International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 330-336
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Isolation and Characterization of *Vibrio* Spp. By Sequencing of 16s rDNA From Coelomic Fluid of Sea Urchin (*Stomopneustes variolaris*)

Ravitheja. S and *Chandra. M

Department of studies in Biosciences, Mangalore University, Dakshina Kannada District, Karnataka - 574199, India.

Received: 14 Oct 2018/ Accepted: 12 Nov 2018/ Published online: 01Jan 2019
Corresponding Author Email: drchandram1@gmail.com

Abstract

Aim of the present study was to molecular characterization of bacterial species isolated from coelomic fluid of sea urchin (*Stomopneustes variolaris*) collected from St. Mary's Island, Malpe beach, West Coast of India. Two morphologically different species of bacteria viz., *Vibrio parahaemolyticus*3KCL and *Vibrio* sp. SOVt012 were isolated and identified by biochemical tests and confirmed through molecular characterization approach. Bacterial 16S rDNA gene was amplified using suitable primers. The amplified 16S rDNA gene sequence was compared with the sequence in NCBI sequence database. The average number of bacterial colonies was recorded with a count of 98 for $1x10^7$cfu/mL. Halophilic nature was determined by streaking the isolates on nutrient agar plate containing 0.5% NaCl. Only *Vibrio parahaemolyticus*3KCL showed the growth but *Vibrio* sp. SOVt012 didn't show the growth and it is confirmed as a true halophile. Limit of halotolerance was analysed for both the isolates on nutrient broth with higher NaCl concentrations and found to be growing well and as a result they are highly halotolerant. The antibacterial activities of cell free isolates were performed by well diffusion method against human bacterial pathogens viz., *S. typhi, S. pyogenes, E. coli* and *E. faecalis.* However, a zone of inhibition was not observed against any pathogens. The results indicated that even though coelomic fluid of sea urchin associated with bacteria they didn't show any antibacterial activity, but it is the reservoir of few halotolerant and halophilic *Vibrio* spp. This data will be used to provide information on the magnitude of such pathogens in sea urchin.

Keywords
Coelomic fluid, *Stomopneustes variolaris*, 16S rDNA sequencing, *Vibrio* spp.

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INTRODUCTION

Sea urchin (*Stomopneustes variolaris)* (Lamarck, 1816) is belonging to the family Stomopneustidae and distributed from Kanyakumari to Vishakhapatnam shores of India. It is most commonly found in the shallow sub tidal zone on rocky bottoms. It is commonly known as slate pencil sea urchin and it is the single living genus and species [1]. Sea urchins have long been harvested for their gonads (roe). They are popular in Korean and Japanese cuisine and are also a traditional food in Chile. The world annual consumption of sea urchins has been steadily increasing over the last decades and sea urchins are one of the important fisheries in several areas of the world [2]. However, after removal of the edible gonads the residual shells with spines are generally discarded as food waste without further utilization. But the shells are containing various bioactive components [3, 4]. So far, about 1000 species of sea urchins have been identified [5] but only 16 species of sea urchins are harvested for food worldwide.

Symbiotic microbial communities are common in marine invertebrates and include several species of bacteria, microalgae, virus and archaea [6]. The study of interactions between marine organisms and their microorganisms is providing interesting insights into phenomena of symbiosis and pathogenicity. In fact, it is known that even in the marine environment the bacteria establish relationships with guests and in some cases, organisations promoting the acquisition of nutriment, tissue development or immune system [7]. The prokaryotic microbiota of marine invertebrates is constituted, for the most part, by obliged symbionts [8] but there are also optional and many of them produce specific extracellular compounds [9]. They live in a complex community and need some interactions to growth, produce energy and protection. Moreover, microorganisms are not only a source of contamination and disease but also serve for a correct development. The internal organs such as gonads and gut are bathed in the coelomic fluid and surrounded by the tests. This coelomic fluid is mediating the main immune functions in echinoderms, including antimicrobial activities [10]. This coelomic fluid is mediating the main immune functions in echinoderms, including antimicrobial activities.

Vibrio spp. is among the infectious agents that can result in deterioration of meat or represent a potential disease source for humans. The risk of disease from ingesting pathogens found in raw meat is significantly higher than cooked meat, although both can be contaminated [11]. Meat can be contaminated during the production process at any time, from the slicing of prepared meats to cross-contamination of food in a refrigerator. All of these situations may lead to a greater risk of disease. From public health point of view, *Vibrio spp.* represents a greater portion of the food borne illnesses across the coast cities worldwide [12]. Some *Vibrio spp.* poses a significant health threat to humans who suffer from immune disorders and liver diseases. It enters human hosts via wound infections or consumption of raw shellfish (primarily oysters), and infections frequently progresses to septicaemia and death in susceptible individuals [13]. This could be due to food contamination with *Vibrio spp.* shed from seafood or prevalent usage of undercooked seafood/meat or surface contamination during marine shipping of such foods. Despite the vast majority of environmental *V. parahemolyticus* isolates are avirulent, it is leading cause of gastroenteritis linked to seafood consumption in the United States [14]. Many species of sea urchin are harvested for food worldwide. Hence an attempt was made to isolate and characterize the isolated bacteria from coelomic fluid of sea urchin (*Stomopneustes variolaris*) that may cause food borne illness.

II. MATERIALS AND METHODS

Collection of samples

Sea urchins (*Stomopneustes variolaris*) were carefully collected from St. Mary's Island also known as Coconut Island and Thonsepar, a set of four small islands in the Arabian Sea off the coast of Malpe in Udupi, Karnataka, India. They are known for their distinctive geological formation of columnar basaltic lava. Sea urchins were collected at low tides by scuba diving at depths of 8-15 m during mid-summer in the month of April 2017. They were transferred to the ice boxes and brought to the laboratory.

Sample processing

Sea urchins were washed thoroughly under running tap water to remove debris and followed by distilled water. Coelomic fluid was obtained from a pool of five animals and this fluid was collected by inserting a sterile disposable 10 ml syringe into peristomial membrane. The sample was then centrifuged at 8000 rpm for 10 minutes, the supernatant was collected in sterile falcon tubes, a 5 ml aliquot was separated and remaining supernatant was diluted 1:1 with sterile 2.5% saline. This was done in order to enrich the organisms present in the sample since a higher salt concentration would mimic their natural habitat (sea

water). The sample was preserved at 4 ^{0}C for further study.

Isolation of bacteria by Spread plate method

To isolate bacteria, Zobell marine agar (ZMA) was used. Zobell Marine Agar was formulated by Zobell and has a composition that mimics seawater and thus helps the marine bacteria to grow abundantly [15]. Inoculated agar plates were incubated at room temperature.

0.1 mL of enriched coelomic fluid sample was spread on Zobell marine agar (ZMA) plates to obtain marine bacteria. The enriched samples were spread in triplicates. All the plates were incubated at room temperature and the bacterial growth was observed at overnight incubation on ZMA.

Morphological study of bacterial isolates

Gram's staining

Gram staining was done according to the procedure [16]. The isolate was smeared in the slide and heat fixed. The crystal violet dye was added, kept for 1 minute and washed in running water. Gram's iodine was added, kept for 1 minute and washed in running water then the smear was decolorized with 90% ethanol and finally the counter stain safranin was added and after a minute washed in running water. It was observed under the light microscope (Olympus clinical microscope model CH20i, Japan).

Determination of CFU/mL of the sample

The enriched sample of coelomic fluid was tenfold serially diluted up to 10^{-6}. 0.1 mL of the final three dilutions i.e., 10^{-4}, 10^{-5} and 10^{-6} of samples were spread on sterile ZMA plates and incubated at room temperature for overnight. Countable colonies were observed on 10^{-4} dilution plates of the coelomic fluid sample.

Biochemical Characterization of bacterial isolates:

The bacterial isolates were subjected to biochemical analysis according to Bergey's Manual of Determinative Bacteriology [17]. The details of each test are given below.

Indole test:

The isolated organism was inoculated in 1 mL of sterile tryptone water and incubated overnight. After 24 hours, 1 mL of xylene was added to the tube and shake vigorously and allowed it to stand for 5 minutes. 0.5 mL of Kovac's reagent was added down the side of the tube without shaking. Development of pink colour indicates positive test.

Methyl Red test

The acidic pH of the medium was detected by adding methyl red indicator to an overnight culture of the test organisms in glucose phosphate broth. Development of pink or red colour indicates a positive test.

Voges - Proskauer test

The test organism was inoculated in sterile glucose phosphate broth and incubated overnight. Added 0.5 mL of α-napthol, mixed well and then added 0.5 mL 40% KOH. Development of red coloured ring within 5 minutes indicates a positive reaction.

Citrate Utilization

For performing the test, the media are inoculated with the test organism and incubated overnight for the presence of turbidity in liquid basal broth (Koser's citrate). Presence of turbidity in Koser's citrate indicates positive test.

Sugar Utilization/Fermentation test

For this test, organism was inoculated in a culture medium containing a specific carbohydrate at 1% concentration in a peptone water base. The acid production was detected (after overnight incubation) by adding a suitable pH indicator such as Phenol Red and the gas formed was detected using a Durham's fermentation tube.

Catalase test

To determine the catalase production by a bacterium, a few drops of 3% hydrogen peroxide can be added to the culture organism. If effervescence is observed the enzyme is catalase positive.

Oxidase test

For this test, a loopful of bacterial growth was placed on a Whatmann filter paper and add oxidase reagent (1% solution of N, N, N', N'- tetra-dimethyl-phenylenediamine dihydrochloride) on it. Species which are positive for oxidase enzyme turn purple whereas negative ones show no colour change.

Halophilic nature of bacterial isolates

The bacterial isolates were maintained in a high salt environment (2% - 2.5%) throughout the experiment. Therefore, in order to determine whether these bacteria were halotolerant or true halophiles, they were streaked on regular nutrient agar plate containing 0.5% NaCl.

Limit of halotolerance

In order to determine the limit of halo tolerance, both the isolates were inoculated in a series of tubes containing nutrient broth with higher NaCl concentrations ranging from 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5% and 6%. These tubes were incubated overnight at room temperature.

Molecular characterization and Identification of bacterial isolates

The two bacterial isolates were identified using 16S rDNA. For bacterial isolates, a large-scale colony PCR reaction of 100 µl was set up using specific primers to obtain a product of 709 bp. 16S forward primer sequence- 5'GTG TAG CGG TGA AAT GCG 3' and 16S reverse primer sequence- 5' ACG GGC GGT GTG TAC

AA 3' were incorporated in the reaction mixture. PCR products were purified and sequenced using a PCR purification kit (Cosmo Genetech, Republic of Korea), the purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% Agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. The sequenced results were then uploaded to the National Centre of Biotechnology Information (NCBI) GenBank (http: //www. ncbi.nlm.nih.gov/) and BLAST function was performed to identify the bacterial isolates.

Antibacterial activity of cell free broth

An attempt was made to observe if any of the bacterial isolates produced an extracellular antibacterial substance. In order to perform this, experiment the bacterial isolates were grown in nutrient broth with 2.5% NaCl at incubation temperature of 37 ^{0}C and 18 rpm speed in shaker for overnight. These liquid cultures were then centrifuged at 8000 rpm for 10 minutes and the supernatant was collected in fresh tubes. This cell free broth was hypothesized to be the source of antibacterial activity. A well diffusion assay was set up using 100 µl of the cell free broths as test sample and 100 µg/mL Ampicillin was used as positive control. *S. typhi, S. pyogenes, E. coli* and *E. faecalis* were used as test organisms. The test organisms were obtained from Institute of Microbial Technology, Chandigarh, India (IMTECH).

III. RESULTS

Two morphologically distinct bacterial colonies were observed on ZMA plate after overnight incubation at room temperature (fig.1). In coelomic fluid sample, colonies were 4-5 mm in diameter, completely transparent just like water droplets, circular with entire margin, butyrous and flat, while other colonies were translucent, large with 10-13 mm diameter, irregular shape, flat and butyrous.

Colony forming units (CFU) of coelomic fluid was represented in the table1. In dilution 10^4, number of bacterial colonies yielded from coelomic fluid was 92 and 105 in plate I and plate II respectively. The average number of colony was 98 for 1x10^7cfu/mL.

Isolated bacterial colonies were streaked again on ZMA plates to get clear appearance of bacteria and marked as C2 and C3 on Petri plates for easy identification (fig. 2).

Colony morphology is an important characteristic of bacterial isolation. Based on the colony morphology and Gram-negative staining, isolated organisms were identified as *Vibrio* sp. (C2) (species unidentified) and *Vibrio parahaemolyticus* (C3) from the culture (Fig. 4).

The result of the biochemical characterization of bacterial isolates of coelomic fluid sample was represented in the Table 1. All the biochemical tests showed positive results in both bacterial isolates except Voges Prausker test which showed negative result.

Molecular characterization of bacterial isolates

Based on the colony morphology and 16s rDNA sequencing, isolates were identified as *Vibrio* sp. SOVt012 (C2) and *Vibrio parahaemolyticus* 3KC1 (C3) from the culture.

Halophilic and halotolerent nature of the bacterial isolates from coelomic fluid was determined. The result showed a dense growth in both cultures when inoculated in a series of tubes containing nutrient broth with higher salt concentrations ranging from 2.5%, 3%, 3.5%, 4.5%, 5%, 5.5% and 6%. Thus, these bacteria were concluded to be highly salt tolerant. When streaked on regular nutrient agar containing 0.5% NaCl, only *Vibrio paraheamolyticus* showed the growth but Vibrio sp. SOVt012 didn't show the growth so it was confirmed that *Vibrio* sp. SOVt012 is a true halophile as it required high salt concentration.

Cell free broth of isolated strains were subjected to antibacterial activity against bacterial human pathogens like *S. typhi, S. pyogenes, E. coli* and *E. faecalis* but none of the strains were active against the test organisms. This indicates that the isolated organisms didn't have antibacterial property.

IV. DISCUSSION

Our results represent the first report of isolation and identification of *Vibrio spp*. by sequencing of 16S rDNA from coelomic fluid of sea urchin (*Stomopneustes variolaris)* viz., *Vibrio* sp. SOVt012 and *V. parahaemolyticus*3KC1 using Zobell marine agar. *Vibrio* species are commonly associated with polluted water, seafood, and other aquatic animals as the main source of contamination [18]. *V. parahaemolyticus* inhabits marine and estuarine environments [19]. Food borne infections with *Vibrio* spp. are common in coastal cities where retail markets are close to the sea basin [20]. Its presence imposes potential health risks to the public due to its association with gastroenteritis, wound infection, and septicemia [21, 22].

As a result, one of the major risks involves the consumption of raw or undercooked seafood, meat and meat products that may be contaminated by food borne pathogens present in the marine/retail markets [23, 24]. Such risks are further increased if

the food is mishandled during handling, slaughter, transportation, and processing where pathogens could multiply exponentially under favourable conditions [25]. The occurrence of *V. Parahaemolyticus* was examined in samples of finfish and *Penaeus monodon* from wholesale fish markets in Kolkata, India, by standard culture technique [26]. The prevalence, pathogenicity, and serotypes of *V. parahaemolyticus* were investigated in shrimp from Chinese retail markets [27]. Many authors reported *Vibrio* spp. isolated from different varieties of food including sea foods [28, 29 and 30]. *Vibrio parahaemolyticus* was isolated and characterized from coastal water in the Eastern Province of Saudi Arabia [31]. The association of these species may be due to the irresponsible and unhygienic act of washing chicken, beef and camel meats with sewage contaminated water/seawater [32]. Molecular confirmation of the retrieved *Vibrio* isolates was done using partial amplification of 16S rDNA sequencing. Biochemical tests and Gram-negative staining were also conducted to confirm the identification of *Vibrio* spp. This study is evidenced with the work demonstrated by many workers [33, 34 and 35] on different samples of seawater, marine fish, shellfish, etc. and causes outbreaks worldwide. Antibacterial activity of cell free broth of isolates showed negative result against test organisms. This indicated that coelomic fluid of sea urchin was the reservoir of some halotolerent and halophilic *Vibrio* species.

V. CONCLUSION

Sea urchins are commonly found in shallow sub tidal rocky bottom, feed on algae and organic matter on sea beds. Sea urchins are one of the important fisheries in several areas of the world. In this study we isolated and identified two *Vibrio* species from coelomic fluid through biochemical and molecular approach. *Vibrio* species are commonly associated with polluted water, sea foods and other aquatic animals which cause food borne diseases. It is concluded that the detail study of characterization of microbes is required with respect to safety in consumption of sea foods from the public health point of view.

ACKNOWLEDGEMENT

Authors are thankful to the department of Biosciences, Mangalore University for providing the laboratory facility to carry out this work.

Table 2. Biochemical characterization of bacterial isolates of coelomic fluid of sea urchin (*Stomopneustes variolaris*).

Bacterial isolates	Biochemical Tests						
	Indole test	Methyl red	Voges Prausker	Citrate utilization	Sugar utilization /Fermentation test	Catalase test	Oxidase test
Vibrio Sp. SOVt012 (C2)	+	+	-	+	+	+	+
Vibrio parahaemolyticus 3KC1 (C3)	+	+	-	+	+	+	+

'+': Positive, '-': Negative

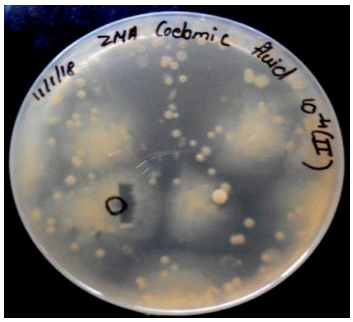
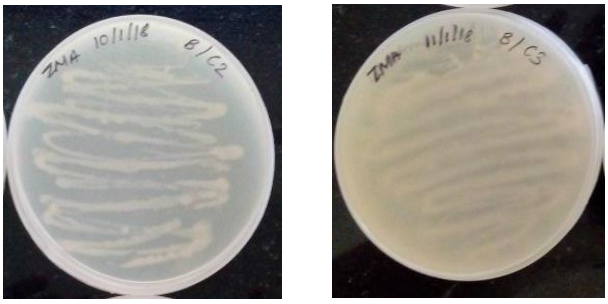


Fig. 1. Colonies in coelomic fluid on 10^{-4} dilution.



C2 C3

Fig.2: Bacterial isolates of coelomic fluid (subcultured).

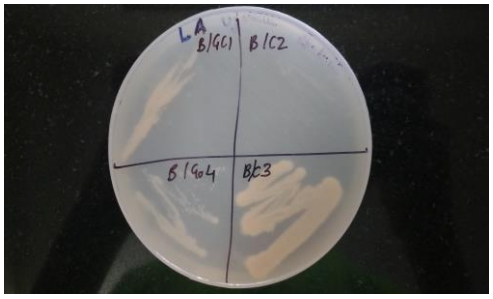
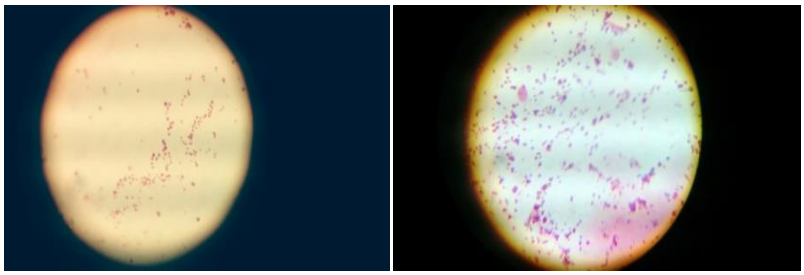


Fig.3. Halophilic nature of *Vibrio* sp. SOVt012 (C2)



C2 C3

Fig.4. Bacterial species in Gram negative staining.

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