Prevalence of *Vibrio cholerae* and Other Vibrios from Environmental and Seafood Sources, Tamil Nadu, India

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Authors’ contributions

This work was carried out in collaboration between all authors. Author KS designed, coordinated this work and finalized the manuscript for publication. Author AB participated in seafood and environmental sample collection, isolation, identification, confirmation of vibrios and wrote the first draft of the manuscript. Author SDK managed the literature searches. All the authors read and approved the final manuscript.

ABSTRACT

**Aims:** The aim of this study was to investigate the prevalence of diarrhoea causing human pathogen *V. cholerae* and other vibrios from different environmental and seafood samples in Tamil Nadu, India.

**Place and Duration of Study:** Laboratory of Clinical Microbiology, Department of Bio-Medical Science, School of Basic Medical Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India between 2012 and 2013.

**Methodology:** Seafood, water and plankton samples were collected at different locations of Tamil Nadu, India. All the samples were primarily enriched with alkaline peptone water (APW). 2-3 loopful of overnight cultures were streaked onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plates. Suspected *Vibrio cholerae*, *V. parahaemolyticus* and other vibrios were picked up and identified by using standard biochemical and serological characterization and also by molecular methods.

**Results:** Among the various samples that includes freshwater, coastal water, plankton and various seafoods, only plankton and seafood samples were found to be harbored with *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis*. The remaining samples were negative for...
vibrios. All *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis* strains possessed outer membrane protein W (*ompW*), thermostable direct haemolysin (*tdh*) and toxin regulatory protein (*toxR*) gene respectively. Hemolytic activity of *V. cholerae* exhibited different reaction isolated from seafood and plankton. The median lethal dose (LD<sub>50</sub>) of some *V. cholerae* strains was generally high.

**Conclusion:** The result of the study suggested that the seafoods may act as an important reservoir of pathogenic vibrios and pose threat to human health.

**Keywords:** *V. cholerae*; *V. parahaemolyticus*; *V. fluvialis*; Diarrhoea, Hemolytic; LD<sub>50</sub>.

### 1. INTRODUCTION

*Vibrio* spp. are gram negative aquatic bacteria that are widely distributed in sea and brackish water environments, both as free-living organisms and bound to a variety of substrates, including suspended mineral particulates, plants and the exoskeleton of zooplankton [1,2]. Only a few species, including *Vibrio cholerae, V. parahaemolyticus, V. mimicus, V. vulnificus*, and *V. fluvialis* have been linked to human food-borne infections. The diarrhoeal disease, cholera, which has caused epidemics and pandemics, continues to be a global threat to public health and is caused by infection with *V. cholerae* [3]. Every year millions of cholera episodes occur throughout the world especially in developing countries and thousands of cases are reported to be fatal [4]. In 2011, WHO reported 589,854 cholera cases from 58 countries with death rate increased from 7543 to 7816 [5]. Based on the biotyping, phage-typing and serotyping, *V. cholerae* has been classified into different serogroups, of which only the serogroups O1 and O139 are known to cause cholera [6]. In 1993, *V. cholerae* O139 came into the lime light by causing explosive cholera epidemics in the Indian Sub-continent [7].

Clinical strains of *V. cholerae*, associated with pandemic and epidemic human cholera, produce a potent enterotoxin, encoded by the *ctxAB* locus in combination with central regulatory gene *toxR* [8]. However, the ability of pathogenic vibrios to cause disease depends on the expression of various virulence-factors like toxin-coregulated pilus (*tcpA*), outer membrane protein (*ompU*), haemolysin (*hlyA*), Zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), heat stable enterotoxin (Nag - ST-stn) and Type III secretion system (TTSS) [9-11]. It is universal conviction that most of the isolates from environmental sources do not carry cholera toxin (CT). However, many experimental studies have proven that non-toxigenic environmental isolates have the ability to agglutinate with O1 antiserum. As such, even though these isolates are non-toxigenic, they still have the ability to cause mild diarrhoea. Many food-borne disease outbreaks attributed to environmental strains have involved isolates that were negative for *ctxAB* but possessed heat-stable enterotoxin (*stn*) gene and TTSS, which determines the virulence of particular non-O1 and non-O139 *V. cholerae* [12].

A number of non-O1 and non-O139 *V. cholerae* isolated from environmental water samples have been demonstrated to cause fluid accumulation in rabbit and colonization in mice despite the absence of genes encoding *CT* and *TCP* [3]. Hence, it is pertinent to differentiate pathogenic and non-pathogenic *Vibrio* strains, since most of the non-toxigenic vibrios may exist as a part of the normal bacterial flora of estuarine and coastal water. Colonization of vibrios in plankton (copepods) has been well documented and acts as an aquatic reservoir of *V. cholerae*. Outbreaks of cholera attributed to seafood have been reported in many countries, including the Philippines, Latin America and, recently, Haiti [13].
contaminated with these pathogenic vibrios not only plays an important role in the transmission of diseases but also act as a reservoir in the marine realms. Hence, it is necessary to survey the presence of toxigenic and non-toxigenic vibrios from various sea foods and their environment. The intent of the present study was to isolate and to assess the vibrios in the aquatic environment and seafood samples along the South East Coast of Tamil Nadu, India.

2. MATERIALS AND METHODS

2.1 Environmental and Seafood Samples

A total of 840 samples were collected from different environmental and sea food sources. Seafood samples were collected from different landing centers along the Tamil Nadu coast and also from fish market. All the seafood samples were transferred into new polythene bags and sealed tightly. Fifteen fresh water samples (pond and river water) and fourteen coastal water samples (Mimisal, Mandapam and Rameswaram coasts) were also collected 5 cm below the surface water along the South East Coast of Tamil Nadu, India. Plankton samples were also obtained by towing autoclaved horizontal – tow plankton net (Bolting silk No.20, mesh size 0.076 mm) for 15 minutes at the subsurface. Water and plankton were transferred into sterile polyethylene bottles after collection. Utmost care was taken for all the samples to avoid cross contamination. All the samples were transported to the laboratory in an ice chest (4°C) and bacteriological examinations were made immediately [14].

2.2 Isolation and Identification of Vibrios

Briefly 25 g of seafood samples were homogenized with 225 ml of APW (Hi-Media, India) and incubated for overnight at 37°C. Each 500 to 1000 ml of water sample were concentrated on 0.45µm pore diameter membrane filter and enriched in APW. Similarly plankton (copepods) was also concentrated by centrifugation and transferred into APW and incubated for overnight at 37°C. A Loopful of enriched APW broth culture was streaked on to TCBS, (Hi-Media, India) plates [15]. All the plates were incubated at 37°C for 24 h. Sucrose positive colonies showing yellow and green slightly flattened with opaque centre and translucent peripheries from each plate were subcultured either on trypticase soy agar (TSA) or nutrient agar (NA) (Hi-Media, India) with 1 and 3% NaCl for further biochemical confirmation. All the strains were subjected to a series of biochemical tests [16]. Serological identification was also carried out by agglutination with commercial specific antisera according to the manufacturer’s instructions (Denka, Seiken Corp., Tokyo, Japan) [10].

2.3 Template DNA Preparation

Template DNA preparation was carried out by boiling method [17]. Cultures were grown in APW at 37°C for 24 h and centrifuged at 12,000 RPM for 3 min. Supernatant was carefully removed and the pellet was resuspended with 500 µl of sterile Milli-Q water, boiled at 100°C for 15 minutes and immediately incubated on ice for 10 min. The mixture was then centrifuged at 12,000 RPM for 3 minutes and the supernatant with template DNA were then transferred into sterile tubes and used for PCR amplification.
2.4 Multiplex PCR Assay

Multiplex PCR assay for identification of *V. cholerae* and *V. fluvialis* were carried out for the targeting genes encoding for *ompW* and *tox-R* [17,18]. All PCR reactions were performed in a reaction mixture volume of 30μl. Each 30μl of the reaction mixture contained the following reagent: 3.0μl of 10 x PCR buffer, 2.0μl of 1.24mM dNTP’s, 10 p mol of each primers, 5U of Taq polymerase and 3μl of DNA template (Table 1). The reaction volume was adjusted to 30μl with sterile triple distilled water. The cycling profile was as follows: initial denaturation at 96°C for 4 min and 30 cycles of denaturation at 95°C for 30s, annealing at 66°C for 20s, extension at 72°C for 30s and final extension 72°C for 30s.

2.5 Uniplex PCR Assay

Detection of thermostable direct haemolysin (*tdh*) gene in *V. parahaemolyticus* was performed in a reaction volume of 25µl of the reaction mixture contained the following reagents: 2.5µl of 10 x PCR buffer, 2.0µl of 2.5mM dNTP’s, 10 p Mol of each primer, 5 U of Taq DNA polymerase and 3µl of DNA (Table 1). The reaction volume was adjusted to 25µl using sterile triple distilled water. The cycling profile was as follows: initial denaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 2 min, annealing 50°C for 2 min, extension at 72°C for 30s and final extension at 72°C for 7 min [19].

**Table 1. PCR primers used in the present study**

| S.No | Primers | Primer sequence (5' - 3') | Amplicon |
|------|---------|---------------------------|----------|
| 1.   | *ompw*-F’ | CACCAAGAAGGTGACTTTATTGTG | 588 bp   |
|      | *ompw*-R’ | GAACATTATAACCCCGCG         |          |
| 2.   | VF-F’   | TTgATCgCgACCgTCgAAAgCg     | 321 bp   |
|      | VF-R’   | TggCgTgCTgAACAATCACTCg     |          |
| 3.   | *tdh*- F’ | CCAAATACATTTTACTTGG        | 199 bp   |
|      | *tdh*- R’ | GGTACTAAATGGCTGACATC       |          |

2.6 Gel Electrophoresis

All PCR products were combined with 4µl of loading buffer and 10µl of these mixtures were loaded into a horizontal agarose gel in 1xTAE buffer containing 0.5 µg/ml of ethidium bromide. Electrophoretic separation was done at 100v for 40 min along with 1000 bp PCR ladder as molecular weight markers. The gel was visualized under UV transilluminator and recorded as JPEG file by using a gel documentation system (Gel Doc 2000, Bio-Rad).

2.7 Virulence Studies

*V. cholerae* isolated from different environmental and seafood samples were selected and tested for their hemolysis production and median lethal dose in mice. Ability of *V. cholerae* and *V. fluvialis* strains to produce hemolysin was tested on blood agar supplemented with 5% human or sheep blood and spot inoculations were made on blood agar plates. For *Vibrio parahaemolyticus* Wagatsuma agar (Hi-Media, India) incorporated with blood erythrocytes were used. The zone of hemolysis was read after 18-24 h incubation. Median lethal dose (LD$_{50}$) of the culture was calculated by the technique of Reed and Muench [20]. Briefly, selected *V. cholerae* strains activated by growing in trypsinase soy broth (TSB) (Hi-Media, India) for 24 h at 37°C were inoculated to brain heart infusion (BHI) broth (Hi-Media, India)
and incubated at 37°C for 24 h. Undiluted and five tenfold dilutions of cultures were prepared. Six mice each weighing about 20-24g, were selected for each dilution and intraperitoneal injections were made with 1 ml of culture. The culture was also diluted and plated in duplicate on TSA plates to know the number of cells per ml of the original culture. The inoculated mice were observed for lethality for every one hour for the first ten hours and then after 24 h.

3. RESULTS

3.1 Prevalence of Vibrios from Marine Realms

Seafoods and various environmental samples were analyzed for the presence of *Vibrio cholerae*, *V. parahaemolyticus* and *V. fluvialis*. Of 840 sample examined, 227 (27.0%) were positive for vibrios (Table 2). Among the environmental samples, plankton sample (copepods) yielded the highest number of suspected vibrios, (80.0%) followed by freshwater (73.3%) and coastal water (64.2%). Among seafood samples, bivalves were found to yield vibrios most frequently (51.8%). This was followed by finfishes (34.0%) and crustaceans (21.4%) collected from fish market. However, the incidence of vibrios was low in finfishes (11.1%) and crustaceans (17.2%) obtained from landing centre (Table 2).

3.2 Biochemical Characterization of the Isolates

A total of 635 *V.cholerae*, 174 *V. parahaemolyticus* and 24 *V. fluvialis* were confirmed through a series of biochemical reactions (Table 3). The distribution of *V. cholerae* was 16 from fresh water, 9 from coastal water and 119 from plankton (copepods) in environmental samples. Among seafood, samples from fish market was found to be associated highest number of *V.cholerae* and it was shared by finfishes (N 225) and crustaceans (N 154), whereas finfishes (N 40),crustaceans (N 29) and bivalves (N 43) from landing centre was found to low in number. *V. parahaemolyticus* was confirmed biochemically from 174 samples, including coastal water (N 3), plankton (N 50), finfishes (N 90), crustaceans (N 24) and bivalves (N 7) from landing centre and fish market. No *V. parahaemolyticus* was obtained from fresh water and crustaceans of landing centre. The distribution of *V. fluvialis* was also found in plankton (N 12) followed by crustaceans (N 6), finfishes (N 5) and bivalves (N 1) from landing centre and fish market. None of *V. fluvialis* was isolated from the coastal water and freshwater samples. All the isolates were biochemically typical with respect to cytochrome C oxidase, string test, oxidation/fermentation reaction in triple sugar Iron (TSI), sulfur reduction-indole motility medium (SIM), aminoacid decarboxylase, and fermentation of different sugars. Some environmental isolates showed variable results in Voges-Proskauer, inositol and mannitol sugars (data not shown). Biochemical results revealed the presence of *V.cholerae* in all samples whereas the *V.parahaemolyticus* and *V.fluvialis* were found to be associated only with seafoods.

3.3 PCR Assay

All the isolates were identified as vibrios through various biochemical test, were confirmed by multiplex PCR targeting *ompW* gene for *V. cholerae* and tox-*R* gene for *V. fluvialis*. *V. parahaemolyticus* were confirmed by *tdh*-gene based uniplex PCR assay. In the present study, only 218 were *V. cholerae*, 69 were *V. parahaemolyticus* and 7 were *V. fluvialis*. (Table 3, Figs. 1 and 2).
3.4 Serological and Virulence Properties

All the isolates were non-agglutinable vibrios (NAG) or non-cholera vibrios. These strains showed no agglutination with either O1 or O139 antiserum. All *V. cholerae* non- O1 which showed hemolysis on blood agar plates and non-hemolytic strains were selected at random and tested for their Median Lethal Dose (LD$_{50}$) on mice and the results are given in Table 4. The LD$_{50}$ values ranged from $2.6 \times 10^5$ to $6.9 \times 10^7$. In hemolytic strains, the values fluctuated between $4.2 \times 10^5$ and $6.9 \times 10^7$.

Table 2. Prevalence and distribution of vibrios isolated from different environmental and seafood samples along the Tamil Nadu Coast between 2012 and 2013

| S.NO | Source (N) | Type of sample examined | No.of positive sample obtained |
|------|------------|-------------------------|--------------------------------|
| 1.   | Fresh water | Pond water              | 5 (73.3%)                      |
|      |             | River water             | 10                             |
| 2.   | Coastal water | Mimal area            | 5 (64.2%)                      |
|      |             | Mandapam area           | 6                              |
|      |             | Rameswaram area         | 3                              |
| 3.   | Plankton | Copepods               | 10 (80.0%)                     |
| 4.   | Landing centre | Finishes              | 224 (11.1%)                    |
|      |             | Crustaceans             | 116 (17.2%)                    |
|      |             | Bivalves*               | 83 (51.8%)                     |
| 5.   | Fish market | Finishes               | 238 (34.0%)                    |
|      |             | Crustaceans             | 140 (21.4%)                    |
|      |             | Total                   | 840 (27.0%)                    |

*Collected fresh specimen in estuarine region only
Numbers in parentheses indicate percentage positive

Table 3. Biochemical and PCR confirmation of isolates from environmental and seafood samples

| S. No | Sample         | No. of strains confirmed by biochemical tests* | No of positive as confirmed in PCR |
|-------|----------------|---------------------------------------------|-----------------------------------|
|       |                | *V. c* | *V. p* | *V. f* | *V. c* | *V. p* | *V. f* |
| 1.    | Fresh water   | 16     | 0      | 0      | 0      | 0      | 0      |
| 2.    | Coastal water | 9      | 3      | 0      | 0      | 0      | 0      |
| 3.    | Plankton      | 119    | 50     | 12     | 8 (6.7%) | 14 (28.0%) | 2 (16.6%) |
|       | Landing centre|        |        |        |        |        |        |
| 4.    | Finishes      | 40     | 2      | 0      | 27 (67.5%) | 0      | 0      |
|       | Crustaceans   | 29     | 0      | 3      | 0      | 0      | 0      |
|       | Bivalves      | 43     | 7      | 1      | 21 (48.8%) | 1 (14.2%) | 0      |
| 5.    | Fish market   | 225    | 88     | 5      | 89 (39.5%) | 40 (45.4%) | 2 (40.0%) |
|       | Crustaceans   | 154    | 24     | 3      | 73 (47.4%) | 14 (58.3%) | 0      |
|       | Total         | 635    | 174    | 24     | 218 (34.3%) | 69 (39.6%) | 7 (29.1%) |

* V. c – *Vibrio cholera*; V. p – *Vibrio parahaemolyticus*; V. f – *Vibrio fluvialis*

*a The biochemical test included oxidase, string test, Triple sugar Iron (TSI), Sulfur reduction –Indole-Motility (SIM), Methyl Red, Voges-Proskauer (Vp), Lysine, Arginine and Ornithine decarboxylase (LAO) and salt tolerance tests
Fig. 1. Multiplex PCR analysis for identification of *V. cholerae* and *V. fluvialis* isolated from seafood samples. Lane M - DNA marker (1000 bp), Lane 1: *Vibrio cholerae* O1 Classical 569B (positive control) Lane 2: *V. fluvialis* IDH 2036 (positive control) Lane 3 – 5: non-O1/non-O139 *V. cholerae* from seafoods (Vc 1, Vc 4 & Vc 26) Lane 6: *V. fluvialis* from copepods (Vf 43), Lane 7 and 8: non-O1/non-O139 *V. cholerae* from seafoods (Vc 67 & Vc 69)

Fig. 2. PCR analysis of *tdh* gene in *V. parahaemolyticus* (199 bp). Lane M: 1000 bp marker, Lane 1: *V. parahaemolyticus* positive control, Lane 2: negative control, Lane 3 – 16: *V. parahaemolyticus* isolated from seafoods

Table 4. Haemolysis and mouse virulence of some strains of *V. cholerae* from seafoods.

| S.No | Strain No. | Hemolytic activity | LD_{50} dose on mice |
|------|------------|--------------------|---------------------|
| 1    | Vc 6       | +                  | 4.5 *10^5           |
| 2    | Vc 19      | -                  | 3.2 *10^6           |
| 3    | Vc 47      | +                  | 4.2 *10^5           |
| 4    | Vc 49      | -                  | No death            |
| 5    | Vc 91      | +                  | 6.9 *10^7           |
| 6    | Vc 131     | +                  | 4.6 *10^8           |
| 7    | Vc 139     | +                  | No death            |
| 8    | Vc 140     | +                  | 6.3 *10^7           |
| 9    | Vc 168     | -                  | No death            |
| 10   | Vc 170     | -                  | 5.0 *10^7           |
| 11   | Vc 175     | +                  | 5.7 *10^7           |
| 12   | Vc 200     | -                  | 2.6 *10^5           |
4. DISCUSSION

In this study, several species of finfishes (Indian Mackerel, sardines, mullets cat fishes, clupeiodes, milk fish, silver bellies, ribbon fishes, therapions, gobids, carngids, gizzard shad, flat fishes and half beaks) crustaceans like shrimp and crabs and bivalves like oyster, clams and mussels collected from both fish market and landing centre were found to harbor more *V. cholerae*. From the available reports it is well known that vibrios are found to be associated with seafoods, evokes immediate concern from public health point of view, as these vibrios get transmitted through consumption of such contaminated seafood. Outbreaks of cholera involving seafood have also been reported in the Philippines and Latin America, some parts in Asia and African regions [21,22]. In our study, a total of 840 samples (15 fresh water, 14 coastal water, 10 plankton, 423 seafoods from landing centre and 378 seafoods from fish market) were analyzed for *V. cholerae* and other human pathogenic vibrios. From the findings, it is evident that seafood from fish market such as finfishes (39.5%) and crustaceans (47.4%) were positive for *V. cholerae* (Table 3). Seafoods were found to be the carriers of *V. cholerae* and the ubiquitous distribution of this pathogen in such seafoods renders it practically impossible to eliminate this pathogen from the marine realms. This in agreement with the findings of Saravanan et al., Ottavani et al. and Karunasagar et al. who reported the *V. cholerae* and other vibrios in various seafood samples and plankton [23, 24]. Similarly, highest percentage of *V. parahaemolyticus* was found in crustaceans (58.3%) and finfishes (45.4%) from fish market followed by plankton (28.0%) and bivalves (14.2%) from landing centre which supports the findings of Ottavani et al. who isolated the *V. parahaemolyticus* from plankton and crustaceans [25]. Eventhough little information is available on *V. fluvialis*, it is very interesting to note that the presence *V. fluvialis* with tox-R gene in finfishes (40.0%) and plankton (16.6%), which correlates with the findings of Tsai et al. who concluded that *V. fluvialis* as a causative agent for necrotizing fasciitis and septicemia in the Gulf of Mexico and South East Asia, associated with minor trauma [26]. Moreover, the complete ecological association of this pathogen with plankton and the pathogenesis of this organism remain enigmas.

Prevalence of *V. cholerae* in seafood has been studied by various research groups. However, in some cases conventional biochemical tests were not able to distinguish *V. cholerae* from *V. mimicus* and *V. parahaemolyticus* from *V. vulnificus* due to the sharing of serological markers and phenotypic characters of these vibrios. Therefore PCR-based detection which targets the specific markers of different Vibrio spp. is necessary for identification of these vibrios [27-29]. In our study, from PCR-based analysis that strains of *V. cholerae* showed 100% specificity of all non-O1 *V. cholerae* strains tested. Therefore, the presence of *ompW* in *V. cholerae*, a species-specific genetic marker used for *V. cholerae* detection in naturally contaminated seafood. Our result also parallels with Ottavani et al. who analyzed the prevalence of non-O1 and non- O139 *V. cholerae* strains from various seafoods [24]. On the other hand, *tdh* gene can be used to identify pathogenic *V. parahaemolyticus* isolates. In this context, the presence of *tdh* gene from various seafood isolates was observed which indicates the high risk of contamination for seafood consumers. Previous studies have also been reported the similar findings of pathogenic *V. parahaemolyticus* that harbouring *tdh/trh* gene among the seafood isolates [30]. Moreover, recent reports revealed that *V. fluvialis* was found to be as mixed pathogen with other vibrios, the patients who acquired the infection from contaminated food and water [31]. In this study, the presence of *V. fluvialis* with tox-R gene was found in copepods (16.0%) and finfishes (40.0%). To our knowledge, this is the first report of *V. fluvialis* with tox-R gene from environmental region of South East Coast of India.
Attempts to measure the virulence of a pathogen are often made by experiments with susceptible animals like the mouse and guinea pig which can be reasonably well standardized in terms of age, sex, weight and some genetic factors: when death is the end results, mortality rates are easily measured. Our results in all the isolates revealed that most of the strains were produced hemolysin among seafood isolates. Proteases and hemolytic activity have been related to virulence in *V. cholerae* [32,33] and as already suggested it could be relevance in those illness different from cholera by non-01 *V. cholerae*. In addition, it is known that *V. cholerae* non-O1 and non-O139 produce El Tor-like hemolysin, but little information is available on hemolysin production in environmental strains [34,35]. The prevalence of putative virulence traits, genetic diversity and pathogenicity of *V. cholerae* non-O1 and non-O139 using animal models has been extensively studied by various researchers [36]. Virulence genes are dispersed among environmental strains of *V. cholerae* and may be ferried and the fact that most of virulence genes are located on mobile elements [37]. Our results is comparable to those reported elsewhere [38-40]. In this study, non-haemolytic strains, however, needed a much larger number of cells than haemolytic strains to kill the mice. The LD50 data of the study suggest that mortality results from the gastrointestinal infection which generally a good indicator of virulence genes. Recent studies have cited the importance of mice and rabbit to study the various virulence genes among vibrios which showed that these lab animals were used to test the ability of a particular organism to produce enterotoxin and hence their diarrheagenic potential [41].

5. CONCLUSION

The present study clearly establishes a fact that seafoods and other environmental samples act as a reservoir of vibrios and causes diseases to the human. The most important means of controlling human infection lies in simple hygienic measures aimed at preventing multiplication of the vibrios in seafood, and contaminated of cooked food from raw seafood and other sources. By regular monitoring the virulence genes and genetic changes of this pathogenic *V. cholerae* and other vibrios are essential and also studying virulence gene pool among the environmental isolates in a locality are useful for tracing the origins of newly emerging epidemic clone in disease surveillance and for understanding the genetic evolution of the organisms.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hervio-Heath D, Colwell RR, Derrien A, Robert-Pillot A, Fournier JM, Pommepuy M. Occurrence of pathogenic vibrios in Coastal areas of France. J Appl Microbiol. 2002;92:1123-1135.
2. Jores J, Stephan R, Knabner D, Gelderblom HR, Lewin A. Isolation of *Vibrio vulnificus* and a typical *Vibrio* from surface water of the Baltic Sea in Germany. Berliner and Munchener Tierarztliche wochenschrift (Berlin). 2003;116:396-400.

3. Faruque SM, Sack DA, Colwell RR, Takeda GB. Emergence and evolution of *Vibrio cholerae* O139. Pro Natl Acad Sci USA. 2003;1304-1309.

4. Gaffga NH, Tauxe RV, Mintz ED. Cholera: A new homeland in Africa. Am J Mid Hyg. 2007;77:705-713.

5. World Health Organization (WHO). Weekly Epidemiological record.2012;87(31-32):289-304. Accessed 3 August 2012. Available: http://www.who.int/wer.

6. Sarkar BL, Bhownick TS, Das M, Rajendran K, Nair GB. Phage types of *Vibrio cholerae* O1 and O139 in the past decade in India. JPN J Infect Dis. 2011;64:312-315.

7. Ramamurthy T, Garg R, Shame R, Bhattacharya SK, Nair GB, Shimade T, et al. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India. Lancet. 1993;314:703-704.

8. Malti D, Das B, Saha A, Nandy RK, Nair GB, Bhadra RK, et al. Genetic organization of pre-CTX and CTX prophage in the genome of an environmental *Vibrio cholerae* non-O1, non O139 strain. Microbiol. 2006;152:3633-3641.

9. Faruque SM, Nair GB. Molecular ecology of toxigenic *Vibrio cholerae*. Microbiol Immunol. 2002;46:59-66.

10. Kumar P, Jain M, Goel AK, Bhaduria S, Sharma SK, Kamboj DV, et al. A large cholerae outbreak due to a new cholerae toxin variant of the *Vibrio cholerae* O1 E1 Tor biotype in Orissa, Eastern India. J Med Microbiol. 2009;58:234-238.

11. Chatterjee S, Ghosh K, Raychoudhuri A, Chowdhury G, Bhattacharya MK, Mukhopadhyay AK. Incidence, virulence factors, and clonality among clinical strains of non – O1, non – O139 *Vibrio cholerae* isolation from hospitalized diarrheal patient in Kolkata, India. J Clin Microbiol. 2009;47(4):1087-1095.

12. Alam A, Miller KA, Chand M, Butler JS, Dziejman M. Identification of *Vibrio cholerae* type III secretion system effector proteins. Infec Immun. 2011;79(4):2718-2740.

13. Weber JT, Mintz ED, Caizares R, Semiglia A, Gomez ZI, Sempertegui R. Multiply-resistant epidemic cholerae in Ecuador; transmission by water and Sea food. Program Abstracts. 32nd Inter science. Antimicrob Agent Chemother. 1992;944:268.

14. Rice EW, Baird RB, Eaton AD, Clesceri. Standard methods for the Examination of water and wast water, 22 nd ed. American Public Health Association (APHA), American Water Work Association (AWWA) and Water Environment Federation. 2012.

15. Di pinto A, Terio V, Novellol, Tantilo G. Comparison between thiosulphate- citrate bile salt sucrose (TCBS) agar and CHROM agar vibrio for isolating *Vibrio parahaemolyticus*. Mol Cel Probes.1992;6:477-487.
20. Reed LJ, Muench H. A simple method estimating fifty percent end points. Am J Hyg. 1938;27:493-497.

21. Ang GY, Balqia CY, Elina K, Azura HT, Hani MH, et al. Molecular evidence of cholera outbreak caused by a toxigenic Vibrio cholerae O1 El Tor variant strain in Kelantan, Malaysia. J Clin Microbiol. 2010;48:3963-3969.

22. Fraser B. Haiti still gripped by cholera as election looms. World Report; 2010.

23. Saravanan V, Sanath kumar H, Karunasagar I, Karunasagar I. Putative virulence genes of Vibrio cholerae from seafood and the coastal environment of South West India. Int J Food Microbiol. 2007;119:329-333.

24. Ottaviani D, Leoni F, Rocchegiani E, Santarelli S, Masini L, Trani VD, et al. Prevalence and virulence properties of non-O1 non-O139 Vibrio cholerae strain from seafood and clinical samples collected in Italy. Int J Food Microbiol. 2009;132:47-53.

25. Ottaviani D, Leoni F, Rocchegiani E, Canonico C, Potenzionis S, Santarelli S, et al. Prevalence, serotyping and molecular characterization of Vibrio parahaemolyticus in mussels from Italian growing areas, Adriatic Sea. Environ Microbiol Res. 2010;2:192-197.

26. Tsai YH, Hsu RWW, Huang KC. Systemic Vibrio infection presenting as necrotizing fasciitis and sepsis-a series of thirteen cases. J Bone Joint Surg. 2004;86A:2497-2502.

27. Karunasagar I, Rosalind G, Karunasagar I, Malathi GR. Virulence of seafood associated strains of non O1 Vibrio cholerae. Quality assurance in the fish industry. 1992;211-216.

28. Hasan F, Kamuzzaman M, Meklones JJ, Faruque SM. Satellite phage TLC pro phage enables toxigenic conversion by CTX phage through diff site alteration. Nature. 2010;467:982-985.

29. Balckston GM, Nordstrom JL, Bowen MD. Use of the real time PCR assay for the detection of the ctxA gene of Vibrio cholerae in an environmental survey of Mobile Bay. J Microbiol Meth. 2007;68:254-259.

30. Kanjanasopa D, Pimpa B, Chowpongpang S. Occurrence of Vibrio parahaemolyticus in cockle (Anadara granosa) harvested from the South Coast of Thailand. Songk J Sci Technol. 2011;33:295-300.

31. Kobayashi K, Ohnaka T. Food poisoning due to newly recognized pathogens. Asian Med J. 1989;32:1-12.

32. Zhang XH, Austin B. Haemolysins in Vibrio species. J Appl Microbiol. 2005;98:1011-1019.

33. Restrepo D, Huprikar SS, Vanhorn K, Bottone EJ. O1 and non-O1 Vibrio cholerae bacteremia produced by haemolytic strains. Diag Microbiol Infe Dis. 2006;54:145-148.

34. Singh DV, Matte MH, Matte GR, Jiang S, Sabenea F, Shukla BN, et al. Molecular analysis of Vibrio cholerae O1 and non O139 strains. Clonal relationship between clinical and environmental isolates. Appl Enviorn Microbiol. 2006;66:4022-4028.

35. Stypulkowska Misiurewicz, Pancer H, Roszkowiak A. Two unrelated cases of septicaemia due to Vibrio cholerae non-O1, non-O139 in Poland, July and August 2006,EuroSurveillance11:48. Available:http://www.eurosurveillance.org/edition/v13n11/080313_3.asp.

36. Bag PK, Bhownik P, Hajra TK, Ramamurthy T, Sarkar P, Majumder M, et al. Putative virulence Traits and pathogenicity of Vibrio cholerae non-O1, non-O139 isolates from surface water in Kolkata, India. Appl Environ Microbiol. 2008;74:5635-5644.

37. Chakraborty S, Mukhopadhayay A, Bhadra R, Ghosh AN, Shimada R, Yamasaki S, et al. Virulence genes in environmental strains of Vibrio cholerae. Appl Environ Microbiol. 2000;66:4022-4028.
38. Nair GB, Sarkar BL, De SP, Chakrabarthi MK, Bhadra RK, Pal SC, et al. Ecology of Vibrio cholerae in the fresh water environs of Calcutta, India. Microbial Eco. 1998;15:203-215.
39. Venkateswaran K, Nakano H, Okabe T, Takayma K, Matsuda O, Hashimoto H. Occurrence and distribution of Vibrio spp., Listonella spp., and Clostridium botulinum in Seto Inland sea of Japan. Appl Environ Microbiol. 1989;55:559-576.
40. Sathiyamurthy K, Purushothaman A, Ramaiyan V. Studies on human pathogen Vibrio cholerae. In recent advances in toxicology research ed. Gopalakrishnakone P, Tan CK, Singapore: Venom and Toxin research group, National University of Singapore. 1992:421-430.
41. Faruque SM, Chowdhury N, Kamrussazaman M, Dziejman M, Rahman MH, Sack AD, et al. Genetic diversity and virulence potential of environmental Vibrio cholerae population in a cholera-endemic area. PNAS. 2004;104:2123-2128.

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