



Original Research Article

Isolation and Identification Vibrios from marine Seafood Samples

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A B S T R A C T

Key words

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Isolation of
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activity;
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resistant pattern.

In this report encompasses the incidence of *V. alginolyticus* in sea foods as a human pathogen, in contribute to the onset of sporadic and epidemic outbreaks of diarrhoeal disease in humans. The following aspects were covered during the present investigation. Isolation of *v. alginolyticus* in sea food samples collected from Chennai, kanchipuram and Sriperumbudur local fish market. Antibiotic resistant pattern of isolated *V.alginolyticus*. Hemolytic activity of randomly selected in *Vibrio alginolyticus*. High lights of the pattern investigations are, *V.alginolyticus* was readily recoverable in all the 2 samples tested viz., Finfishes and crustaceans. All the biochemical test were carried out. Among 2 categories of samples, crustaceans harboured high number (80%) and finfishes (73%) of *v.alginolyticus*. Antibiotic resistant pattern was observed in Penicillin, Erythromycin, tetracycline, streptomycin, amoxyclav ampicillin, vancomyvin, and novobiocin. No resistant pattern was observed in Validixic acid, Gentamycin, Nitrofurantoin, Chloramphenical. A total of 15 strains were tested for hemolytic reaction 12 strains were positive for hemolytic production in blood agar plates and the remaining 3 strains were failed to lyses blood erythrocytes.

Introduction

The genus *Vibrio* includes more than 35 species, mostly in marine and natural habitats of sea water, and this species is broadly distributed throughout the world. There are halophylic marine bacteria of this genus known to cause in humans. *Vibrio parahaemolyticus* cause food borne gastroenteritis associated with seafood. Other halophylic *Vibrios*, *V.alginolyticus*, *V.fluvialis*, *V.metschnikovii* are also pathogenic for humans, while *V.anguillarum* is a pathogen fish and other marine animals (Selmin Ozer *et al.*, 2008).

Vibrios are gram-negative bacillus, curved, motile with a single polar flagellum and facultative anaerobic organisms that are natural inhabitants of the marine environments. In the United States, transmission of *Vibrio* infections in primarily through the consumption of raw or under cooked shellfish. Patients with liver disease are at particularly high risk for significant morbidity and mortality associated with these infections (Nicholas *et al.*, 2000). *Vibrio alginolyticus* is able to multiply in salty water at elevated water

temperature (Schets *et al.*, 2006). The ability to *Vibrio alginolyticus* to utilize sucrose has been used (Renate, *et al.*, 1987). *V. alginolyticus* mainly occur in marine and estuarine environment (Qian *et al.*, 2007).

It is one of the main *Vibrio* pathogens affecting marine animals such as marine fish, shrimp and shellfish. *V.alginolyticus* cause wound infection, ear infection, gastroenteritis and septicemia, food intoxication, Hemorrhaging, dark skin ulcer from the skin surface and large economic damage (Qian *et al.*, 2007). Internally fish accumulate fluid in the peritoneal cavity and some cases in hemorrhaging livers (Carmen Balbona *et al.*, 1998). Association with *V.alginolyticus* infections include chronic diarrhea a patient with AIDS, Conjunctivitis and post traumatic intracranial infection (louisinna). *V.alginolyticus* is a species with a broad geographical distribution in marine and estuarine waters especially in bathing areas (Larsen *et al.*, 1981). *V.parahaemolyticus* and *V.alginolyticus* were responsible for intestinal disease the inhabitants of the littoral localities of the Crimea. In the United States (i.e., the Gulf States Alabama, Florida, Texas, and Louisiana), raw oysters consumption is an important cause of *Vibrio* associated gastroenteritis among adults without underlying illness (Stefania Zanetti *et al.*,1998).*V.alginolyticus* is involved in epizootic outbreaks in cultured gilt-head sea bream in Mediterranean aquaculture causing fish mortality (Carmen Balebona *et al.*, 1998).

V.alginolyticus as a pathogen of shellfish rather than fish (Colorni *et al.*, 1981) adequately the recovery of *V.alginolyticus* from diseased sea bream (*Sparus aurata*)in Isrel. The characteristics were in complete agreement with the description of *V.alginolyticus* (Shewan and veron 1974). It

is usually isolated in the spring &summer from marine sources. i.e.: depend on water temperature greater than 10°C. *V.alginolyticus* as well as *V.parahaemolyticus* should be considered in tissues infections exposure to ocean water (Sally JoRubin *et al.*, 1975). *Vibrio alginolyticus* is frequently involved in epizootic out breaks in cultured gilt-head sea bream in Mediterranean aquaculture, causing fish mortality and important economic losses. The natural disease caused by *V.alginolyticus* in sea bream includes the septicemia, hemorrhaging, dark skin and ulcers on the skin surface in some cases. Internally, fish accumulate fluid in the peritoneal cavity and in some cases have hemorrhagic livers. This organism associated with edible sea food, rather than its distribution in natural aquatic environment, evokes immediate concern from public health point of view, as this pathogen gets transmitted through consumption of such contaminated food. This aim of the study was to characterize the virulence potential of *V.alginolyticus* strains responsible for sea food contamination. The present study has therefore been under taken in the following aspects to isolate potentially pathogenic *Vibrio* such as *Vibrio alginolyticus* from sea foods and their resistant pattern and hemolytic activity of randomly selected isolates.

Materials and Methods

Collection of seafood samples

20 different sea food samples such as Finfishes and crustaceans (shrimp and crab) were collected from different sites in Chennai, Kanchipuram, and sriperumbudur fish market. All sea food samples were transported in individually labeled and sealed new plastics bags to avoid contamination. The samples were placed in sealed containers with dry ice and

transported frozen to the laboratory for bacterial analysis. The time between sample collection and analysis was approximately 24 hours.

Sample processing for bacteriological examination of sea foods

Finfish and crustaceans were washed thoroughly with sterile distilled water prior to bacteriological examination. The heads and tails of the fishes were cut into small pieces using sterile scissors and the guts were removed. The Crustaceans and finfish samples were then homogenized in blenders, and 25g of each homogenate was placed in 225ml of alkaline peptone water (APW) pH 8.6, and incubated at 37°C for 24 hours. At the end of incubation period, two loopful of culture from pellicle of each flask (Enrichment broth APW) were then streaked on to Thiosulfate citrate bile salts sucrose (TCBS) agar plates and incubated at 37°C for 24 hours. Suspected typical colonies of *Vibrio alginolyticus* (yellow, large (diameter>5mm)colonies, sucrose positive) were selected, growing colonies were subcultured on Trypticase soy agar (TSA) (Merck) supplemented with 3% NaCl and incubated at 35-37°C for 24 hours. (Halophilism tests were performed on Trypticase soya agar (TSA) and supplemented with NaCl 0%, 1%, 3%, 6%, 8%, and 10%). Additional characterization tests included Gram staining, motility test, Biochemical test, catalase, cytochrome oxidase activity test, Triple sugar iron test, ornithine, Arginine, lysine, valine, leucine, dehydrolase test, Nitrate reduction test, Gelatin hydrolysis test, starch hydrolysis test, and glucose, lactose, mannitol, maltose and sucrose fermentation test were carried out. The identification of bacteria was confirmed based on *Bergey's Manual of determinative bacteriology*, 9th edn (1994).

Antibiotic resistant pattern

V.alginolyticus strains were isolated from seafood samples were grown in nutrient broth containing 2% NaCl. Muller-Hinton (MHA) agar medium (Hi media) was used for antibiotic resistances pattern. Disc diffusion assays for antibiotic susceptibility were conducted following the method described by Anon (1970). All isolated were tested against the following discs (mcg) (Hi media) Gentamycin 30; Chloramphenical 30; Tetracycline 30; Novobiocin 30; Vancomycin 5; Erythromycin 15; Penicillin, Amoxyclav 10; Nitrofurantoin 300; Ampicillin 25; Vancomycin30; Nalidixic acid 50; The 24 hours broth culture of *V.alginolyticus* were swabbed on MHA agar plates and the disc were placed by using alcohol dipped and flamed forceps on the surface of MHA agar medium. All the plates were left for 20 minutes and then incubated in an inverted position at 37°C for 18-24 hours. The results were recorded by measuring the zone of inhibition.

Hemolytic activity

V. alginolyticus strain to produce hemolysin was tested on blood agar supplemented with 5% sheep anticoagulant blood. All the selected cultures were seeded in blood agar plates, and the plates were incubated at 37°C for 24 hours. Hemolytic activity was determined.

Results

Isolation of *Vibrio alginolyticus*

A total number of 20 sea food samples (Finfishes and crustaceans) were collected from Chennai, Kanchipuram and sriperumbudur fish market. Result revealed that 11 finfish samples were positive for *Vibrio alginolyticus* out of 15 samples

Table.1 Bio-chemical characteristics of *V.alginolyticus*

TEST NAME	RESULTS
Gram staining	Gram Negative rods
Motility	Motile
Indole	+Ve
Methyl Red	+Ve
Voges proskauer	+Ve
Citrate Utilization test	+Ve
Triple sugar iron agar	Acid/Acid, No gas, and H ₂ S
Catalase	+Ve
Oxidase	+Ve
Nitrate reduction test	+Ve
Gelatin hydrolysis test	+Ve
Starch hydrolysis test	+Ve
Carbohydrate Fermentation test	
Glucose	+Ve
Maltose	+Ve
Mannitol	+Ve
Sucrose	+Ve
Lactose	-Ve
Amino acid decarboxylase test	-Ve
Arginine	
Lysine	+Ve
Ornithine	+Ve
Leucine	-Ve
Valine	-Ve
Trypticase soy agar	+Ve
Halophilisms test	-Ve
0% NaCl	
1% NaCl	+Ve
3% NaCl	+Ve
6% NaCl	+Ve
8% NaCl	+Ve
10% NaCl	+Ve

Table.2 Incidence of *V.alginolyticus* in different groups of finfishes and crustaceans collected from fish market

S.No	Samples	Total no. of samples	Total no.of Positive for vibrios	No.of strains Isolated for <i>V.alginolyticus</i>	Total no.of Negative sample
1.	Finfishes	15	11(73%)	11	4
2.	Crustaceans (Shrimp & crab)	5	4(80%)	4	1

collected. Where as in crustaceans 4 samples were positive for *Vibrio alginolyticus* out of 5 samples collected. However, in Finfish samples and crustaceans *V.alginolyticus* was isolated (Figure .1) and Biochemical test were carried out (Table-1). Among 2 categories of sample, crustaceans harboured higher number (80%) and Fin fishes (73%) of *V.alginolyticus* (Table-2)

Antibiotic resistant pattern

The pattern of resistance to various antimicrobial substances in *V.alginolyticus* isolated from sea foods samples is given in (Table 3; Figure 2). Among the strains tested against 12 antibiotics resistant pattern was observed in penicillin (10 strains), Ampicillin (6 strains) Erythromycin (6 strains), Tetracycline (8 strains), Streptomycin (7 strains), Amoxyclav (6 strains), Vancomycin (9 strains), Novobiocin (6 strains). No resistant pattern was observed in Nalidixic acid, Gentamycin, Nitrofuratoin, Chloramphenicol.

Hemolytic activity

Among the 15 strains tested, 12strains (80%) showed positive hemolytic activity on blood agar plates. Strains number SF6, SF12 and SF14 failed to produce hemolytic on blood agar plates (Table 4).

Table.3 Antibiotic resistant pattern of *Vibrio alginolyticus* isolated from sea food samples

S.No	St.No	P	E	Na	G	Nf	C	T	S	AM	A	V	N
1.	SF1	+(14)	+(20)	+(21)	+(17)	+(14)	+(13)	-	+(14)	-	+(15)	-	+(19)
2.	SF2	-	-	+(20)	+(15)	+(17)	+(14)	-	+(07)	-	+(18)	-	+(18)
3.	SF3	+(21)	+(21)	+(17)	+(17)	+(18)	+(23)	+(17)	-	+(08)	-	+(08)	-
4.	SE4	-	-	+(14)	+(13)	+(11)	+(15)	+(18)	-	+(10)	-	+(12)	-
5.	SE5	+(14)	+(17)	+(13)	+(20)	+(19)	+(16)	+(20)	+(13)	-	-	+(09)	+(25)
6.	SF6	-	+(14)	+(14)	+(14)	+(17)	+(20)	+(28)	-	+(30)	-	-	-
7.	SF7	-	+(13)	+(15)	+(13)	+(15)	+(18)	-	+(14)	+(28)	+(15)	+(01)	-
8.	SF11	-	-	+(14)	+(15)	+(16)	+(25)	-	-	+(15)	+(13)	-	+(15)
9.	SF12	-	-	+(18)	+(16)	+(18)	+(26)	-	+(16)	-	+(17)	-	-
10.	SF14	+(15)	-	+(19)	+(20)	+(21)	+(22)	+(06)	+(12)	+(16)	+(21)	-	-
11.	SF16	-	+(14)	+(21)	+(14)	+(20)	+(11)	-	-	+(18)	-	-	+(18)
12.	SF17	-	-	+(18)	+(15)	+(24)	+(23)	-	-	+(14)	+(14)	-	+(21)
13.	SF18	+(18)	+(15)	+(17)	+(18)	+(30)	+(16)	-	-	+(13)	+(18)	+(07)	+(22)
14.	SF19	-	+(16)	+(14)	+(14)	+(15)	+(14)	+(14)	+(15)	-	-	+(15)	+(04)
15.	SF20	-	+(18)	+(12)	+(16)	+(13)	+(23)	+(18)	+(20)	-	-	-	+(10)

Measured zone diameter (mm) and susceptibility status of isolates, + = Sensitive, - = Resistant, **Antibiotics** P = Penicilin, E =Erythromycin, NA =Nalidixic acid, G= Geyamycin, C = Chloramphenicol, T =Tetracyline, NF =Nitrofurantion, S =Streptpmycin, AM =Amoxyclav, A =Ampilicin, V =Vancomycin, NO =Novobiocin.

Table.4 Hemolytic activities of randomly selected Isolates of *Vibrio alginolyticus*

S.No	Strain No	Name of the Organisms	Source	Hemolysin On blood Agar Plates
1.	SF1	<i>Vibrio alginolyticus</i>	Fin fishes	+
2.	SF2	<i>Vibrio alginolyticus</i>	Fin fishes	+
3.	SF3	<i>Vibrio alginolyticus</i>	Fin fishes	+
4.	SF4	<i>Vibrio alginolyticus</i>	Fin fishes	+
5.	SF5	<i>Vibrio alginolyticus</i>	Crab	+
6.	SF6	<i>Vibrio alginolyticus</i>	Fin fishes	-
7.	SF7	<i>Vibrio alginolyticus</i>	Finfishes	+
8.	SF11	<i>Vibrio alginolyticus</i>	Shrimp	+
9.	SF12	<i>Vibrio alginolyticus</i>	Crab	-
10.	SF14	<i>Vibrio alginolyticus</i>	Finfishes	-
11.	SF16	<i>Vibrio alginolyticus</i>	Finfishes	+
12.	SF17	<i>Vibrio alginolyticus</i>	Finfishes	+
13.	SF18	<i>Vibrio alginolyticus</i>	Finfishes	+
14.	SF19	<i>Vibrio alginolyticus</i>	Finfishes	+
15.	SF20	<i>Vibrio alginolyticus</i>	Crab	+

+ = Positive for hemolysin production, - = Negative for hemolysin production

Discussion

Vibrio alginolyticus, a halophilic gram-negative bacterium that inhabits marine and estuarine environments. The human diseases caused by *V.alginolyticus* include gastroenteritis, soft tissues, otitis media, food intoxication and septicemia. Furthermore, it is one of the main *Vibrio* pathogens affecting marine animals, such as marine fish, shrimp and shell fish, which leads to large economic damage. Thus, it is essential to explore an effective protective pathway against by this microorganism. *V.alginolyticus* was reported to be the most prevalent species with in *Vibrio* spp in mussels and sea water samples. According to another study, toxigenic *V.alginolyticus* strains were isolated as a predominant species from Chennai coastal waters and sea food has been produced and consumed by

bathing in this area. Therefore, researchers have stated that these potentially pathogenic isolates may significantly contribute to the onset of sporadic and epidemic outbreaks of diarrhoeal disease in humans. The clinical significance of these potentially pathogenic *vibrio* strains are in association with gastroenteritis and invasive septicemia and usually spread to human via consumption of raw or under cooked shell fish, wound infections acquired by contact with fish, shell fish and sea water. In the present study, the several species of Finfishes and crustaceans were collected from the market found to harbour *Vibrio alginolyticus* and other vibrios. From available reports it is well known that sea foods were found to be they carrier of vibrios and the ubiquitous distribution of

this pathogenic Bacteria in sea foods. Bacterial adhesion to host surface has been described as one of the initial steps in microbial pathogenesis. It has been suggested that hydrophobicity is a determining factor in the adhesive process and in the survival of pathogen in cells. The strains of *V.alginolyticus* assayed did not agglutinate sea bream erythrocytes were agglutinated. Similarly, chen and Hanna reported that only two of the vibrio species which they tested, *V.alginolyticus* and *V.anguillarum*, attached to cells and tissues from giant tiger prawn.

The results obtained in this study, the isolated gram negative halophilic bacterium *Vibrio alginolyticus* requires at least 3% NaCl and can tolerate up to 10% NaCl for growth. Sea water and sea food is an important vehicle of transmission of this species and other vibrios and the possible spread of vibrios to marine invertebrates. Antibiotic susceptibility of *Vibrio alginolyticus* were determined by disc diffusion on Muller Hinton agar (Merck) supplemented with 3% NaCl. The diameter of the national committee for clinical laboratory standards. Antibiotic resistant pattern was observed in Ampicillin, Tetracycline, and Chloramphenicol. (Table -3) the prevalence of antibiotics resistant microorganisms is ecologically very important and this character is plasmid borne. R plasmid is responsible for antibiotics resistant characters. These R plasmids have been found in *V.alginolyticus* and other vibrios and carry transferable drug resistance. The data suggest that antibiotic resistant *Vibrio alginolyticus* may survive better than sensitive organisms in surface water. In general, resistance of bacteria to antibiotics may be due to enzymatic destruction of antibiotics impermeability

of the cell wall to the antibiotics destruction of antibiotics impermeability of the cell wall to the antibiotics additional of chemical group to antibiotics of the 15 strains vibrio tested 12 strains 80% were positive for hemolytic activity. In this present investigation concluded that the evolution of the risk of *V.alginolyticus* to marine environmental health and sea food safety. *V.alginolyticus* infections have been associated with gastroenteritis after consumption of contaminated sea food and it can also infect open wounds exposed to warm sea water. There are many reports about the presence of *V.alginolyticus* in sea food samples in Chennai. In this respect, the presence of *V.alginolyticus* isolated in sea food collected in the Chennai coastal region indicates a public health problem. Nevertheless, these organisms were also detected in several coastal regions of the world, in both temperate and tropical areas and there should be an awareness of the potential for zoonotic diseases when handling sea animals such as fish and shrimps or their tissues.

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