensitivity/ resistant patterns also assessed by Bauer–Kirby method.

Key words: *Etroplus suratensis*, *Vibrio vulnificus*, Lethal dosage and AST.

INTRODUCTION

Fishes are one of the most primitive man's main foods in the earlier days as a hunter food gatherer. It is the only sector which offers animal protein to a broad-cross section of the society thereby is in an advantageous position to ensure nutritional value. The fish meal is highly proteinaceous in nature, when compared to other animal foods. Pickering and Steward, 1984 reported that overcrowding acts as an aquaculture related chronic stress or which reduces growth besides affecting the immune system in several species of fish like Atlantic salmon, Carp and Sea bream. Due to over exploitation by human population our natural water resources have also become highly polluted through indiscriminate discharge of industrial, sewage and agricultural wastes. Microbial pathogens are associated with the marine and fresh water aquatic animals.

Vibriosis is an economically important disease of fish, marine invertebrates and large marine mammals and is responsible for the high mortality rates in aquaculture worldwide. The genus *Vibrio* spp. cause fatal diseases commonly in *Epinephelus* spp. *Auguilla* spp. *Lates calcarifer, Liza macrolepis, Orechromis* spp. Among *Vibrio* spp., *Vibrio vulnificus* is one of the fish pathogen in marine and brackish waters (Thampuran *et al*., 1998). Vibrionaceae members distributed in estuarine, marine environment and heterotrophic bacteria. They are halophilic, gram-negative, comma-shaped bacteria and *V. vulnificus* cause diseases in human as well as terrestrial and aquatic animals. The common names for *Vibrio* infections of fishes include "red-pest" of eels and "salt- water furunculosis", "red-boil" and "pike-pest" Peggy *et al*. (1996). Oliver (1986) reported the production of extracellular enzymes and cytotoxicity by *V. vulnificus. Vibrio vulnificus* produce toxin such as cytotoxin, cytolysine also produce lipopolysaccharide (Tison *et al*., 1986 and Yoshid, 1985).

The present study mainly focused on isolation and identification of *V. vulnificus* from the diseased fishes. A standard procedure was followed for identification of *V. vulnificus* (Bergey's Determinative bacteriology, 1990). Virulence of *V. vulnificus* was assessed by Lethal Dosage (LD_{50}) method.

MATERIAL AND METHODS

Isolation of the bacterium

Blood samples and tissue homogenates from the injected fishes were used to isolate the bacterium. The blood samples were removed aseptically and then the clean supernatant of tissue homogenates were plated on to Zobell's Marine agar. The colonies were stained and then identified as *Vibrio* spp. based on morphology and motility. The identified colonies were subsequently maintained in Alkaline peptone water (Larsen *et al*., 1998) or Brain Heart Infusion Broth (BHIB). Furtherly it was plated on a selective medium, such as, Thiosulfate Citrate Bile salt Sucrose Agar (TCBS-Hi-media), *Vibrio vulnificus* selective medium (VVM), and Sodium dodecyl sulfate - Polymixin B- Sucrose medium, (Arias *et al*., 1999; Jofre *et al*., 2000 and Bryant, 1987). Identification of *V. vulnificus* done by Bergey's Manual Bacteriology (1990).

Antibiotic Sensitivity Test

Sensitivity / Resistant pattern of isolate of *Vibrio vulnificus* was done by Kirby – Bauer method (Bauer and Kirby, 1966). All the test cultures were inoculated into tryptone soy broth TSB and incubated at 37ºC for 5 hours. Each strain of *V. vulnificus* spreaded over the Muller Hinton Agar (MHA) plates, the panel of antibiotics were selected and placed aseptically over the MHA plates. The plates were incubated at 37°C for 18- 24 hours. Then the plates were examined for the presence of zone of inhibitions and results were interpreted according to the standard chart (Hi-media, India).

Determination of Lethal Dosage

Five different dose regimes (10^{-4}-10^{-8} CFU / fish) were used to kill fifty percent of the challenged fishes, *Etroplus suratensis* that had been collected from backwater of Muttukadu and acclimatized at 37°C for 30 days prior to experimentation. Twelve fish per dose, six in each replicate were challenged through intramuscular injection, at the base of the dorsal fin with 0.1 ml suspension of the bacterium (*Vibrio vulnificus*) were inoculated. Injected fishes were kept under observation for 7-10 days. Cumulative mortalities were used in calculating the LD_{50} (Reed and Muench, 1938).

RESULTS AND DISCUSSION

In the present study, the injected fishes were sluggish, necrotic, hemorrhagic spot, boils and blackening of body surface, large sores and

Table 1: Biochemical characters of *Vbrio vulnificus*

S. No	Reactions for the species	*V. vulnificus* from *E. suratensis*
1	Gram's Staining	-
2	Motility	+
3	Gas from Glucose	-
4	Acid from Sucrose	-
5	Acid from Fructose	+
6	Acid from Maltose	+
7	Acid from D-Mannose	+
8	Acids from D-Galactose	+
9	Acid from D -Xylose	-
10	Acid from L-Arabinose	-
11	Indole Production	-
12	Methyl Red	-
13	Voges-Proskauer(VP)	-
14	Citrate utilization	+
15	Urease activity	-
16	Nitrate reduction	-
17	Gelatin hydrolysis	+
18	Starch hydrolysis	+
19	Phospholipids	+
20	Catalase	+
21	Oxidase	+
22	Growth on TCBS agar	+
23	Growth at 0% Nacl	-
24	Growth at 0.5% Nacl	-
25	Growth at 3% Nacl	+
26	Growth at 5% Nacl	+
27	Growth at 6% Nacl	+
28	Growth at 8% Nacl	-
29	Growth at 4° C	-
30	Growth at 30° C	+
31	Growth at 35 ° C	+

granulating lesions with feeble pale gills were identified. The diseased fishes were collected and the samples from kidney and blood were cultured on Zobell's Marine agar, the non-swarming colonies with serrated margins were observed. Typical green colour colonies were observed on TCBS agar. In normal fish *Etroplus suratensis*, the bacterium *Vibrio vulnificus*, isolated from infected fishes were injected intramuscularly and the lesions were observed.

The various biochemical characters of *Vibrio vulnificus* isolated from infected fishes were tabulated in table 1. The characters of *V. vulnificus* it includes, gram negative rod, motile, growth at 0%, 0.5%, 3%, 5%, 6% and 8% NaCl, growth at 4ºC, 30ºC and 35ºC, gelatin and starch hydrolysis positive, oxidase and catalase positive, IMViC – Indole negative, MR negative, VP negative, citrate positive, growth on TCBS Agar, gas and acid from glucose, gas from fructose, maltose, sucrose, galactose, mannose, arabinose and xylose. Stephenie *et al*. (2010) studied the correlation of D- mannitol fermentation with virulence - associated genotypic character in *V. vulnificus*, which was isolated from oysters and water samples. Indole-negative *V. vulnificus* strains isolated from a septicemic patient (Amaro, 1995) and indole-positive *V. vulnificus* isolated from Danish eel farm (Dalsgaard *et al*., 1998). Feifei Han *et al*. (2009) reported the clinical and environmental types of *V. vulnificus* isolated from Louisiana oysters. A culture - free method for the detection of *Vibrio vulnificus* from costal seawater based on loop - mediated isothermal amplification targeting *vcgC* gene (Yongjun Li *et al*., 2010). Based on the present investigation, it is clearly indicated that the isolates from *Etroplus suratensis* were *V. vulnificus.*

This study also showed the optimum temperature and salinity for the growth of organism. Temperature and salinity form a major controlling factor in aquaculture system. The optimum temperature lies between 30°-35°C supports normal growth (Beena, 2000). Fishes were much susceptible to temperature changes, which lead to temperature shock, followed by stress and thus are prone to infections. Salinity also forms important limiting factors in brackish water culture systems. The result of the present study, the sodium chloride concentration lies between 3% - 6% respectively.

Sensitivity and Resistant pattern of *Vibrio vulnificus* (54 - isolates) were studied and tabulated in table 2. A panel of antibiotics were employed, among these Gentamycin (25.5mm), Norfloxacin (25.0mm), Lomefloxacin (25.0mm), Nalidixic acid (23.5mm), Levofloxacin (22.5mm) and Co-trimoxazole (24.0mm) mean values were found to be sensitive and Tetracycline and Cephotaxime fail to inhibit *V. vulnificus* isolates. Antibiotics are frequently used to cure diseases but there is always a risk of bacteria developing resistance and residues in the product (Fjalestad *et al.,* 1993). *Vibrio vulnificus* isolates sensitive with many antibiotics and resistant with Tetracycline and Cephotaxime. Li *et*

Table 2: Sensitive and Resistance pattern of *Vibrio vulnificus* (54 isolates) to *Etroplus suratensis*

S. No	Antibiotics	Symbol	Disc content (mcg)	Zone of inhibition(mm)			Result
				Minimum	Maximum	Mean ± SEM*	
1	Gentamycin	G	10	21	30	25.5 ± 4.5	S
2	Tetracycllin	T	30	-	-	-	R
3	Norflaxacin	NX	10	22	28	25 ± 3 .0	S
4	Nalidixic acid	NA	30	21	26	23.5 ± 3.5	S
5	Cephotaxime	CE	30	18	22	20.0 ± 2.0	R
6	Lomefloxacin	LO	10	22	28	25.0 ± 3.0	S
7	Levofloxacin	LE	5	19	26	22.5 ± 3.5	S
8	Co-trimoxazole	CO	1.25/23.75 mcg	20	28	24.0 ± 4.0	S

*- Standard Error of the mean

al. (1999) reported that Cefriaxone, Nalidixic acid, Chloramphenicol and Sulphamethoxazole were sensitive with isolates of *V. vulnificus* isolated from moribund silver sea bream.

The occurrence of resistance to OXA by fish pathogen been reported to have increased over the recent years. Development of drug resistance by fish pathogen has frequently been reported.

Tables 3(a): Survival pattern of *Etroplus suratensis* injected with *Vibrio vulnificus* (Intramuscular Route)

Dilution Factor	Replicates	Day Post Injection (Dpi)							Total Mortality	Total Survival
		1	2	3	4	5	6	7		
10^{-4}	R1	6	6	6	6	5	5	4	4	8
	R2	6	6	6	6	6	5	4		
10^{-5}	R1	6	6	5	5	4	4	3	7	5
	R2	6	5	5	4	4	3	2		
10^{-6}	R1	6	5	5	4	4	3	3	8	4
	R2	6	6	5	4	3	2	1		
10^{-7}	R1	5	5	4	3	3	2	1	11	1
	R2	6	5	4	4	3	2	0		
10^{-8}	R1	5	5	5	3	2	1	0	12	0
	R2	6	5	4	4	3	2	0		

Table 3(b): Determination of LD_{50} values of *Vibrio vulnificus* isolated from *Etroplus suratensis*

CFU 0.1ml/fish	Mortality	Survival	Cumulative Mortality	Cumulative Survival	Mortality rate	Mortality Percentage
10^8	12	-	42	-	42.42	100.00
10^7	11	01	30	01	30.31	96.80
10^6	08	04	19	05	19.24	79.10
10^5	07	05	11	10	11.21	52.38
10^4	04	08	04	18	4.22	18.18

$$PD^* = \frac{\%\ \text{Mortality next above 50\%} - 50}{\%\ \text{Mortality above 50\%} - \%\ \text{Mortality next below 50\%}}$$

$$= \frac{52.38 - 50}{52.38 - 18.18}$$

$$= \frac{2.38}{34.20} \quad = 0.069$$

Concentration factor for LD_{50} = Concentration factor with > 50% mortality – P.D.

= 5.00 – 0.069 = 4.931

$LD_{50} = 10^{4.93}$

*PD = Proportionate Distance

Disease resistance genes have not been identified in fish. However, the production of transgenic fish with enhanced resistance to specific diseases remains as a possibility for the future (Fjalestad *et al*., 1993). Although the present study recommends the use of sensitive drugs in aquaculture, it is suggested that these antibiotics should be assessed against the set of standards as reported by Austin and Austin (1987) in order to ensure safety.

The lethal dose (LD_{50}) values of *Vibrio vulnificus* were recorded in table 3. The LD_{50} value for the juvenile *Etroplus suratensis* was found to be $10^{4.93}$. Melanisation followed by reddening and swelling at the site of injection were observed as immediate changes, one to three hours post injection with $10^{-7}$and 10^{-8} CFU / fish of *V. vulnificus.* Mean lethal dose 50% LD_{50} represent the number of bacteria needed to kill 50% of the inoculated fishes (Reed and Muench, 1938). This test has given a more quantitative assessment of the vaccine potency than the Relative Percent Survival (RPS) method (Ellis, 1988). Higher lethality due to injection challenge was by direct access of the bacterium to the circulatory system.

Biosca *et al.* (1993) reported the presence of the capsule in *Vibrio vulnificus* biotype 2 and its relationship to virulence for eels. Capsule expression, siderophore production, ability to use hemin and exotoxin production expresses the virulence factors for human (Amaro *et al.,* 1995). Amaro *et al.* (1997) reported that the lipopolysaccharide O-side chain of *V. vulnificus* E is a virulence determinant for eels. Iron acquisition system is involved in the virulence mechanism of *V. vulnificus*. Sung Young Goo *et al.* (2006) reported the presence of *OmpU,* a major outer membrane proteins is an important virulence factor involved in the adherence of *V. vulnificus* to the host cell. Lee *et al.* (2010) reported the presence of I1pA protein of *V. vulnificus* is an important virulence factor involved in the adhesion. The utilization of N-Acetylneuraminic acid and a Sialic acid involved in *V. vulnificus* pathogenesis (Jeong *et al*., 2009). Fouz *et al.* (2002) reported LD_{50} value of *V. vulnificus* to be 1.0 X 10^{-6} CFU / ml and 1.1 X 10^{-7} CFU / ml for eel and tilapia group.

The virulence of the bacterium depended on the type of species, its susceptibility and also the some extent and environmental conditions. The results of the present study revealed that *Vibrio vulnificus* can become a potential threat to the culture of brackish water species. The present study fulfilled that this kind of study will certainly promote the identification and prevention of Vibriosis from the aquaculture industries.

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