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RESEARCH ARTICLE

DISTRIBUTION AND PHENOTYPIC IDENTIFICATION OF PATHOGENIC *VIBRIO ALGINOLYTICUS* AND OTHER VIBRIO SP ISOLATED FROM CHENNAI EAST COAST REGION, SOUTH INDIA

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ABSTRACT

The objective of the present study was aimed to evaluate the distribution and phenotypic identification of pathogenic *Vibrio alginolyticus* and other Vibrio sp. Total halophilic and Vibrio sp were isolated from marine water and sediment samples of Chennai East Coast Region [ECR]collected from Thiruvanmyur to Mahabaliburam by standard spread plate method. Each suspected colonies were identified by physio-chemical properties and pure strain were subjected to phenotypic identification as described in the literatures, finally the 16S rRNA sequence of the isolate was deposited in the GenBank database under accession number JN863235.Quantitative distribution of total halophilic and total vibrio counts of water and sediment samples were varied from 68 x 10^3 to 22x10^3cfu ml^{-1} and 13 x 10^3 to 8 x 10^3cfu ml^{-1}, 87x10^3to28x10^3cfu g^{-1} and 21 x 10^3 to 9 x 10^3cfu ml^{-1} respectively. Qualitatively 23Vibriosp were isolated from both water and sediment samples. Of them *Vibrio alginolyticus* was the predominant species hence it was confirmed by phenotypic analysis. *Vibrio alginolyticus* is an opportunistic pathogen; our analysis showed that these results are very important for assessing risk of infection after bathing tourists especially at Thiruvanmyur, Kovelam and Mahabaliburam.

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INTRODUCTION

In the last two decades, research on medical, environmental and taxonomic aspects of Vibrio species has been expanded greatly. Microorganisms belonging to the genus Vibrio are gram negative curved bacilli, facultative anaerobes, oxidase and catalase positive, non-spore forming with a polar flagellum which gives them great mobility. They are aquatic bacteria, most are halophilic and any of them may also live in alkaline environments. Vibrio's are not only an economic threat to marine shrimp farming, but represent a public health concern as they are easily transmitted to humans through the consumption of sea foods (Blackstone *et al*., 2003; Renata *et al*., 2010). This bacterium is normally found in the marine environment and the disease outbreaks occur when fish are exposed to infectious agents in the presence of stress factor (Austin and Austin, 2007). Many species of this genus are pathogens, symbionts or parasitic. As a representative of the halophilic Vibrio's, *Vibrio alginolyticus* is isolated from coastal waters and sediments all over the world (Chan *et al*., 1986; Eiler *et al*., 2006; Hervio-Heath *et al*., 2002) and is considered to be part of the normal marine micro flora.

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However, *V. alginolyticus* is ahalophilic (salt-tolerant) gram negative bacterium recognised as bacterial pathogen of humans, and the incidence of infection significantly increases during the summer months. *V. alginolyticus*are ubiquities in seawater and tends to cause superficial wound and ear infections (otitis media, otitis externa, endophthalmitis) and gastrointestinal infections (Gomez *et al*., 2003; Li *et al*., 2009; Reina *et al*., 1995). Most reports of *V. alginolyticus* wound infections results from exposure of cuts or abrasions to contaminated seawater. *V. alginolyticus* associated infections may be resolved using appropriate antibiotics; however, very rarely these infections can progress to bacteraemia and necrotising fasciitis, particularlyin immunocompromised. This bacterium also belongs to the most important opportunistic pathogens of aquatic animals, Including fish, shellfish, crustaceans, coral and echinoids causing serious disease and damage in cultured fish and important economic losses (Austin *et al*., 1993; Balcázar *et al*., 2010; Gonzalez-Escalona *et al*., 2006).

V. Alginolyticus shows an enormous intra specific variable and its pathogenicity. In fact, it seems that the pathogenesis of *V. alginolyticus*are the sum of the concentrated action of multiple virulent factors, including the iron uptake system

(Litwin and Calderwood, 1993), extracellularhaemolysin (Aguirre-Guzmán *et al*., 2004; Lee *et al*., 1996) and protease (Rui *et al*., 2009) are suggested as the major contributors to the pathogenicity in this species (Ming-Xia Chen *et al*., 2011).Studies of the distribution, dynamics of this species have rarely been addressed in India. When we examined the diversity of Vibrio in East Coast water and sediment, we found that *V. alginolyticus* –like species was the dominant Vibrio species. Since *V. alginolyticus* was the dominant we decided to investigate the distribution of this bacterium in order to obtain valuable information for the aquaculture industry and for the health warning system.

Moreover *V. alginolyticus* is considered the most frequents pecies living freely in seawater and sediments and can survive in seawater under starvation condition while maintain their virulence (Ben Kahla-Nakbia, 2007). Thepresent study has therefore been under taken in the following aspects to isolate potentially pathogenic Vibrio such as *V. alginolyticus* from sea water, sediments and their resistant pattern and haemolytic activity of randomly selected isolates.

MATERIALS AND METHODS

Sampling method

For the present study, marine Water and Sediment samples were collected from different sites in Chennai East Coast Region from Thiruvanmyur to Mahabaliburam. The sampling site was 10 km in distance from one site to another. The samples were taken into sterile bags, kept on ice during transport to the laboratory. Temperature, salinity and pH were measured by a pH meter.

Sample preparation and quantification of Vibrio

A series of 10 fold dilutions of each water and sediment samples were plated on Thiosulfate Citrate Bile Salt sucrose (TCBS) and Tryptone Soy Agar (TSA) (Hi Media Ltd., Mumbai) for total Vibrio and total halolophilic counts respectively by the standard spread plate method. The inoculated plates were incubated at 35 ºC for 18 - 24 hours.

Isolation and identification of Vibrio

From each TCBS plate suspected yellow color colonies were picked out, streaking on to Tryptone Soy Agar plus 2% NaCl followed by incubation for 24 hours at 35ºC to obtain pure cultures. One of thedominant colonies was then selected and restreaked onto TSA to obtain pure cultures for identification tests. Morphological, gram staining, biochemical testing with the Arginine dihydrolase oxidase test, Catalyzed reaction, Nitrate reduction, Amino acid decarboxylase reactions were used to identify the species.

Antibiotic Susceptibility Test

Purified culture of the test organism were picked with wire loop and introduced into a test tube containing 4 ml of Tryptone Soy broth. These tubes were then incubated for 20-24 h to produce a bacterial suspension of moderate cloudiness. For the sensitivity test, large petriplates were used with Mueller - Hinton Agar. Plates were dried before use. The bacterial broth suspension was streaked evenly on to the surface of the medium with sterile cotton swab. After the inoculum dried the antibiotic discs (Hi Media Ltd., Mumbai) were placed on the agar with flamed forceps and gently pressed down to ensure contact. The discs used were Ampicillins (10 µg), Chloramphenicol (30µg), Erythromycin (15µg), Gentamycin (10µg), Kanamycin (30µg), Methicillin (5µg), Nalidixic acid (30µg), Streptomycin (10µg), Oxytetracycline (10 µg) and Tetracycline(30µg). The results were interpreted according to Bauer *et al*.

Kanagawa Phenomenon

The ability to hemolyse erythrocytes was tested on Blood agar. A colony of each of the isolates was sub cultured on to freshly prepared blood agar (Nutrient agar containing human blood) plates and streaked to obtain discrete colonies. The plates were incubated at 37º C for 24 h after which the colonies were examined visually for haemolytic activity.

Extraction of genomic DNA

Bacteria were grown at 28ºC on Tryptone soy broth for 24 h and harvested by centrifugation at 7000 rpm for 20 min at 4ºC.The Nuclic acid of pelleted bacteria was extracted using a Genomic DNA purification kit (Genei, Bangalore) and stored at -20ºC, until used for polymerase chain reaction (PCR) tests.

Polymerase Chain Reaction (PCR) and Phylogenetic analysis

To identify the bacterium at the gene level, Polymerase Chain Reaction (PCR) was performed to amplify a partial 16S rDNA gene of the bacteria. The 16 SrDNA fragment was amplified using the primer set 27F(5-AGA GTT TGA TCC TGG CTC AG – 3) and 1492R(5-GGT TAC CTT GTT ACG ACT T-3). The cycle sequencing reaction was performed using BigDye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (from Applied Biosystems, P/N: 4337457). The sequencing reaction - mix was prepared by adding 1ul of BigDye v3. 1, 2ul of 5x sequencing buffer and 1ul of 50% DMSO. To 4ul of Sequencing reaction –mix was added 4 Pico moles of primer (2ul) and sufficient amount of DNA.

The constituted reaction was denatured at 95°C for 5 minutes. Cycling began with denaturing at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension for 4 minutes at 60°C and the cycle repeated for a total 30 cycles in a MWG thermocycler. The reaction was then purified on sepheadex plate (Edge Biosystems) by centrifugation to remove unbound labelled and unlabelled nucleotides and salts. The purified reaction was loaded onto the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 hours. A similar search for the nucleotide sequence of 16S r DNA of the test isolate was carried out using a Blast search at NCBI (http://www.ncbi.nlm.nih.gov).

Nucleotide sequence accession number

The 16S rRNA sequence of the isolate was deposited in the GenBank database under accession number JN863235.

RESULTS AND DISSCUSSSION

Among the 40 different Vibrio species recorded from wild and cultured fish, nine species viz., *Vibrio alginolyticus, V. anguillarum, V. damsels, V. Harvey, V. ordeal, V. Pelagius, V. salmonicida, V. Splendidus*and *V. Vulnificus* were reported as pathogens infecting the marine fish (Farto *et al*., 2002; Toranzo *et al*., 2005). Among Vibrio species*V. alginolyticus* is considered as the major pathogen causing severe infections leading to massive mortality in various fish species throughout the world. However, the virulence of the *V. alginolyticus* to fish could not be firmly established because the virulencevaries from specie to species, and in some cases even vary within the same fish species (Jung and Shin, 1996).

Moreover, the onset of fibrosis by *V .alginolyticus* is always associated with deteriorating culture conditions or physical damage of cultured fish; which lead to consider this an opportunistic pathogen (Austin and Austin, 2007). Furthermore, it is one of the main Vibrio pathogens affecting human diseases include gastroenteritis, soft tissues, otitis media, food intoxication and septicemia. Thus, it is essential to explore an effective protective pathway against by this microorganism.

In our study Total HalophilicBacterial Count (THC) varied from 68 x 103 to 22 x 103cfu ml-1 and Total VibrioCount (TVC) from$13x10^3$ to $8x10^3$cfu ml^{-1} (Table 1). Density of Vibrio in the coastal water of Korea was reported to be 0.2 x 10^1 to $9.0x10^3ml^{-1}$(Jung and Shin, 1996). Sreeja and Ravindran, 1999 reportedwas 0.2 x 10^1 to 9.0 x 10^3 ml^{-1} in coastal water and 0.8 x 10^1 to 3.0 x 10^1 ml^{-1} in open water off Mangalore coast of India. Vibrio as a dominant flora with incidence 35% preponderance in seawater and sediment collected from Madras coastal waters was also established (Prabhu *et al*., 1991). Wide range in the values of quantitative occurrence of Vibrio in water can be due to the variations in the physical-chemical parameters prevailing in the sampling station and the time of collection (SanthaSudha *et al*., 2012).THC and TVC of sediment samples were tabulated in Table 1.

Station No 1.Thiruvamayur; 2.Nainarkuppam; 3.Kovalam; 4.Edayur; 5.Mahabaliburam

In sediment sample, as in seawater, Vibrio load is significantly related to total halophilic bacteria. THC was also high, ranging from 87 x 10^3 to 28 x 10^3cfu g^{-1} and TVC from 21 $x10^3$to $9x10^3$cfu ml^{-1}. This corroborates with the earlier report of the total flora from sediment of Cochin area (Chandrika and Nair, 1994).

Similarly, Vibrio comprising 35% of total flora was reported from water and sediment of Madras coast (Prabhu *et al*., 1991). In the present study mean percentage of Vibrio in sediment was observed high when compared to water. Generally sediment provide better micro environment than water and thus rich flora can flourish. It was reported earlier that the flora of sediment were 3 times (Williams and La Rock, 1985) and 10 times (Pagnocca, 1991) higher than the water. This high value might be due to the comparatively higher nutritional status, availability of substrate for attachment or the positive interactive effect of organisms present in the sediment.

The qualitative distributions of individual Vibrio species in our findings from water and sediment samples were presented in Table 2. *V. alginolyticus, V. parahaemolyticus, V. Campbell*and *V. orientalis*were found to be the predominant species; hence a single isolate of *V. alginolyticus* was identified from the samples. A close scrutiny of the literature showed that even if Vibrio is ubiquitous in distribution, the species diversity differed with geographical areas. *V.alginolyticus* was found to be the predominant species in Taiwan Sea (Cheng *et al*., 1995). Seventy-two percentage of the Vibrio population of Tanabe Bay, Japan, was found to be constituted by *V. alginolyticus, V. Campbellii*and *V. Harvey* (Miyazaki and Ezura, 1995). This finding has economic importance also because of the bottom dwelling nature of shrimps and other demersal fishes can carry a higher load of the virus.

Table 1. Bacteriological parameters of water and sediment samples collected from various sites of the Chennai east coast region

	Water		Sediment	
Station No	Total Halophlic bacterial count X 10^3 g^{-1}	Total Vibrio count X 10^3 g^{-1}	Total Halophlic bacterial count X 10^3 g^{-1}	Total Vibrio count X 10^3 g^{-1}
1.	68	13	87	21
2.	60	14	81	18
3.	35	11	48	13
4.	30	9	38	11
5.	22	8	28	9

Table 2. Distribution of different vibrio species in water & sediment samples collected from Chennai east coast region

Sampling Sites	Species
1.Thiruvamayur	*V.alginolyticus, Vibrio* spp, *V.parahaemolyticus, V.fluvialis, V.harvey*
2. Nainarkuppam	*Vibrio* spp,*V. alginolyticus, V.parahaemolyticus, V.fischeri*
3. Kovalam	*V. alginolyticus, V.parahaemolyticus, V.fischeri, V.cholerae, Vibrio* spp
4. Edayur	*V. alginolyticus, Vibrio* spp,*V.parahaemolyticus, V.fluvialis, V.harvey*
5. Mahabaliburam	*V. alginolyticus, Vibrio* spp,*V. alginolyticus, V.parahaemolyticus*

Vibrio has been reported as a major component of sediment fromVellar estuary, India. Higher percentage of pathogenic Vibrios like *V. parahaemolyticus, V. vulnificus*and*V. alginolyticus* in the sediment samples are to be considered in the case of demersal trawling methods of fish capture, as that method can increase in the pathogen load in skin and gill. Sea water and seafood is an important vehicle of transmission of this species and other virus and the possible spread of virus to marine invertebrates (Ramesh *et al*., 1993).

The Vibrio strains isolated and reported in the present investigations manifested typical biochemical characteristics of fibrous. They were Motile, Oxidase and Catalase positive, Gram – negative that reduced Nitrate to Nitrite (Table 3), grew in TCBS agar medium (Figure 2).

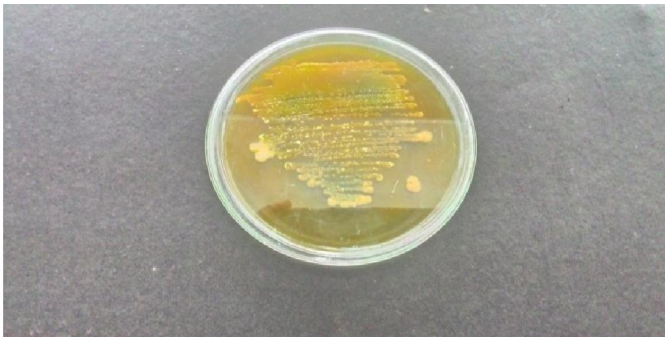

Fig.1. Yellow colour colonies of *V .alginolyticus* on TCBS Agar Medium

Table 3. Morphological & Biochemical Characterization of *V. alginolyticus*

Test	Reaction	Test	Reaction
		Gelatinase	+
Gram reaction	-	Chitinase	+
Motility	+	O/F TEST	O, F
β- galactosidase	-	Utilization:	
Acetone production	+	Glucose	+
Nitrate reduction to nitrite	+	Manitol	+
Catalase	+	Inositol	-
Oxidase	+	Sorbitol	-
Growth in NaC l3-8%	+	Rhamnose	-
Arginine dihydrolase	-	Sucrose	+
Lysine decarboxylase	+	Arbinose	_
Ornithine decarboxylase	+	Cytochrome oxidase	+
Indole production	+	Arabinose	-
Vogesproskauer	-		
Citrate utilization	-		

The method of Bauer *et al*., for testing for resistance to 10antibiotics resulted a variety of resistance patterns for *V. alginolyticus* strain. Based on the resistant, intermediate, and susceptible zone diameters established for enteric bacteria *V. alginolyticus* stains were susceptible to Chloramphenicol, Nalidixic acid resistant to Gentamycin, Erythromycin, Ampicillin, Methicillin, and Streptomycin and had intermediate reaction to Oxytetracycline, Kanamycin and Tetracycline (Table 4).

V. alginolyticus has been reported to be resistant to Ampicillin, Methicillin, Lincomycin, Penicillin and Carbenicillin and to be susceptible to Tetracycline, Chloramphenicol, Gentamycin, Kanamycin, Streptomycin, and Neomycin. Very few of these isolates were susceptible to Tetracycline, Kanamycin, Streptomycin or Neomycin. *V.parahaemolyticus* has been reported to be resistant to the same antibiotics as *V.alginolyticus*. Both of the wide variety of resistant patterns found among *V.alginolyticus* and*V. parahaemolyticus* isolates in study and their intermediate and resistant reactions to several antibiotics create a high potential for refractory infections caused by *V.parahaemolyticus*and*V.alginolyticus* (Molitoris *et al*., 1985).

Table 4. The antimicrobials sensitivity of *Vibrio alginolyticus*

Antimicrobials	Result
	*Vibrio alginolyticus*JN863235
Chloramphenicol (30µg)	S
Oxytetracycline(10µg)	I
Gentamycin (10µg)	R
Erythromycin (15µg)	R
Ampicillins (10µg)	R
Kanamycin (30µg)	I
Methicillin (5µg)	R
Nalidixic acid (30µg)	S
Streptomycin (10µg)	R
Tetracycline (30µg)	I

To identify the organism at genetic level DNA was extracted from *V. alginolyticus* is done according to the protocol described in the commercial kit DNA purification (Genei, Bangalore). The cycle sequencing reaction was performed using Big Dye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (from Applied Biosystems, P/N: 4337457).

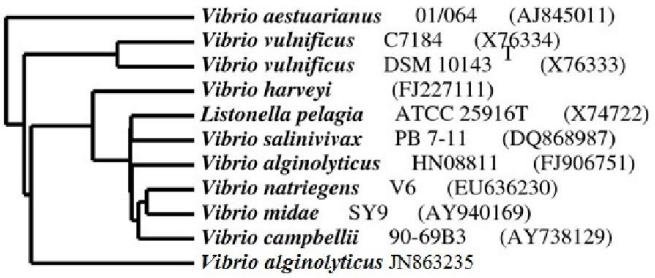

Fig. 2. Phylogenetic tree of the 16S rDNA sequence of strain *V .alginolyticus* JN863235 and the bacteria mostly closed to it

A similar search for the nucleotide sequence of 16S r RNA of the test isolate was carried out using a Blast search at NCBI (http://www.ncbi.nlm.nih.gov).The tree was created from the online tool Blast2tree from the following website: http://bioinfo.unice.fr/blast/blast2treedyn/blast2tree.phpthe sequence of the 16S rDNAamplicon from isolate was determined and deposited at GenBank database under accession number JN863235. This sequence showed 99.9%identity with the sequence of the reference strain of *V. alginolyticus* (GenBank accession number HNO8811) and *V. alginolyticus*16S r DNA(GenBank accession number FJ906751).16S rDNA gene sequencing is considered by many authors to be a very reliable method for identification of any bacteria including marine Vibrio(Gomez-Leon *et al*., 2005).

Conclusion

In summary, our analysis showed that these results are very important for assessing risk of infection after bathing tourists especially at Thiruvanmyur, Kovelam and Mahabaliburam. Prevention is the only way to avoid contact with a contaminated environment. Indeed, the water and seafood monitoring should allow the detection of potentially pathogenic isolates.

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