Journal of Medical and Health Studies (JMHS)
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Isolation and Characterization of Vibrio Pelagius from Marine Tidal Sediment Samples of Thekkurichi South West Coast, Tamilnadu

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ARTICLE INFORMATION

Received: September 15, 2020
Accepted: November 15, 2020
Volume: 1
Issue: 1

KEYWORDS

Micro biota, V. pelagius, biochemical, virulence, pathogenicity

ABSTRACT

Aquatic animals differ from terrestrial animals in the level of interaction between the intestinal micro biota and the surrounding environment. The bacteria present in the aquatic environment influence the composition of the gut micro flora. Vibrio pelagius is the most common surface organisms in surface water of the world. The organism originally isolated from marine and fresh water habitats and in association with aquatic animals. The present study the virulence, the biochemical properties of the toxicity such as proteolytic, haemolytic, haemagglutination and lipolytic were performed. Also V. pelagius had higher proteolytic, haemolytic activity and Haemoagglutination V. pelagius agglutinates 1:16 dilutions. These results reveal that proteases, haemolysins or exotoxins might play foremost role in the pathogenicity of Vibrio pelagius.

1. Introduction

Aquaculture plays a vital role in food production throughout the year. The aquatic environment contains a plethora of obligate and opportunistic bacterial pathogens as well as beneficial and neutral bacterial strains. In recent years, vibriosis has become one of the most important bacterial diseases in maricultured organisms affecting a large number of species of fish and shellfish. Pathogenicity, or virulence, is the ability of a bacterium to cause infection, virulence factor (or mechanism of pathogenesis or virulence mechanism) indicates a bacterial product or strategy that contributes to virulence or pathogenicity. Unlike gram positive bacteria, which naturally release various proteins into culture fluid, gram negative bacteria usually do not release such proteins, probably because their outer membrane acts as a barrier; however, some gram-negative bacteria, including the family *Vibrionaceae,* release a variety of proteins into culture fluid, known as extracellular proteins.

Virulence caused by factors such as the extracellular enzymes and stress proteins can be recognized by the loss of cell adherence, induction of cell lysis, or apoptosis. A clam primary cell culture, despite consisting of a heterogeneous cell population, has been used to study the interaction between cells and a pathogenic *Vibrio* species. Many extracellular bacterial proteases are suggested to play important roles in virulence. This study was aimed at investigating the isolation and characterization of the selected *Vibrio* strains. Specifically, the present work was designed to determine the toxicity of *Vibrio pelagius* ECPs *in vitro* and to evaluate *in vivo* studies in Artemia cellular-immune responses, after exposure to these extracellular products.

2. Literature Review

Bacterial infections are considered to be a major cause of mortality in fish hatcheries and bacteria most frequently associated with disease in farmed fish and shellfish are species within the family *Vibrionaceae* (Thompson *et al.,* 2004). *Vibrio* species are responsible for diseases in many wild and reared aquatic organisms including shrimp, molluscs, fish and other vertebrates (Austin and Zhang, 2006). *Vibrio* species are very common in marine and estuarine habitats and on the surface and in the intestinal contents of marine animals (Fouz *et al.,* 1990).

Vibrio pelagius is a halophilic *Vibrio* species that was first described as *Beneckea pelagia* by Baumann *et al.* (1971) and later as *V. pelagius* (Baumann *et al.,* 1984) and *Listonella pelagia* (MacDonell and Colwell, 1985). Baumann *et al.* (1971) isolated the bacterium from sea water and (Furniss *et al.,* 1978) described *V. pelagius* as one of the most commonly isolated *Vibrios* along the British coast. Similarly, other reports consider *V. pelagius* is an environmental organism without any pathogenic importance (Ringo *et al.,* 1996; Ringo and Vadstein, 1998). This organism has been isolated as the etiological agent of infections in farmed turbot (Scophthalmus maximus) (Angulo *et al.,* 1992), and farmed vaccinated turbot (Santos *et al.,* 1997). Moreover, other authors have demonstrated an association of this species with other infective agents in reared turbot (Bloch *et al.,* 1991) and farmed shellfish (Castro *et al.,* 1992).

Pathogenicity, or virulence, is the ability of a bacterium to cause infection, virulence factor (or mechanism of pathogenesis or virulence mechanism) indicates a bacterial product or strategy that contributes to virulence or pathogenicity (Salyers and Whitt, 2002). Hemolytic activity has been clearly demonstrated in the ECPs *in vitro* and *in vivo* a phenomenon which is responsible for the severe hemorrhaging symptoms found in *Vibrio* infected fish (Thune *et al.,* 1993). Proteases have also been implicated as virulence factors in various *Vibrio* strains pathogenic to fish or shrimp (Liu *et al.,* 1997). The hemoagglutinating ability of fish pathogenic *Vibrio* strains including *V.anguillarum* (Toranzo *et al.,* 1983) increased the knowledge of the disease vibriosis.

3. Methodology

3.1. Bacterial isolates and culture conditions

Marine tidal sediment samples were collected from the Thekkurichi coastal area. The samples were transported to aseptically and stored in the refrigerator at 4^0C until used. The sediment sand samples were washed 3 times with 100 ml of sterile sea water on sterile filters and it was serially diluted up to 10-fold. 0.1ml of this sample was plated on TCBS agar plates, which was then incubated at 37ºC for 24 hours. After incubation Green and yellow colored colonies produced were streaked on the 2.8% Nutrient agar slants, and incubated overnight, at room temperature. The pure culture was stored at refrigerator for further works. The selected isolates were identified by morphological, physiological and biochemical confirmations as well as based on the characteristics described in Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994).

3.2. Characterization of Virulence factors

3.2.1. Preparation of extracellular product

Selected three strains were incubated at 28°C for 24 hr in TSB with shaking. 25 ml of each culture was centrifuged at 10,000 x g for 30 min to remove bacterial cells. Supernatant was passed through 0.45 µm syringe filter. The filtered supernatant was taken as sample 1, the syringe was washed reversely with PBS (pH 7) and the soup said to be sample 2 and sample 3.

3.2.2 Assay of proteolytic activity

The enzyme (0.25 ml) was added to 0. 5 ml casein (1%), 1.25 ml of Tris buffer (pH 7.2) and the reaction mixture was incubated at 30ºC for 30 min. Then added 3ml of 5% Tri Chloro Acetic acid (TCA) incubated at 4ºC for 10 minutes. The precipitates were centrifuged at 4ºC for 15 minutes. The 0.5ml of supernatant was transferred to test tubes and added 2.5 ml of Na2Co3 incubated at 37ºC for 20 minutes. After incubation added 0.5 ml of Folin phenol (1:1) and the absorbance of the sample was measured at 660 nm.

3.2.3. Assay of Haemolytic activity

Haemolytic activity of selected *Vibrio* strains were tested on nutrient agar supplemented with 2% sheep erythrocytes with incubation at 37ºC for 48 h. For haemolysin quantification, strains were cultured in TSB (Tripticase Soy Broth) separately, at 37ºC for 24 h. After incubation the culture was pelleted by centrifugation at 10,000 xg for 10 min supernatant (500 µl) was added to 1ml of 1% suspension of sheep erythrocytes in phosphate buffered saline and incubated at 37ºC for 1h and centrifuged at 5,000 xg for 5 min to remove unlysed erythrocytes. Lysis of erythrocytes was determined by measuring the absorbance of the supernatant at 545 nm.

3.2.4. Haemagglutination assay

This assay was performed in U – shaped micro well plates. Two-fold serial dilution of serum samples were made in TBS (Sritungalucksana *et al.,* 1999). An equal volume of 1.5% RBC was added to each dilution of serum sample. The plates were incubated at 25ºC for 30 min. Haemagglutination titer was recorded as the reciprocal of last dilution, resulting in agglutination after 30 minutes incubation. Negative controls comprised mixed equal volume of RBC and TBS.

3.2.4. Lipolytic activity

A preliminary qualitative analysis for lipolytic activity was conducted by using spirit blue agar plates. The spirit blue agar media was sterilized. After sterilization 1% of tyrosine was poured into the sterilized agar media. Shake well and poured into the sterile Petri plates. After solidification of the plates a single streak of tested organism was made on the agar plate. Plates were incubated at 37ºc for 24 hours.

3.3. *In vivo* virulence studies

The bacteria were grown in TSB medium at 28ºC 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.

4. Results and Discussion

Vibrio pelagius were isolated from the marine tidal sediments and collected from the Thekkurichi coastal area, kanyakumari district, Tamilnadu, India and the strains were confirmed by morphological and biochemical confirmative tests (Table 1) and (Fig 1a). The total proteins estimated from the extracellular proteins of *V. pelagius* were given in the Fig (2). The maximum protein content was observed in the *Vibrio pelagius* (95.33 U/ml). The protease assay and production (U/ml) of *V.pelagius* were given in Table (2). The higher protease value 1681.93 U/ml 1465.64 U/ml was observed in the *V.pelagius* . The haemolytic activity (%) was given in Table (2). From the results, the maximum haemolytic activity was recorded in *V.pelagius* (53.91 %). Haemagglutination assay were carried out against the RBC of human O^{+ve} blood groups and the results were given in (Table 3). According to the results, among the three different isolates, *V.pelagius* agglutinates up to 1: 8 dilutions. The screening test for lipolytic activity of *V.pelagius* was performed on the Spirit blue agar. There is no zone formation was observed around the three strains streaked on the Spirit blue agar. This shows that the negative results for the lipase production.

In *A. franciscana,* the control of nauplii groups survived 91% when no pathogenic inoculation was given. The survival was observed was 36% in *V.pelagius* respectively (Table 4). In *A. franciscana,* the control of adults' groups survived 85% when no pathogenic inoculation was given. The survival was observed was 29% in *V.pelagius* respectively (Table 4). Aquatic animals differ from terrestrial animals in the level of interaction between the intestinal micro biota and the surrounding environment. The bacteria present in the aquatic environment influence the composition of the gut micro flora and vice versa. (FAO, 2000). *Vibrios* are among the most common surface organisms in surface water of the world. They occur in both marine and fresh water habitats and in association with aquatic animals. Some *Vibrio* species are pathogens of fish, eels and frogs as well other vertebrates, invertebrates and can cause Vibriosis, a serious infectious disease in both wild and cultured fish and shellfish (Austin and Austin, 1993).

In this study, among the different colonies developed from the sand sample, dominant colonies were selected. These dominant colonies were identified as *Vibrio* species based on their growth on TCBS agar plates. Further it was confirmed by biochemical tests. Among the total biota isolated from the sand sediments sample, more than seventy percent identified as *V. pelagius.* The present study investigated the pathogenic activities of live cells and extracellular products of *Vibrio pelagius* isolated from the marine tidal sand sediments. Extracellular virulence factors such as haemolysin and proteases have been suggested to play a significant role in diseases caused by *V.pelagius* (Farrell *et al.,* 1991). In this present investigation, *Vibrio pelagius* produced higher proteolytic activity. Thus protease seemed to be one of the most important factors responsible for pathogenicity in *V.anguillarum* (Hiroki Inamura *et al.,* 1985). Hemolytic activity has been considered as important virulence factors contributing to pathogenicty of the infection process (Zhang *et al.,* 2001). The pathogenicity of various *Vibrio* species especially *V.pelagius* has been extensively studied (Austin and Austin, 1993). In the present study, the haemolytic activity was recorded in terms of zones of clearing on blood agar plates, the *V.pelagius* showed higher haemolytic activity compared with other two strains. And in the case of analysis of haemolytic activity revealed that the strongest reactions occurred with the concentrated ECP, maximum haemolytic activity was recorded in *Vibrio pelagius* (53.91 %). Hemolytic activity by *V.pelagius* cells has been suggested to be a virulence factor during infection of fish by contributing to hemorrhagic septicemia and diarrhea (Hirono *et al.,* 1996).

The haemagglutinating activity of bacteria is closely related to the adhesiveness of organisms for the surface of cells and is believed to play an important role in the infectivity of bacterial pathogens (Toranzo *et al.,* 1983). Haemagglutination assay in the present experiment, *Vibrio splendidus* agglutinates upto 1:16 dilutions, *Vibrio pelagius,* and showed haemagglutination up to 1:32

dilutions. But in the *Vibrio pelagius* agglutinates up to 1:8 dilutions only. The occurrence of a great diversity of hemagglutination types suggests the existence of a multiplicity of *V.pelagius* strains in the marine environment (Larsen and Stig Mellergaard, 1984). Artemia can be surface sterilized and the nauplii stages are bacteria free. This allows experiments with *Vibrio* to be performed without interference of any other bacteria (Lightner, 1977). The interaction between the bacteria and *Artemia* in this way was considered important and useful for the study of pathology. The interaction of three species of *Vibrio* with nauplii and adult stage of *Artemia franciscana* were studied. Every interval study showed the rapid decrease in the number of different stages of *Artemia* species. In the present study, nauplii are affected rather more than the adult stage.

5. Conclusion

In this study, among the different colonies developed from the sand sample, dominant colonies were selected. These dominant colonies were identified as *Vibrio* species based on their growth on TCBS agar plates. Further it was confirmed by biochemical tests. Among the total biota isolated from the sand sediments sample, more than seventy percent identified as *V. pelagius.* The present study investigated the pathogenic activities of live cells and extracellular products of *Vibrio pelagius* isolated from the marine sediments. The present study revealed that, the virulence of the pathogenic *Vibrio* isolated from the marine tidal sediments, extra cellular protein, haemolytic and lipolytic activity analysis pave a way of new microorganism characterisation.

Funding

This research received no external funding.

Conflicts of Interest:

The authors declare no conflict of interest.

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Table1. Morphological and biochemical confirmation of pathogenic *Vibrio pelagius*. isolated from the marine tidal sediments

S.No	Biochemical tests	*V. pelagius*
1	Motility	**+**
2	Simple staining	Rod
3	Gram staining	-
4	MR	+
5	VP	-
6	Oxidase	+
7	Catalase	+
8	Indole	+
9	Citrate	+
10	Starch	+
11	Skim milk	+
12	Glucose	-
13	Lactose	+
14	Lysine	-
15	Ornithine	+
16	Arginine	-

Fig 1. *Vibrio pelagius* isolated from marine tidal sediments from Tekkurichi coastal area.

Table 2. Protease and Haemolytic assay for the pathogenic *V.pelagius* isolated from the marine tidal sediments.

S. No	Protease assay (U/ml)	Haemolytic assay (U/ml)
1	1795.32 ± 0.02	36.71 ± 0.003

Fig 2. Total protein estimation (µg) of the ECP of pathogenic *Vibrio pelagius* by Bradford's assay

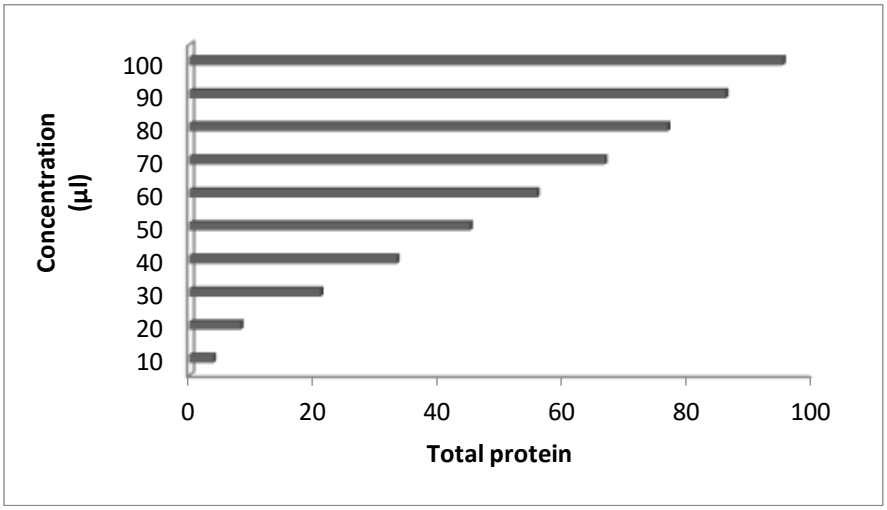

Table 3. Haemagglutination performed by human O^+ blood group against *V.pelagius* by micro well plate assay

S.No	Dilutions	Agglutination
		V. pelagius
1	- ive control	-
2	+ ive control	+
3	1:1	+
4	1:2	+
5	1:4	+
6	1:8	+
7	1:16	+
8	1:32	-

(-) No agglutination takes place **(+)** Agglutination takes place.

Table 4. Survival of *A. franciscana* nauplii challenged pathogenic *V. pelagius* at different time intervals

S.No.	Time (hrs)	Survival (%)			
		***A. franciscana* nauplii**		***A. franciscana* adult**	
		Control	***V. pelagius***	**Control**	***V. pelagius***
1	24	98.33 ± 1.24[a]	78.33 ± 1.24 [b]	94.00 ± 1.63[a]	77.00 ± 2.16 [c]
2	48	95.00 ± 2.44 [a]	65.33 ± 1.24 [b]	91.00 ± 1.24 [a]	65.33 ± 1.24 [b]
3	72	91.33 ± 1.24 [a]	44.60 ± 1.69 [c]	85.33 ± 0.47 [a]	39.33 ± 2.05 [c]

Means with the same superscripts (a-c) do not differ from each other ($P < 0.05$).