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Molecular confirmation of *Vibrio alginolyticus*

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Abstract

Vibrio is a genus of bacteria indigenous to the aquatic environment also contaminant of raw or under cooked seafood. Some bacterial species of this genus are now considered as emerging pathogens, involved in food-borne infections in humans. The chromosomal DNA was isolated from the fresh liquid cultures of *Vibrio* sp *(V.alginolyticus)* by the method. In this study, the *Vibrio* species were isolated from fishes and crustaceans procured from local market in Kanchipuram District. Cultural, biochemical and other phenotypic characteristics of *Vibrio* species studied in previous studies. Among the *Vibrio* species, *V.alginolyticus* was found as most prevalence pathogens in all collected sea foods. In this present study, the identified *V.alginolyticus* strains were confirmed by molecular studies. The PCR amplification of 16S ribosomal RNA gene of the bacterial strains were carried out. in Thermal cycler (Gene AMP 2720 – Applied Biosystem). 16s rRNA gene sequencing was carried out using Beckman Coulter CEQ 8000 auto analyzer from the 16S rRNA products. The 16s rRNA sequences of the strains were computationally analysed by BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/).

Keywords: *Vibrio*, PCR amplification, 16s rRNA, BLAST.

Introduction

Fish and seafood constitute an important food component for a large section of world population. They come after meat and poultry as staple animal protein foods where fish forms a cheap source of protein (Wafaa *et al*., 2011). Sea foods are prone to bacterial contamination, especially filter feeders such as mussels and oysters, which concentrate these bacteria in their filtration systems and therefore, are ideally suited to trap all bacteria and viruses, pathogenic or otherwise, that live in the water (Popovic *et al.*, 2010).

Vibrio is a genus of bacteria indigenous to the aquatic environment also contaminant of raw or under cooked seafood. Some bacterial species of this genus are now considered as emerging pathogens, involved in food-borne infections in humans (Gopal *et al*., 2005; China *et al.*, 2003). *Vibrios* are gram-negative bacillus, curved, motile with a single polar flagellum and facultative anaerobic organisms that are natural inhabitants of the marine environments. The species such as *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. mimicus, V. fluvialis, V. furnissii, V. metschnikovii, V. hollisae* and *V. damselaare* are important human pathogens. They account for a significant proportion of human infections such as gastroenteritis usually associated with consumption of raw or under cooked seafood, wound infections, septicaemia and ear infections (Adeleye *et al.*, 2010).

V. alginolyticus is considered one of the most frequent species living freely in water and sediment and can survive in sea water even in famine conditions while

maintaining their virulence (Ben Kahla-Nakbia *et al.*, 2007; Harriague *et al.*, 2008;). Also, it is recognised as a potential reservoir of many known virulence genes of other *Vibrio* species in the aquatic environment which have been demonstrated to contribute to the onset of wound infections, enteric pathologies, septicaemia and peritonitis in humans by exposure to seawater (Masini *et al.*, 2007).

It is one of the main *Vibrio* pathogens affecting marine animals such as marine fish, shrimp and shellfish, causes wound infection, ear infection, gastroenteritis and septicemia, food intoxication, Hemorrhaging and dark skin ulcer from the skin surface (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. *V.alginolytius* is a species with a broad geographical distribution in marine and estuarine waters especially in bathing areas (Larsen *et al.*, 1981).

Molecular approaches that interrogate the whole genome appears to be a way forward to highlight what may be only minimal differences between strains. PCR method has been widely used in the development of molecular diagnostic techniques for bacteria, because it allows a comparative analysis of genomes between different isolates of the same species by employing distinct molecular markers (Sudeesh *et al.*, 2002). The aim of this study is to investigate the molecular characterisation of *V. alginolyticus* strains to resolve taxonomic ambiguities by 16rRNA PCR amplification and its gene sequences.

Materials and Methods

Extraction of Chromosomal DNA

The chromosomal DNA was isolated from the fresh liquid cultures of *Vibrio sp (V.alginolyticus)* by the method followed by Troyer *et al.* (1990). The bacterial cells were lysed and proteins were removed by digestion with proteinase K. Cell wall debris, polysaccharides and remaining proteins were removed by selective precipitation with N-cetyl-N,N,N-trimethylammonium bromide (CTAB), and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation. The collected DNA was air dried and resuspended in distilled water. The quantity of isolated DNA was confirmed by Agarose gel electrophoresis.

Agarose gel Electrophoresis

Agarose electrophoresis was performed in a horizontal sub-marine apparatus (Medox, India). Agarose (1.5%) was melted with TE buffer and 2µl of ethidium bromide was added at the concentration of 4 mg/ml. TE buffer was used as the tank buffer and electrophoresis was carried out for 30 minutes at constant voltage. The gel was visualized under UV transilluminator and photographed.

PCR amplification

The PCR amplification of 16S ribosomal RNA gene of the bacterial strains were carried out based on the methodology of Coenye *et al*. (1999) in Thermal cycler (Gene AMP 2720 – Applied Biosystem).

Primers used

Forward: 5’ AGAGTTTGATCCTGGCTGAG 3’
Reverse: 5’ AAGGAGGTGATCCAGCCGCA 3’

Reaction mixer

Sterile water	-	38µl
10x assay buffer	-	5µl
dNTPs mix (10mM each)	-	3µl
Template DNA (20-30ng)	-	1µl
Forward primer (100µM)	-	1µl
Reverse primer (100µM)	-	1µl
Taq poly (1U)	-	1µl

Cycling conditions

Cycle 1: 94°C for 10 minutes for denaturation
Cycle 2: (30 Repeats)

Step-1: 94°C for 60 seconds for denaturation
Step-2: 60°C for 60 seconds for annealing the primer
Step-3: 72°C for 60 seconds for chain extension
Cycle 3: 72°C for 5 minutes final extension

Gene Sequencing

16s rRNA gene sequencing was carried out using Beckman Coulter CEQ 8000 auto analyzer from the 16S rRNA products. The amplified products were cleaned up using QIAQuick (Qiagen) Spin column. The cycle sequencing was carried out using DTCS quick start Dye terminator kit (Beckman Coulter).

Reaction mixture: (20µl)

Sterile water (Sigam Aldrich)	-	5µl
Template DNA (10ng)	-	2µl

Sequencing primer (1µM) - 5µl
DTCS master mix - 8µl

Reaction conditions: (25 cycles)

96°C for 10 seconds for denaturation.
55°C for 10 seconds for primer annealing
60°C for 240 seconds for polymerization.

The removal of unbound dye and nucleotide from cycle sequenced product was carried out using Dye Ex spin columns (Qiagen). The purified samples were sequenced by Beckman Coulter CEQ8000 sequencer.

Sequence analysis

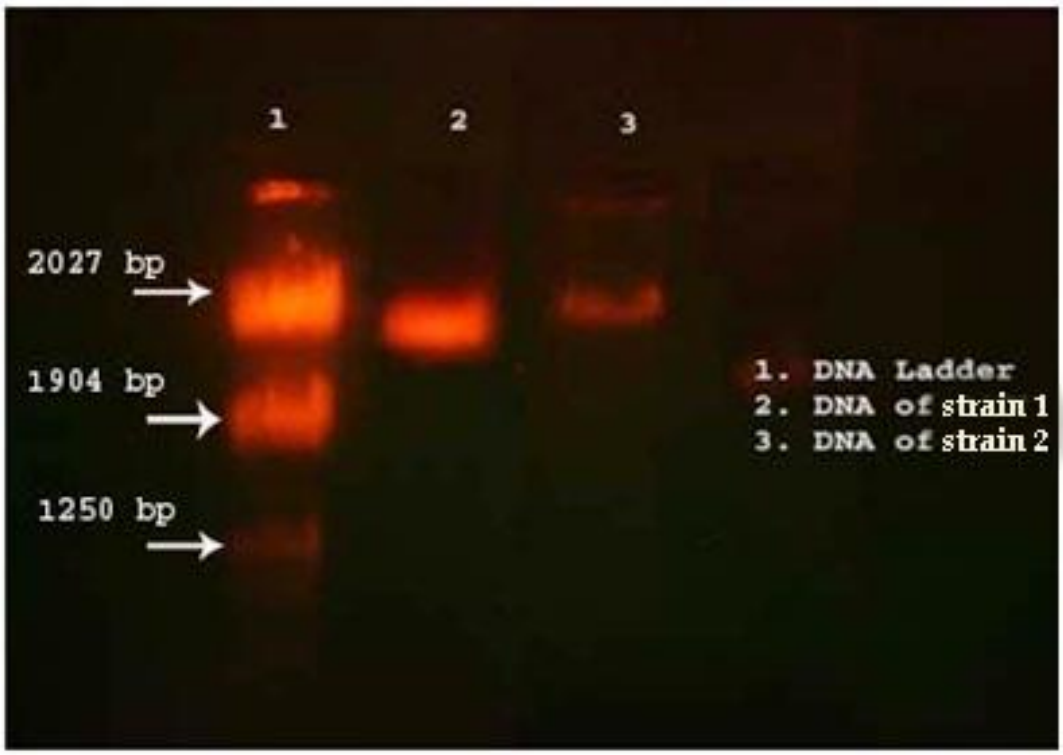
The 16s rRNA sequences of the strains were computationally analysed by BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/). An aligned sequence in FASTA format was taken for this analysis. The BLAST tool was run after uploading of particular sequence into the BLAST tool at a required place. After completion the analysis, the tool showed a list of sequences which are closely matched with the test sequence, the table was carefully observed.

Results

Isolation of chromosomal DNA

The chromosomal DNA of *V.alginolyticus* strains was isolated and the quantity of DNA was confirmed by agarose gel electrophoresis. In the present study, 1.5% agarose was used for electrophoresis. The electric migration of DNA into the agarose gel was visualized under UV transillumination (Figure 1).

Figure 1. Isolated DNA from samples on agarose gel



PCR amplification and gene sequencing

The PCR amplification of 16S ribosomal RNA gene was carried out using Thermal cycler (Gene AMP 2720 – Applied Biosystem) and sequenced by Beckman *CEQ 8000* DNA Sequencer. The 16S ribosomal RNA gene sequences of strain1 and 2 were as follows,

16s rRNA sequence of strain 1

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAA
CGAGTTATCTGAACCTTCGGGGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGAT
GTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTT
CGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCG
ACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA
AGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGYRGTRKWGTTAATAGCYSYATYRTTTGAC
GTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA

16s rRNA sequence of strain 2

TGGAGAGTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACG
AGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAAT
TGCCCTGATGTGGGGAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCAAAGAGGGGG
ACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGTGAGGTAAGGGCTCACCAA
GGCGACCCTAGCGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC
CTTCGGGTTGTAAGCACTTTCAGTCGTGAGGAAGGYRGTRKWGTTAATAGCYSYATYRTTTGACGTTAG
CGACAGAAGAGCACCGGCTTCCGTGCCAGCAGCCGCGTA

Sequence analysis

Nucleotide Sequence of strain 1 contained totally 528 letters of nucleotides. It was analyzed by similarity searching tool BLAST – Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov) using the Program BLASTN 2.2.27. In this analysis the test sequence showed maximum similarity to *Vibrio alginolyticus*. The sequences producing more similarity have been shown (Table 1).

Table 1. Sequences producing significant alignments

Sequences producing significant alignments:

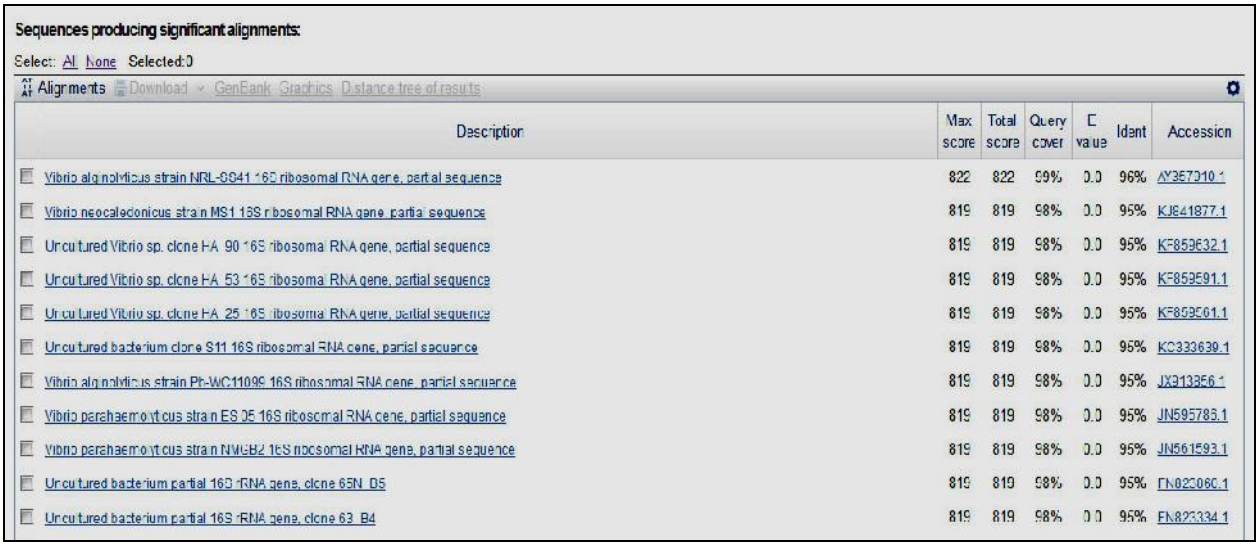
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Vibrio alginolyticus strain NRL-SS41 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	98%	AY357910.1
Vibrio alginolyticus strain NRL-CB9 16S ribosomal RNA gene, partial sequence	893	893	100%	0.0	97%	AY357894.1
Vibrio neocaledonicus strain MS1 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	KJ841877.1
Uncultured bacterium clone S11 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	KC333639.1
Vibrio parahaemolyticus strain ES 05 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	JN595786.1
Vibrio parahaemolyticus strain NWGB2 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	JN561593.1
Uncultured bacterium partial 16S rRNA gene, clone 65N_E5	867	867	99%	0.0	96%	FN823660.1
Uncultured bacterium partial 16S rRNA gene, clone 63_D12	867	867	99%	0.0	96%	FN823061.1
Uncultured Vibrio sp. 16S rRNA gene, clone HG331	867	867	99%	0.0	96%	FN545745.1
Uncultured Vibrio sp. 'Artemia' 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	AF108137.3
Vibrio parahaemolyticus strain CM12 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	EU660325.1
Vibrio natriegens strain CM3 16S ribosomal RNA gene, partial sequence	867	867	99%	0.0	96%	EU660320.1

Nucleotide Sequence of strain 2 contained totally 517 letters of nucleotides. It was analyzed by similarity searching tool BLAST – Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov) using the Program BLASTN 2.2.27. In this analysis the test sequence showed maximum similarity to *Vibrio alginolyticus*. The sequences producing more similarity have been shown (Table 2).

Table 2. Sequences producing significant alignments



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Vibrio alginolyticus strain NRL-SS41 16S ribosomal RNA gene, partial sequence	822	822	99%	0.0	96%	AY357010.1
Vibrio neocaledonicus strain MS1 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	KJ641877.1
Uncultured Vibrio sp. clone HA_90 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	KF859632.1
Uncultured Vibrio sp. clone HA_53 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	KF859591.1
Uncultured Vibrio sp. clone HA_25 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	KF859561.1
Uncultured bacterium clone S11 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	KC333639.1
Vibrio alginolyticus strain Pb-WC11099 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	JX913356.1
Vibrio parahaemolyticus strain ES 05 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	JN595783.1
Vibrio parahaemolyticus strain NWGB2 16S ribosomal RNA gene, partial sequence	819	819	98%	0.0	95%	JN561593.1
Uncultured bacterium partial 16S rRNA gene, clone 65N_D5	819	819	98%	0.0	95%	FN822060.1
Uncultured bacterium partial 16S rRNA gene, clone 63_B4	819	819	98%	0.0	95%	FN822334.1

Discussion

In this study, the *Vibrio* species were isolated from fishes and crustaceans procured from local market in Kanchipuram District. Cultural, biochemical and other phenotypic characteristics of *Vibrio* species studied in previous studies. Among the *Vibrio* species, *V.alginolyticus* was found as most prevalence pathogens in all collected sea foods. In this present study, the identified *V.alginolyticus* strains were confirmed by molecular studies.

Methods of bacterial identification can be broadly delimited into genotypic techniques based on profiling an organism's genetic material and phenotypic techniques based on profiling either an organism's metabolic attributes or some aspect of its chemical composition (Liu and Stahl, 2007). Genotypic techniques have the advantage over phenotypic methods that they are independent of the physiological state of an organism; they are not influenced by the composition of the growth medium or by the organism's phase of growth. Phenotypic techniques, however, can yield more direct functional information that reveals what metabolic activities are taking place to aid the survival, growth, and development of the organism (Logue *et al.*, 2008).

In this study, the chromosomal DNA of *V.alginolyticus* was isolated by the method followed by Troyer *et al.* (1990). A good quantity of DNA was isolated and it was confirmed by Agarose gel electrophoresis. Followed by PCR amplification of 16S ribosomal RNA gene of the bacterial strains was carried out based on the methodology of Coenye *et al*. (1999). The obtained PCR products were subjected to nucleotide sequencing using Beckman Coulter CEQ 8000 auto analyzer and the obtained 16S rRNA sequences were computationally analysed by BLAST – Basic Local Alignment Search Tool. In this analysis the test sequences showed maximum similarity to *Vibrio alginolyticus*. Hence, the strains were confrmed as *V. alginolyticus*.

Vibrio alginolyticus was the major specie isolated from the mussels analyzed showing the relevance to marine ecosystem particularly when associated to the risks for human health (handlers, fisheries, swimmers and seafood consumers) after exposure to aquatic environment. Moreover, the high incidence of this pathogen has been related to gastrointestinal and extraintestinal infections as otitis and cutaneous lesions in humans and animals. This epidemiological aspect is relevant especially to food chain and aquaculture professionals (Carli *et al*., 1993). Virulence genes homologous to *V. parahaemolyticus* and *V. cholerae* virulence determinants are widely distributed among some Vibrio species (*V. parahaemolyticus, V. cholera* non-O1, *V. mimicus, V. hollisae, V. fluvialis and V. alginolyticus*) (Sechi *et al*., 2000).

The uses of molecular technique for the confirmation of *Vibrio* species are useful for knowing the presence of a target *Vibrio* species in a sample. This technique is highly useful for preventive detection. Also, it is an efficient and reliable tool for the food industry.

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