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Toxicity analysis of vibrio species from fish samples

Sandeep Moitra[1*], I Seethalakshmi[2] and L. Jeyanthi Rebecca[1]

[1]Department of Industrial Biotechnology, Bharath University, Selaiyur, Chennai
[2]Life Tech Research Institute, Vadapalani, Chennai

ABSTRACT

Fish and fishery products constitute an important component for a large section of world population, more so in developing countries. Sea foods harbor infectious agents that are present naturally in aquatic environment or introduced through human activities. In the present study fish samples were collected for the isolation of Vibrio strains to study its toxic effect using Vero cell lines.

Keywords: Fish, Vibrio sp., Vero cell lines, toxicity analysis, MTT assay

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INTRODUCTION

Marine ecosystems cover approximately 71% of the Earth's surface and contain approximately 97% of the planet's water. They generate 32% of the world's net primary production [1]. Sea foods harbor infectious agents that are present naturally in aquatic environment or introduced through human activities. Global products of fish and fishery products have more than doubled since 1970, reflecting an increase in capture, and in particular aquaculture production [2]. *Vibrios* of seafood origin have attracted increasing attention from time to time as it is found to be one of the most important causes of human food poisoning. Earlier reports revealed food poisoning due to the consumption of seafood contaminated with *Vibrio* strains, particularly *Vibrio parahemolyticus* [3].

The incidence of this bacterium increased considerably during recent years in US, Japan and Korea [4] and in India it was reported to be doubled in the last five years [5]. The organism has been well recognized as a causative agent of gastroenteritis, wound infection and Septicemia through the consumption of contaminated sea foods [6]. *V. parahaemolyticus* is an enteropathogenic, halophilic *Vibrio* originally isolated in 1951 in Japan as the causative agent of an outbreak of food poisoning due to fish. Gastroenteritis due to this *Vibrio* has since been identified in several countries and it is now considered as important cause of food poisoning throughout the world. It inhabits the coastal seas where it is found in fish arthropods such as shrimps and crabs and molluscs such as oysters. In Kolkata, it has also been found in small pond fishes [7]. The cholera *Vibrio* is a short, curved, cylindrical rod about 1.5x0.2-0.4 µm in size with rounded or slightly pointed ends. The cell is typically comma shaped, but the curvature is often lost on subcurvature. The *Vibrios* stain readily with aniline dyes and are gram negative and non acid fast [8]. The highest *V. cholerae* counts were found in the temperature range of 21 to 28˚ C with few organisms detected below 14 or above 35 ˚C [9].

Vibrio vulnificus is a member of genus Vibrio which is defined as gram-negative, non-sporing rods that are straight or have a single, rigid curve. They are motile and more have a single polar flagellum [10]. *V. vulnificus* causes two types of illness; the first is wound infection following contact of open wounds with sea water, the second type occurs in compromised hosts particularly those with liver disease. [11]. All virulent strains of *V.vulnificus* are an opaque morphotype strain, which indicates that the capsule plays a role in the virulence of the organism [12].

In the present study fish samples were collected to isolate Vibrio sp. They were then identified at the species level using biochemical assay. The amount of inoculum that showed virulence was identified using MTT assay on Vero cell lines.

EXPERIMENTAL SECTION

Collection and preparation of Samples

Fish samples were randomly collected from five different markets (Saligramam, Vadapalani, T.Nagar, Kodambakkam, Saidapet) of Chennai. They were then placed individually in pre-sterilized polythene bags and transported to the laboratory immediately using a portable ice chest. Aseptic procedure was strictly adopted during the analysis. All the fish specimens were rinsed with sterile distilled water to remove the adhering particles on the samples. Using a sterile knife 1gm of flesh was taken and ground using sterile distilled water. The samples were serially from 10^{-1},to 10^{-7}. An aliquot of 0.1 ml of samples were spread on Thiosulphate citrate bile salts sucrose (TCBS) agar medium and incubated at 37°C for 18 to 24 h.

Green, yellow or black color colonies were appeared on TCBS medium. The isolated cultures were then purified by repeated streaking in Nutrient agar and maintained in Nutrient agar slants. The pure cultures were presumptively identified as *Vibrio cholerae, Vibrio parahemolyticus and Vibrio vulnificus* using gram staining and biochemical assays.

Toxicity analysis using Vero cell lines

The three Vibrio species were inoculated in tubes containing nutrient broth and incubated 5 min. An aliquot of 100 µl was taken from each culture and filtered using syringe filter. It was then diluted (1:1,1:2,1:4,1:8,1:16,1:32) with Minimal Essential Media (MEM) without foetal calf serum in a microtitre plate. The Vero cells were cultured in 96 well plates. The wells were washed with 200µl of MEM without FCS after discarding the medium from the cell line. The serially diluted Vibrio cultures were added into the 96 well plates. The MEM was supplemented with 100 µg each of penicillin and streptomycin, 20µg of amphotericin B, 3% glutamine, 7.5% of sodium bicarbonate and foetal calf serum. The plates were then incubated with 5 % CO_2 in a desiccator. The subculturing was done using TVPG (2 % typsin, 0.2 % EDTA and 10 % glucose). The cells were occasionally checked for cytotoxicity using an inverted microscope after the MTT assay [3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide] and the cell viability in percentage was calculated using the formula (Cell viability (%) = Mean OD/Control OD x 100).

RESULTS AND DISCUSSION

In the present study three Vibrio species were predominantly identified from the collected fish samples. The results of the colony morphology and biochemical assay leading to its identification are summarized in Table-1 and Table-2. The cytotoxic effect of *Vibrio* strains was tested on Vero cell lines. No detectable impairment of proliferation or metabolic activity of Vero cells was noted in the control. In contrast, the culture filtrate of *Vibrio* strains grown in either media exhibited variable cytotoxicity. The MTT test was used for quantitatively assessing the effect of the culture filtrates on the above cells in addition to direct observation of cytotoxicity development.

Table-1: Colony morphological characteristics of *Vibrio* strains

S. No	Preliminary tests	Results		
		V. cholerae	***V. parahemolyticus***	***V. vulnificus***
1	TBCS agar	Yellow colonies	Green colonies	Light yellow colonies
2	Gram staining	Gram negative	Gram negative	Gram negative
3	Motility	Non motile	Motile	Motile

Table-2: Biochemical assay for identification of *Vibrio* strains

S. No	Biochemical tests	Results		
		V. cholerae	***V. parahemolyticus***	***V. vulnificus***
1	Citrate test	Positive	Positive	Negative
2	Lysine	Positive	Positive	Positive
3	Arginine	Positive	Negative	Negative
4	Ornithine	Negative	Positive	Negative
5	Methyl red	Negative	Positive	Positive
6	Voges Proskaur	Positive	Negative	Negative
7	Indole	Negative	Negative	Positive
8	Urease	Positive	Positive	Positive

All the three tested strains of *Vibrio* in this study exhibited evidence of increased cytotoxicity to Vero cells. This was due to the presence of virulence genes such as hemolysin and cytolysin genes. The cytotoxicity was evident up

to 10^{-4} dilution where it was 55.31 % for *Vibrio cholerae* strains, 46.80 % for *Vibrio parahemolyticus* strains and 66.66% for *Vibrio vulnificus* grown in medium without antibiotics (Table-3). As the Vero cells showed more cytotoxicity to crude extract of *Vibrio*, this study may demonstrate an easier and efficient selection of cell line for cytotoxicity study. Further investigations are needed to elucidate the specific virulence factor(s) and susceptibility that cause cytotoxicity on vero cells. Vero cells may be a speculative reason for their higher susceptibility.

Table-3: *In vitro* cytotoxicity evaluation of different *Vibrio* species against Vero cell lines

S. No	Organisms	Dilution							
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	Control
1	*V. cholerae*	10.63	29.78	40.42	55.31	59.57	72.34	82.97	100
2	*V. parahemolyticus*	17.02	23.40	36.17	46.80	76.59	89.36	95.74	100
3	*V. vulnificus*	25.49	37.25	49.01	60.78	68.62	82.35	96.09	100

CONCLUSION

Sea food samples were collected from various markets located in Chennai and examined for the occurrence of *Vibrio* strains. The food samples were tested for the prevalence of *Vibrio cholerae* by enrichment and isolation techniques. The analysis revealed that, the organism can cause severe health hazard and suitable control methods should be adopted to prevent any outbreak. The culture filtrate of different organisms was tested against Vero cell line to assess its cytotoxicity. From these studies it can be concluded that 10^{-4} dilutions shows greater toxic potential which might be due to the presence of more virulence factors produced by the pathogens.

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