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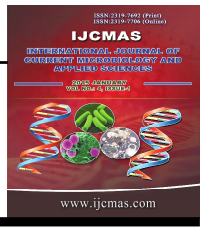

Original Research Article

Isolation of *Vibrio parahaemolyticus* from the gut of Octopus (*Octopus spp.*) and mussel (*Perna indica*)

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Seafood, Microbial contamination, tdh gene, Musscle and Octopus

A B S T R A C T

Over exploration of the population is main reason for food scarcity. Seafood play key role in the world is any form of sea life regarded as food by humans. Seafood prominently includes fish and shellfish. Shellfish include various species of molluscs, crustaceans, and echinoderms. All the seafood's are main harbours for various kinds of microbes among this filter feeding animals like mussels are the main source of the microbial contamination. Among all microbes Vibrios are the dominant in seafood. In the present study is investigated on Isolation of *Vibrio parahaemolyticus* from the Gut of Octopus (*Octopus spp*.), Mussel (*Perna indica*) and also detection of tdh gene. For this study nearly 20 sea food samples are analysed for microbes but the among the 2 species such as Octopus (*Octopus spp*.) and Mussel (*Perna indica*) were identified with contamination of *Vibrio parahaemolyticus* and no tdh gene is present so, the identified *Vibrio parahaemolyticus* is non-pathogenic.

Introduction

Seafood constitutes an important food component for a large section of world Population. They come after meat and poultry as staple animal protein. Sea food are prone to bacterial contamination, especially in filter feeding bivalves such as mussel and oysters which constitute a filtration system and, therefore, are ideally suited to trap all bacteria and viruses (Adebayo- Tayo 2011). They are also found in invertebrates like Octopus, Squids, Cuttlefish, Crabs, Shrimp, Lobsters etc (Adeleye et al., 2010).

Vibrios are most commonly associated with sea food as natural contaminants. They are Gram-negative, facultative anaerobic, motile, curved rods with a single polar flagellum. Among the members of this genus, 12 species have been reported pathogenic to humans and 8 of them are associated with food borne infection of the gastro intestinal tract. Most of the foods borne infections are caused by *V. cholerae, V. parahaemolyticus, V. vulnificus*(Oliver and Kaper, 1997; Dalsgaard, 1998), *V. alginolyticus, V. mimicus, V. fluvalis*and *V.*

damsela.

Most of the *Vibrios* secrete enterotoxins in food or in the gastrointestinal tract. The level of Vibrio in raw seafood was found to range from 50 to 10 CFU/g. *V. alginolyticus*is one of the most frequently found species, followed by *V. parahaemolyticus, V. cholerae, V. mimicus, and V. vulnificus*, in that order. Processed and ready-to-eat products are contaminated with at least one of the potentially pathogenic *Vibrios* at significant frequencies (25 and 17.5 % of samples, respectively), with the level as high as 103 to 104 per gram in some samples (Thararat et al., 2009).

Vibrio parahaemolyticus

V. parahaemolyticus was first identified as a food borne pathogen in Japan in 1950s. By late 1960s and early 1970s, *V. parahaemolyticus* was recognized as a cause of diarrheal disease worldwide, although most common in Asia and the United States. However, not all environmental strains are considered pathogenic, producing a Thermo-stable Direct Haemolysin (TDH) and/or a Thermo-Stable Related Haemolysin (TRH) considered as universal virulence markers (Neelapathi et al., 2011). *V. parahaemolyticus* continues to be the top causative agent among all the reported food poisoning outbreaks in Hong Kong.

The illness caused by *V. parahaemolyticus* food poisoning is a gastroenteritis characterized by watery diarrhoea and abdominal cramps in most cases, along with nausea, vomiting, fever and headache. The incubation period is usually between 12 and 24 hours and the disease usually resolves in three days. The infection is typically acquired through consumption of contaminated seafood, which could be raw or inadequately cooked, or that have been cross-contaminated by improper handling. Low temperature controls during storage favors bacterial proliferation. The total dose of greater than one million may cause the disease and this dose may be markedly lowered by coincidental consumption of antacids or presumably by food with buffering capacity.

Studies carried out in Japan estimated that 2

x10^5 to 3 x 10^7cells have to be ingested for

the indication of the disease. *V. parahaemolyticus* is a slightly halophilic bacterium with optimum growth with NaCl concentrations of 2 to 4% and poor growth is exhibited in media below 0.5% NaCl. The bacterium is inactivated rapidly in distilled water and growth at levels of 10% NaCl is inhibitory. The organism grows at a temperature range between 5 and 43°C, with optimum growth at 37 °C. The optimum pH range for growth is 7.8 to 8.6, although it can grow in the pH range of 4.8 to 11.

Pathogenicity of *V. parahaemolyticus* is caused by many virulence factors such as β-haemolysis, adherence factors, various enzymes and the products of the *tdh, trh, tlh* and *ure*genes. The Pathogenicity of *V. parahaemolyticus* has been associated with Kanagawa phenomenon (KP), the production of TDH (Thermo-stable Direct Haemolysin) which is responsible for the β-haemolysis on wagatsuma agar (Drake et al., 2007). Most of the clinical isolates are KP positive, whereas only 1% to 2% of environmental isolates are KP positive (Drake et al., 2007). *Tdh* is a virulent gene responsible for the production of Thermo-stable Direct Haemolysin (TDH). TDH has been implicated as one of the major virulence factors for the enteropathogenecity of *V. parahaemolyticus* and widely found in pandemic strains (Nelapati 2011). Thus *tdh*

PCR of *V. parahaemolyticus* is suitable for detection of toxigenic strains of *V. parahaemolyticus* that produces Thermo-stable Direct Haemolysin. PCR for the detection of this gene produces a 72 bpamplicon in strains carrying the virulence gene (Ghosh et al. 1998). *Vibrios* are found to play significant role in the degradation of complex materials. Apart from seafood *Vibrios* have been well studied from coastal water, sediment column and in aquaculture products.

Scope of Study

Present study is focused on the isolation and identification of *Vibrio parahaemolyticus* from Mussel (*Perna indica)* and gut of Octopus (*Octopus spp*) of Andaman. Present work also focuses on the Pathogenicity of *Vibrio parahaemolyticus* which is studied by amplifying *tdh* (thermolabile direct haemolysin) gene using Polymerase chain reaction (PCR).

Materials and Methods

Sampling

Sampling was carried out at Sippighat (92O 41′ 58.82″ E, 11O 36′ 33.34″ N) and Science Centre (92O 45′ 24.68″ E, 11O 39′ 17.23″ N) during the low tide (Map). Fresh samples of Mussel *(Perna indica)* and Octopus *(Octopus sp.)* were collected by hand picking and transferred into sterile Ziploc bags and placed in an ice box. This was brought to the laboratory for further bacteriological examination.

Methodology

A standard methodology was followed for the study. Bacteriological enumeration was carried out within an hour of sampling. Both the samples are washed three times with sterile filtered sea water. The Mussel (*Perna indica)* was opened aseptically, shucked and homogenated in a mortar and pestle. The Octopus *(Octopus spp.)* was dissected, the gut was removed and homogenated with the in mortar and pestle. 1ml of the homogenate from both mussel and octopus were transferred to glass vials containing 9ml of Alkaline peptone water (APW) (Himedia – M618S) containing 1% NaCl adjusted to pH of 8.6 and incubated at room temperature for 3-4 hrs (Elliot, 1998). 100µl of inoculum from APW enrichment was transferred to empty sterile Petri-plate, to this 15ml of Thiosulphate- Citrate -Bile salts- Sucrose Agar medium (TCBS, Himedia- M189) was poured and incubated for 18-24 hrs at 37ºC (Farmer et al 1992). Seven (three from *Perna indica* and four from *Octopus spp.*) individual *Vibrio parahaemolyticus* like colonies which were green in color were picked from both the samples and streaked onto fresh TCBS agar plates and incubated at 37ºC for 18-24 hrs.

Sucrose fermenting colonies showed yellow whereas non fermenting colonies were green in colour. Well isolated green colonies were picked and each colony was divided into two parts. One part was streaked on to a non-selective media (Nutrient Agar with 1% NaCl) and the other part was stabbed and streaked on the slant of TSI (Triple sugar Iron agar) tube as a test for biochemical confirmation of *Vibrio parahaemolyticus.* The culture from the non-selective media was used for Gram staining, Oxidase and String tests.

Gram Staining (Farmer 1992)

Gram staining is a method to differentiate Gram positive and Gram Negative bacteria based on cell wall components. Gram staining was done using Himedia Gram Stains- KIT (K001).

Oxidase Test (Farmer 1992)

This is one of the cost efficient methods for the detection of *Vibrio*s. 1% Oxidase reagent was prepared by dissolving 10mg of N, N, N, N- tetramethylparaphenylenediamine (TEMED) (Sigma) in 1ml distilled water (10mg/ml) and wrapped with aluminum foil to protect from light. A Sterile filter paper strip was placed on a glass slide and few drops of oxidase reagent was added and soaked. Growth from the non-selective media plate (1% NA) was picked with sterile tooth pick and rubbed on the filter paper strip. O*+xidase positive colonies turn purple within 10-30 seconds or at a maximum of one minute.

String Test (Farmer 1992)

String test is one of the valuable test to differentiate and detect *Vibrio* from *non - Vibrios*. Most *Vibrios* are string positive, but many other species like *Aeromonas*, are negative. Sodium deoxycholate (String reagent) is a detergent that lyses Gram negative organisms. When cells are lysed, DNA is released into the suspending medium, making it very viscous and able to form "strings of DNA" when touched with a loop that is raised from the surface of the liquid. Growth from the non-selective media plate (1% NA) was picked by a tooth pick and made into a heavy suspension in one drop of 0.5% sodium deoxycholate solution (5g of Sodium deoxycholic acid – Sigma, in 1 litre of distilled water). Every ten seconds the tooth pick is raised to observe if a "string of DNA" has been formed. String positive colonies become viscous and string of DNA was obvious within 60 seconds. If no string was formed it infers string test negative.

Triple Sugar Iron Test (Farmer 1992)

Triple Sugar Iron Test (TSI) is used to detect bacterial ability to ferment Lactose, Sucrose and Glucose and their ability to produce H2S. TSI is a protein medium that contains the following sugars: 1% lactose, 1% sucrose and 0.1% glucose. When glucose is used, the agar turns to yellow (acid). After continued incubation due to decrease in glucose concentration the slant turns alkaline (Red), with butt remaining acid (Yellow) which results in an alkaline over acid reaction (K/A). When lactose and /or sucrose are used, the entire slant will turn yellow (acid) giving an acid over acid reaction (A/A). If these two sugars are not used, the entire slant appears red (alkaline), yielding an alkaline over alkaline reaction (K/K). *Vibrio parahaemolyticus* is known to produce an alkaline-over-acid reaction (K/A) in TSI (Warburton, 2006). As mentioned earlier, half of the green colony is stabbed and streaked on a TSI agar slant (Difco) and incubated for 18-24hrs at 37ºC. Positive results are observed with K/A reaction.

DNA Extraction by Phenol Chloroform method (Murray and Thompson 1980)

3 ml of broth culture was transferred into a microfuge tube containing 1ml of High TE Buffer, centrifuged at 10,000 rpm at 15ºC for 10 minutes. Supernatant was removed and the pellets were washed three times with normal saline. 400µl of TE buffer along with 40µl of lysozyme was added, vortexed and incubated at 37oC for 2 hours. After incubation 56µl of SDS followed by 5µl of proteinase K was added and incubated at 65o C for 3 minutes in water bath. The microfuge tubes were removed from water bath to which 80 µl of 5 M NaCl and 64 µl of CTAB was added, vortexed and again incubated at 65o C for 30 minutes in water bath. After incubation equal amount of chloroform and isoamyl alcohol (24:1) was

added, shaken slowly for 10 minutes and centrifuged at 10,000 rpm for 15 minutes. The aqueous layer (supernatant) was carefully pipetted and transferred to another microfuge tube to which 1ml of absolute alcohol (molecular Biology Grade 99.99%) was added and the tube was placed at -20oC for 1 hour for the DNA to precipitate. After 1 hour, this was centrifuged at 12000 rpm for 10 minutes at 4oC and the supernatant was discarded. Pellets were washed with 1 ml of 70% ethanol and centrifuged for 10 minutes at 12000 rpm at 4oC. Ethanol from the tube was discarded and resuspended with TE buffer (10mM of Tris pH 8.0 and 1mM of EDTA), treated with 30µg mL-1 of RNAse and incubated in water bath for 40 minutes at 37oC.

Amplification of *tdh* gene using Polymerase Chain Reaction to study pathogenicity

Tdh is a virulence gene responsible for the production of Thermo-stable Direct Haemolysin (TDH). *Tdh* PCR of *V. parahaemolyticus* is suitable for detection of toxigenic strains of *V. parahaemolyticus* that produces thermo stable direct haemolysin (Tada, et al., 1992). The presence of a 72 bp fragment of *tdh* amplicon was tested by PCR assay using primer pairs 5'- CCA TCT GTC CCT TTT CCT GC – 3' (sense); 5'- CCA AAT ACA TTT TAC TTG G -3' (antisense). TDH has been implicated as one of the major virulence factors for the entero-pathogenicity of *V. parahaemolyticus* and widely found in pandemic strains.

The *tdh* gene was amplified by PCR using primer. PCR amplification carried out in total volume of 50µl containing 2µl of template DNA, 1µl of Taq-DNA polymerase, 5µl of buffer with MgCl, 1µl of dNTP mix, 1µl of *tdh* forward primer, 1µl of reverse primer and made up to a final volume of 50µl using 39µl of double-12 sterilized distilled water (Ghosh et al., 1998). Amplification was carried out in Thermal cycler (Applied Biosystems).

PCR conditions were as follow: initial denaturation at 94oC for 5 min followed by 25 cycles of denaturation at 94o C for 90 sec, annealing at 50o C for 90 sec, elongation at 72o C for 90 sec and final extension at 72o C for 10 min. The amplified DNA fragments were separated on 1% Agarose gel (Ghosh et al., 1998). The gel was observed in Gel Documentation System (GelDoc, Biorad).

Results and Discussion

Seven isolates of *Vibrio parahaemolyticus like organisms (VPLO)* which showed green colored colonies on TCBS agar medium were obtained. Out of these seven isolates, three were from *Perna indica* and four were from *Octopus* spp. gut. The results obtained are tabulated (Table 1 and 2) and all the seven isolates were stabbed in TSI slants and the results are tabulated (Table 3)

Sucrose fermenting bacteria formed Yellow colored colonies on TCBS agar plate due to the production of acid which is identified by the pH indicator Bromothymol blue and thymol blue. Whereas Sucrose non-fermenting bacteria formed Green colored colonies. Results showed that all the seven isolates were non sucrose fermenters and all of them were gram negative. On subjecting to Oxidase and String tests it was confirmed that all these isolates were Vibrios and further TSI test showed that three had given K/A reaction (Alkaline slant over acidic butt) on TSI slant. These results confirmed that the three isolates (SGMGb, OGUb, OGUc) are Vibrio parahaemolyticus. Further, these three V. parahaemolyticus isolates were screened for pathogenicity using tdh PCR. The PCR results (Fig.2)

showed the absence of tdh gene, thus the isolates were non-pathogenic.

In conclusion the present work aimed to study the isolation and identification of Vibrio parahaemolyticus isolated from Mussel (Perna indica) collected from Sippighat and the gut of Octopus (Octopus spp.) collected from science center. Samples were dissected and seven isolates of Vibrios were obtained. Out of seven three isolates obtained from mussel homogenate and four from Octopus gut .Gram staining showed that the seven isolates were Gram negative. These seven isolates were subjected to biochemical tests such as Oxidase test and String test which confirmed them as Vibrios. Through Triple Sugar Iron (TSI) test three isolates (SGMGb, OGUb and OGUc) were confirmed as Vibrio parahaemolyticus. After biochemical tests the isolates were screened for pathogenicity (presence of tdh gene) using tdh PCR. The amplified PCR product was run in a gel to detect the band of desirable gene. In gel electrophoresis desirable gene band was not found, which finally concluded that the three Vibrio parahaemolyticus isolates (SGMGb, OGUb and OGUc) were tdh negative and hence non-pathogenic. The occurrence of non-pathogenic Vibrio parahaemolyticus isolated from two main food samples of the marine ecosystem shows that the waters of these places are free from this particular pathogenic micro-organism.

Table.1 Results of colony growth on TCBS and 1% NA and Gram staining.

S.NO	Sample	TCBS	1 %NA	Gram staining
1	SGMGa	G	+	-
2	SGMGb	G	+	-
3	SGMGc	G	+	-
4	OGUa	G	+	-
5	OGUb	G	+	-
6	OGUc	G	+	-
7	OGUd	G	+	-

SGMG Perna indica; OGU: - Octopus spp. Gut.
G: green; +: Positive; - : Negative.

Table.2 Details of Oxidase and String Test

S.NO	Sample	Oxidase test	String test
1	SGMGa	+	+
2	SGMGb	+	+
3	SGMGc	+	+
4	OGUa	+	+
5	OGUb	+	+
6	OGUc	+	+
7	OGUd	+	+

SGMG Perna indica; OGU Octopus spp. gut; +: Positive

Table.3 Triple Sugar Iron (TSI) test

S.NO	Sample	TSI
1	SGMGa	A / A
2	SGMGb	K / A
3	SGMGc	Black ppt
4	OGUa	Black ppt
5	OGUb	K / A
6	OGUc	K / A
7	OGUd	A / A

A / A = Acid slant / Acid butt; K / A = Alkali slant / Acid butt.
K= Alkaline (Pink); A= Acid (Yellow).

Fig.1 Map showing sampling sites

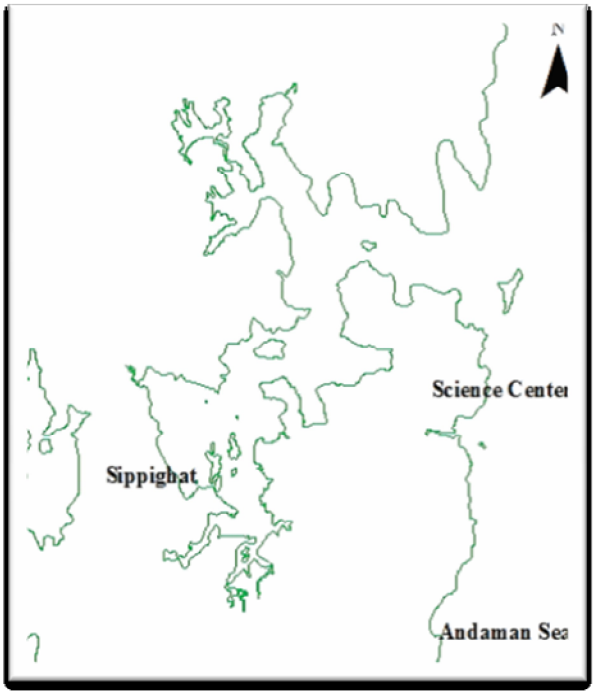
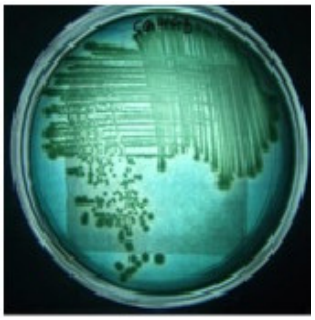


Plate.1 Samples

***Perna indica* from Sippighat**

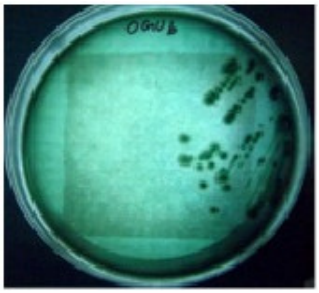
Octopus from Science centre (*Octopus spp.)*

Growth ON TCBS Medium

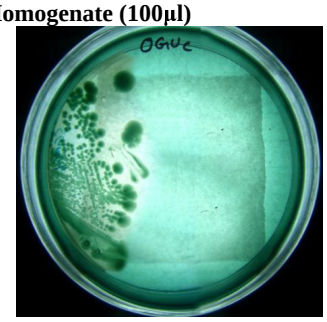


SGMGb

Perna indica Gut Homogenate (100µl)

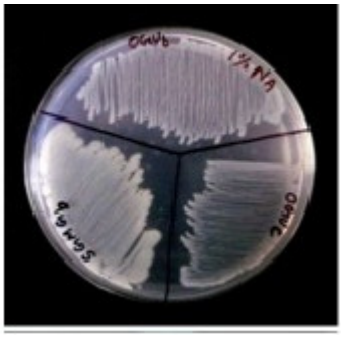


OGUb

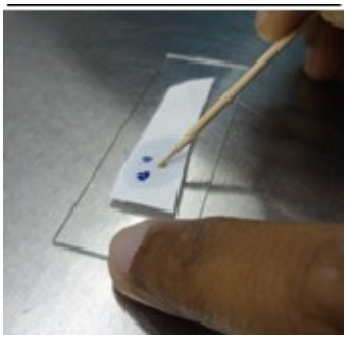


OGUc

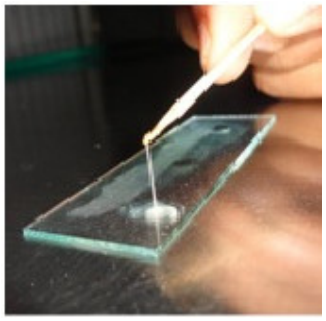
Octopus spp. Gut Homogenate (100µl)



Growth on Nutrient Agar with 1% NaCl



Oxidase Test



String Test (A String of DNA)

Triple Sugar Iron Test (TSI)

Control SGMGb (K/A)

Control OGUb (K/A)

Control OGUc (K/A)

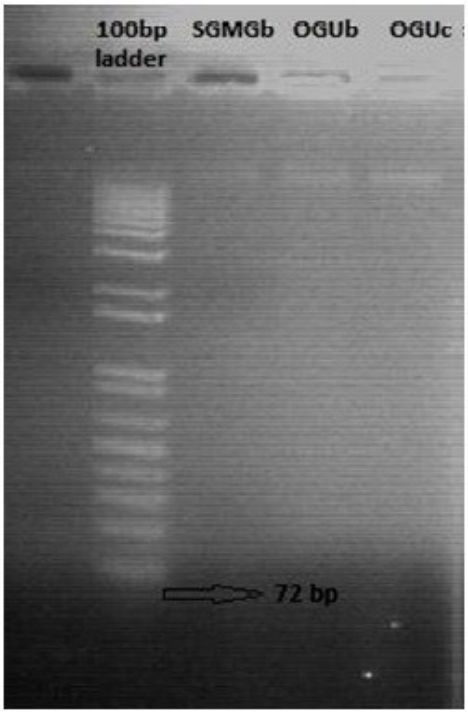


Fig.2 Geldoc image of tdh PCR.

References

Adebayo- Tayoet, B.C, I.O. Okonko, C.U. Esen, N.N. Odu, C.C. Onoh and N.J.P. Igwiloh, 2011. Incidence of potentially pathogenic Vibrio Spp. In fresh seafood from Itu creek in Uyo, AkawaIbom state, Nigeria. *World Applied Sciences Journal*.Vol 15: 985-991.

Adeleye, I.A, F.V Daniels and V.A Enyinnia, 2010.Characterization and Pathogenicity of Vibrio Spp. Contaminating Seafood, In Lagos, Nigeria.*Internet Journal of Food Safety*.Vol 12: 1-9.

Elliot, E.L., C.A. Kaysner, L. Jackson and M.L. Tampli, 1998.Vibrio cholera, V. parahaemolyticus, V. vulnificus, and other Vibrio spp.In Bacteriological Analytical Manual, 8th Edition, and Revision A, 1998.Chapter 9.Published by AOAC International, Gaithersburg.

Farmer, J.J. and F.W. Hickman, 1992.The Family Vibrionaceae the Prokaryotes, 2nd Edition.Vol 3: 2939-3000.

Ghosh, A.R. and S.C. Sehgal, 1998.Haemolysin production by environmental isolates of Vibrio parahaemolyticus from Andamans. *Indian Journal of Medical Research.*Vol 107: 151-154.

Go´mez-Leo´n, J., L. Villamil, M.L. Lemos, B. Novoa and A. Figueras, 2005. Isolation of Vibrio alginolyticus and Vibrio splendidus from Aquacultured Carpet Shell Clam (Ruditapesdecussatus) Larvae associated with mass mortalities. *Applied and Environmental Microbiology* .Vol 71: 98–104.

Nelapati, S., K. Nelapati and B K. Chinnam, 2012. Vibrio parahaemolyticus- An emerging food-borne pathogen-A Review.Veterinary World.Vol 5: 48-62.

Rajapandiyan, S., K. Sudha, Kanth and D. Arunachalam, 2008. Prevalence and distribution of Vibrio vulnificus in fishes caught off Chennai, Indian Ocean. *African Journal of Microbiology Research.*Vol 3: 622-625.

Thararat Chitov, SutipongWongdao, WarayutThatum,TananchanokPuprae, and Pranee Sisuwan, 2008.Occurrence of potentially pathogenic Vibrio species in raw, processed and ready-to-eat seafood and seafood products.Maejo*Int. J. Sci. Technol.* Vol. 3: 88-98.