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Isolation and Characterization of Vibrio Species from Shrimp and *Artemia* Culture and Evaluation of the Potential Virulence Factor

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Abstract

The intensive cultivation conditions for marine shellfish larvae may easily cause microbial problems. Vibrio species are commonly present in disease affected shrimp farms, seawater and sediments. Vibriosis has resulted in severe economic losses to aquaculture worldwide and affects many farm-raised fishes, shrimps, crustaceans and Artemia. *V. harveyi* and closely related bacterial species are commonly found in estuarine and coastal marine habitats and can readily be isolated from different environmental sources. The lethal toxicity of extracellular products (ECPs) produced by *V. harveyi V. anguillarum* and *V. parahaemolyticus* isolated from shrimp and *Artemia* culture. Also the virulence factors such as protease, proteolytic activity, and phospholipase and lipase activity and haemolytic activity was studied the virulence strains compared with the non-virulent Vibrio strains. This paper addresses the virulence and epidemiology of vibrio pathogen; pathogenesis of its disease.

Keywords: *V. harveyi*; *Artemia*; Economic losses; Extracellular products; Virulence

Introduction

Indian aquaculture advanced from a traditional practice and developed into an important food production sector, contributing to national economies and providing better livelihoods for rural and farming families. Increasing world trade liberalization and globalization as well as improved transportation efficiency contributed to a great extent for the farmer to be part of a production chain for the delivery of the safe and high quality products to the end users. The aquaculture sector has become a key supplier of aquatic food, provider of direct and indirect employment, and a great source of foreign trade earnings. However, the higher growth of shrimp farming operations has become a potential cause of many problems. Over exploitation of brood stock fishes is one of the important issues. In addition, the expansion of shrimp culture is accompanied by local environmental degradation and the occurrence of diseases of both infectious and non-infectious etiologies [1].

Disease has become a severe constraint to aquaculture leading to subsequent increase in the cost of production year by year. Frequent occurrence of diseases is the major cause of insufficient reduction in aquaculture. In fact, vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in all producing countries. The major species causing vibriosis in shrimp are *Vibrio alginolyticus, V. anguillarum, V. harveyi,* and *V. parahaemolyticus* [2]. Vibrio infections can spread rapidly when fish are confined in heavily stocked, commercial systems and morbidity may reach 100% in affected facilities.

Vibrio species are very common in marine and estuarine habitats and on the surface and in the intestinal contents of marine animals [3]. Lavilla-Pitogo [4] has reported eight bacterial genera that have been associated with the diseases in penaeid culture systems. Only two groups occur quite commonly: filamentous bacteria and Vibrios, with the latter being more important. Many *Vibrio* species have been reported in penaeids: *V. alginolyticus, V. anguillarum, V. cholerae (non-01), V. damsela, V. fluvialis*, *V. nereis, V. splendidus, V. tubiashii, V. vulnificus, V. parahaemolyticus* and *V. harveyii* [4]. Among the several species of vibrios, *V. harveyi*, *V. penaecida*, *V. parahaemolyticus* and *V. vulnificus* [4-6] are the most important pathogens in shrimp. Artemia, a nauplii, however, are also considered a possible vector for the introduction of viruses and bacteria into rearing systems and it has been speculated that Artemia can act as a reservoir or carrier for bacterial pathogens such as *Vibrio, Erwinia, Micrococcus, Staphylococcus and Bacillus* [7-10]. *Vibrios* are important bacterial pathogens for animals reared in aquaculture [11-13] and several virulence factors involved in the potential pathogenic capacity of *Vibrio* species. Some strains of *Vibrio* secrete haemolysins and cytotoxins and were also strong exotoxin producers in some *Vibrio* strains, iron – acquisition systems play on essential role in their pathogenicity [14].

However, the virulence factors of *V. harveyi* are not completely understood [15]. Considered that proteases, phospholipase, haemolysins or exotoxins might be important for pathogenicity [16]. Considered that cysteine protease was the major exotoxin. It has been reported that the biological activities in the ECP may contribute to the development of disease in terms of enhancing bacterial nutrition or as aggressins enabling the bacteria to counteract the host defence systems [17]. In this study, live cells and ECPs from a wide range of *V. harveyi* isolates were examined for pathogenicity, shrimp and *Artemia* culture, and identification of putative virulence mechanisms.

Materials and Methods

Source of bacterial cultures

Three pathogenic *Vibrio* strains were selected for this study from a variety of sources. *V. harveyi, V. anguillarum* and *V. parahaemolyticus* were isolated from infected *Artemia franciscana* culture tank at CMST campus. And they were isolated from infected semi-intensive

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shrimp farms at Marakkanam, Kancheepuram district of Tamilnadu. The collected shrimp and Artemia samples were kept in icebox and transported to the laboratory and stored at -20°C. The infected samples were washed 3 times with 100 ml of sterile sea water on sterile filters. It was homogenized in a sterile glass homogenizer with sterile water and the samples were serially diluted up to 10 fold. One hundred micro liters of these samples were plated on TCBS agar medium. All the plates were incubated between 28 and 30°C. After 2 to 7 days, colonies growth was observed and was selected based their morphological appearance, physiological and biochemical confirmations as well as based on the characteristics described in Bergey's Manual of Systematic Bacteriology [18].

Bacteria and extracellular products (ECP)

Stock cultures of *Vibrio harveyi, V. parahaemolyticus V. anguillarum* strain were grown on tryptic soy agar (TSA; supplemented with 1% NaCl) for 24 h at 25°C. Two swabs of these bacteria suspended in 5 ml phosphate buffered saline (PBS) pH 7.2, were spread onto cellophane overlying TSA (11% NaCl) and grown for 24 h at 25°C. The ECP was harvested and added 10 ml PBS to the surface of the cellophane overlying TSA (11% NaCl) and spread completely. The harvested bacterial suspension was then centrifuged at 25,000 *g* for 60 min at 4°C; the pellet was discarded. The supernatant fluids were passed through a 0.22-µm filter, and the ECP was stored in 1-ml aliquots at 4°C. Total protein was measured by the method of Bradford with bovine serum albumin as standard.

Enzyme activities of bacteria and ECP

Caseinase, gelatinase and phospholipase activities of ECP were detected by placing 25 ml of sample in wells cut in agarose 1% in PBS, pH 7.2 that contained either 1% casein, 1% gelatin, 0.2% starch, 2.5% egg yolk, 1%, respectively. After incubation at 28°C for 24 h, the diameter of the lytic halo around each well was measured. Similarly, enzyme activities of bacteria were measured by spot inoculating bacteria on agar plates containing the respective substrates as described above. The diameter of the lytic halo around each colony was measured.

Hemolysin test

Bacterial strains were grown overnight in microbial broth at 25°C in incubator and the initial OD_{550} was measured using (UV-Spectrophotometer). A separate falcon tube was taken and about 200 µl of sheep blood was added and well mixed in 20 ml of autoclaved Marine agar. The mixture is then poured on a plate and left 15 min for drying avoiding any air bubbles. Three different drops of 10 µl diluted bacterial solution is then spotted on the plate. Finally, the plates were covered with parafilm and kept in incubator at 28°C for 48 hrs. After incubation, hemolytic zones were observed. The test was repeated two times.

Proteolytic assay

The enzyme casein dissolved at 1% in 0.1 mol/lt of Glycine sodium hydroxide buffer(p H 9.6). To that 1 ml of extra cellular protein to 1 ml of the above solution was added. It was incubated at 30°C for 10 minutes. The reaction was stopped by adding 0.1 mol of 1 mol/lit of Trichloro acetic acid. Then it was centrifuged at 6000 rpm for 10 minutes. Finally the supernatant was collected and the optical density was measured at 380 nm.Tri chloro acetic acid was used as control [19].

In vivo virulence studies

The bacteria were grown in TSB medium at 28°C for 18 hours. The cells were centrifuged at 5000 rpm for 10 minutes. The pellets were washed with sterile saline (0.85% (W/V) NaCl) by centrifuging at 5000 rpm for 10 minutes. Pellet was collected and mixed with Phosphate Buffered Saline (PBS pH 7.4) challenged with different stages (nauplii, and adult) of Artemia species *A. franciscana* at the rate of more than 10^7 Cfu/ml in the culture tank. The overall survival rate after challenge were assessed every 24 hours interval of the culture period. The protocol of total Vibrio count (TVC) of the wild collected *A. franciscana*, the samples were homogenized, serially diluted, toxicity inhibition tests was described as spread onto Petri dish containing the TCBS agar. The plates were incubated at 37°C for 24 hours. Triplicates were maintained. After incubation the numbers of colonies formed were counted.

Data Analysis

Data obtained from virulence factors as well as cumulative mortality were analysed using one way ANOVA ($P < 0.05$ as significant level). Means were also compared using SNK test.

Results

Characterization of bacterial strains

Three major *Vibrio* species, such as *V. harveyi V. anguillarum* and *V. parahaemolyticus* were isolated from the infected shrimp farms at Marakkanam, Kancheepuram district of Tamilnadu and *Artemia franciscana* culture tank at CMST campus. These strains were confirmed by morphological and biochemical confirmative tests (Table 1). The infected shrimp showed symptoms such as lethargy, loss of balance, whirling movement and general weakness within 6 h after challenge with bacteria. Hemolymph failed to clot and slight reddening of pleopods was also noticed. To standardizing the growth, the selected strains were cultured and reached the stationary phase within 2 hours in both the Tryptic soy broth and Nutrient broth.

Characterization of *Vibrio* ECP

The total proteins estimated from the extracellular proteins of as *V. harveyi V. anguillarum* and *V. parahaemolyticus* were given in the Table 1. The maximum protein content was observed in the *Vibrio harveyi* (101.72 U/ml). The moderate value was observed from *V. anguillarum* and *V. parahaemolyticus* (94.48, 94.49 U/ml respectively). The ECP of the strain *V. harveyi V. anguillarum* and *V. parahaemolyticus* were harvested after 24 h of incubation of the culture at 25°C. The virulence factors were tested in different enzymatic activities such as extracellular protein preparation. caseinase, gelatinase and phospholipase activity (Table 2).

Haemolytic activity

The haemolytic activity of *V. harveyi V. anguillarum* and *V.*

S. no	Concentration (µl)	ECP Samples		
		V. anguillarum	*V. harveyi*	*V. parahaemolyticus*
1	10	5.76 ± 0.08	10.56 ± 0.08	3.88 ± 0.089
2	20	16.36 ± 0.12	21.30 ± 0.01	8.28 ± 0.24
3	30	25.50 ± 0.08	31.89 ± 0.04	21.10 ± 0.12
4	40	35.10 ± 0.47	41.62 ± 0.02	33.32 ± 0.08
5	50	49.30 ± 0.06	52.54 ± 0.05	45.06 ± 0.03
6	60	57.46 ± 0.09	61.16 ± 0.01	55.82 ± 0.08
7	70	66.95 ± 0.04	71.08 ± 0.08	66.70 ± 0.08
8	80	76.53 ± 0.01	81.88 ± 0.01	76.82 ± 0.08
9	90	85.68 ± 0.08	92.05 ± 0.01	86.12 ± 0.08
10	100	94.48 ± 0.08	101.72 ± 0.03	95.33 ± 0.09

Table 1: Total protein estimation (µg) of the ECP of pathogenic *Vibrio* sp by Bradford's assay.

S. no	Strain	Enzyme activities								
		Caseinase			Gelatinase			Phospholipase		
		C. zone (mm)	C. dm (mm)	Ratio	C. zone (mm)	C. dm (mm)	Ratio	C. zone (mm)	C. dm (mm)	Ratio
1	*V. harveyi*	16.5 ± 1.0	15.5 ± 1.0	1.1 ± 0.0	16.5 ± 1.0	15.5 ± 1.0	1.1 ± 0.0	16.5 ± 1.0	15.5 ± 1.0	1.1 ± 0.0
2	*V. anguillarum*	13.2 ± 1.0	7.7 ± 0.2	1.7 ± 0.2	13.2 ± 1.0	7.7 ± 0.2	1.7 ± 0.2	13.2 ± 1.0	7.7 ± 0.2	1.7 ± 0.2
3	*V. parahaemolyticus*	17.3 ± 0.5	9.8 ± 1.2	1.8 ± 0.5	17.3 ± 0.5	9.8 ± 1.2	1.8 ± 0.5	17.3 ± 0.5	9.8 ± 1.2	1.8 ± 0.5

Clearing zone (mm) - C. zone, Colony diameter (mm) - C. dm.

Table 2: Enzyme activities towards skim milk powder of pathogenic *Vibrio strains.*

S. no	Strain	Incubation time (hour)					
		18	24	36	48	60	72
1	*V. harveyi*	1568.14 ± 0.04	1795.32 ± 0.02	2864.01 ± 0.09	2019.18 ± 0.05	2061.05 ± 0.01	1645.82 ± 0.02
2	*V. anguillarum*	1415.18 ± 0.02	1681.93 ± 0.01	2316.05 ± 0.02	1946.46 ± 0.01	2012.49 ± 0.06	1418.34 ± 0.05
3	*V. parahaemolyticus*	1256. 41 ± 0.07	1465.64 ± 0.01	2156.14 ± 0.06	1785.54 ± 0.4	1921.71 ± 0.01	1258.26 ± 0.01

Table 3: Protease assay for pathogenic *Vibrio sp* isolated from infected shrimp and *Artemia* species.

parahaemolyticus was tested on the Blood agar plates containing 2% human blood. The isolates were varied in their ability to lyses the RBC's of human blood. According to the results, *V. harveyi* showed higher haemolytic activity than the *V. anguillarum* and *V. parahaemolyticus,* Figure 1 shows the results of isolates.

Protease assay

The protease assay and production (U/ml) of *Vibrio* species such as *V. harveyi V. anguillarum* and *V. parahaemolyticus* were given in the Table 3. The protease is responsible for prawns lost balance and showed whirling movement before succumbing to death. The heat-inactivated protease fractions did not cause any mortality. The lowest protease value 1256. 41 U/ml was observed in the *V. parahaemolyticus* and higher protease value was observed in *V. harveyi* (2864.01 U/ml). In the case of *V. anguillarum* the moderate value was observed (1681.93 U/ml) in 24 hours incubation.

Survival of *A. Franciscana* nauplii, adult Challenged *Vibrio* strains at different time intervals

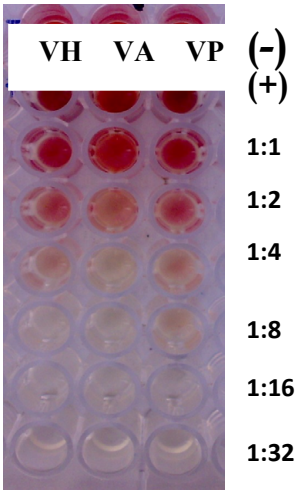
In *A. franciscana,* the control of nauplii groups survived 91% when no pathogenic inoculation was given. The survival was observed was 36, 39 and 44% in *V. anguillarum, V. harveyi* and *V. pelagius* respectively (Figure 2). In *A. franciscana,* the control of adults groups survived 89% when no pathogenic inoculation was given. The survival was observed was 28, 19 and 44% in *V. anguillarum, V. harveyi* and *V. parahaemolyticus* respectively (Figure 3).

Total *Vibrio* count after Challenging in *A. fransciscana*

The total plate count of different stages was given in the Table 4. In nauplii stage, the lowest load $1.7 \times 10^7 \pm 2.05 \times 10^3$ in the *V. anguillarum*, and the highest load was $1.8 \times 10^7 \pm 4.08 \times 10^3$ in the *V. parahaemolyticus*. In adult stage, the lowest load $2.1 \times 10^7 \pm 3.68 \times 10^3$ in the *Vibrio harveyi*, and the highest load was $2.5 \times 10^7 \pm 1.63 \times 10^3$ in the *V. parahaemolyticus*.

Discussion

Disease problem in aquaculture are currently an important constraint to growth of aquaculture, which has impacted both socio-economic development and rural livelihoods in some countries [20]. Some *Vibrio* species are pathogens of fish, eels and frogs as well other vertebrates and invertebrates [21] and can cause Vibriosis, a serious infectious disease in both wild and cultured fish and shellfish [22].The disease can cause significant mortality (>50%) in fish culture facilities



VH: *Vibrio harveyi,* VA: *Vibrio anguillarum,* VP: *Vibrio parahaemolyticus*

Figure 1: Haemagglutination activity of the *V. harveyi, V. anguillarum* and *V parahaemolyticus* on the micro well titre plate.

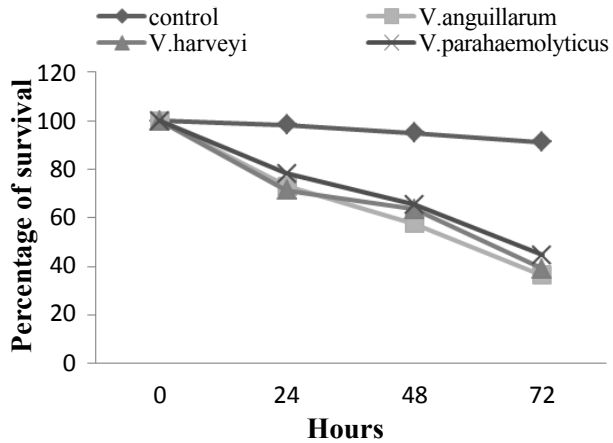


Figure 2: Survival of *A. franciscana* nauplii challenged Vibrio strains at different time intervals.

once as outbreak is in progress. The species associated with disease in fish and shell fish include *V. anguillarum* (isolated most commonly from

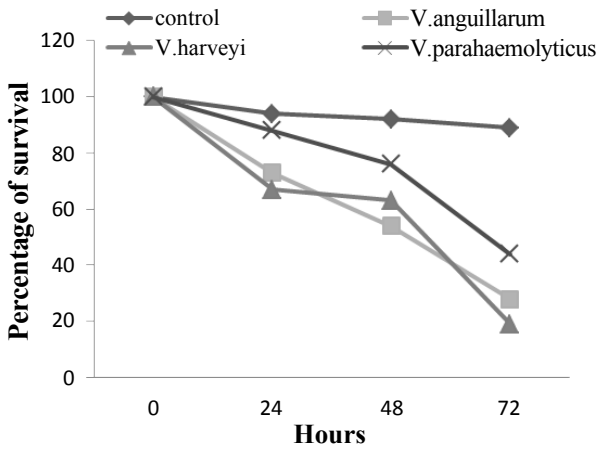


Figure 3: Survival of *A. franciscana* adult challenged Vibrio strains at different time intervals.

S.no	Strains	Total Vibrio count (Cfu/g)	
		***A. franciscana* nauplii**	***A. franciscana* adult**
1	*V. harveyi*	$2.33 \times 10^3 \pm 1.0 \times 10^1$	$2.1 \times 10^7 \pm 3.68 \times 10^3$
2	*V. anguillarum*	$1.7 \times 10^7 \pm 2.05 \times 10^3$	$1.8 \times 10^7 \pm 4.08 \times 10^3$
3	*V. parahaemolyticus*	$1.75 \times 10^3 \pm 0.5 \times 10^1$	$2.5 \times 10^7 \pm 1.63 \times 10^3$

Table 4: Total bacterial count and Total Vibrio count (cfu/g) of the *A. fransciscana*.

marine and brackish water shellfish) *V. harveyi, V. alginolyticus* and *V. parahaemolyticus* have infected in juveniles and adults [23]. Usually most of the studies have focused on the isolation, characterization and identification of the bacterial strains present in tissues and cultural water at the time of infection. However, pathogenesis studies on this vibrio species originally isolated from *Artemia franciscana* and Tiger prawn (*Penaeus monodon*) in the animals have not been previously described. Therefore, a pathogenic *Vibrio* strain's isolated from diseased prawn and *Artemia* was used to investigate its extracellular virulence factor(s) in this study.

Our finding suggests that, there is diversification of result concerning the effect of extracellular products on virulence factor production of *Vibrio harveyi*. Previous report showed that, *V. harveyi* are able to generate various virulence factors i.e. extracellular products (ECP's) responsible for pathogenesis [24,25]. However, in case of *V. harveyi* proteases, phospholipase, hemolysins and cytotoxins are considered as major pathogenicity determinants [24].

Artemia is one of the most important live feed organisms used in the shrimp industry. The importance of brine shrimp was realized as early as 1930, when the high value of its nauplii was described as an ideal food for the larvae of crustaceans in aquaculture. The dry cysts of *Artemia* can be surface sterilized and the nauplii which hatch out are bacteria free. After this stage, pre adult and adult stage is hatched out. This allows experiments with Vibrio to be performed without the interference of any other bacteria [26]. The ability to simplify the interactions between bacteria and Artemia in this way was considered important and useful for the study of pathology in this very important crustacean.

Proteases present in the ECP of bacteria have been implicated as virulence determinants in fish diseases [27] and in shrimp vibriosis [28]. The bacteria, as well as its ECP, showed high protease activity. Extracellular products secreted by the bacteria were found to be highly toxic to tiger prawn. Similar findings have been reported for *V. alginolyticus* [28]. Also, protease fractions to be highly toxic to tiger prawns. This is the first report of the toxicity of the extracellular protease of *V. parahaemolyticus* to tiger prawn. This finding points to the possible role of proteases as virulence determinants of the bacteria. The results obtained from the present study showed that the pathogenic and virulence characteristics of *V. anguillarum, V. harveyi* and *V. parahaemolyticus* are associated with growth standardization, the range of different exotoxins (Haemolysin), and exoenzymes (Protease and lipase). Consistent with the result of caseinase and glelatinase obtained in this study, positive regulation of protease has also been reported before for the *V. harveyi* e.g., *vhp* metalloprotease gene [29].

The high variability in the chemical composition of ECP from different isolates, suggested intraspecific heterogeneity in the taxon, as has been reported for other fish pathogens [11]. Certainly, the proteolytic activity was variable among the isolates, and there was not any correlation with the total protein content. Overall, the ECP displayed fewer enzymic activities than live cells, suggesting that many of these activities were associated with the cell envelop. Alternatively, it is conceivable that some substrates were internalized. It remains for further study to determine the relationship, if any, between cell-associated and diffusible water soluble pathogenicity factors of *V. harveyi*. Also, the relevance of laboratory-produced ECP preparations to natural infections needs to be addressed. Further studies will also need to evaluate different methods for the detection and quantification of culturable and non-culturable Vibrio spp. pathogenesis at molecular level.

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