Fishery Technology 57 (2020) : 128 - 136

Phenotypic and genotypic characterization of nonO1/ nonO139 *Vibrio cholerae* from aquaculture farm sediments

P. S. Reethy* and K. V. Lalitha
Microbiology, Fermentation & Biotechnology Division, Central Institute of Fisheries Technology, Cochin, India.

Abstract

Toxigenic *Vibrio cholerae* is a major public health problem in developing countries andlysogenic conversion of naturally occurring nontoxigenic *V. cholerae* strains by a lysogenic filamentous bacteriophage CTXÖ has also been demonstrated. The aim of this study was to analyse phenotypic and genotypic characteristics of *V. cholerae* strains from sediments in aquaculture settings. Based on biochemical, molecular and serological assays confirmed the presence of fourty nine nonO1/nonO139 *V.cholerae* strains from fifty six sediment samples. PCR assays revealed that all *V.cholerae* strains harboured *toxR, hly*A *and ompW* genes. But none of the virulent genessuch as *ctxA, tcpA, ace and zot* were present in all tested strains. Antibiotic susceptibility to different antibacterial agents was also performed by disc diffusion assay. This result has shown 26.5% of the non-O1/non-O139 *V.cholerae* isolates were multidrug resistant for three classes of antibiotics. Genetic diversity of nontoxigenic *V.cholerae* isolated from aquaculture farm sedimentswas determined by ERIC PCR.The genetic diversity studies revealed clonal relationship among the various non-O1/non-O139 isolates.

Keywords: Non-O1/Non-O139*Vibrio cholerae*, Aquaculture Farm Sediments,Multidrug resistance, ERICPCR, BOX PCR.

Introduction

Vibrio cholerae non-O1 and non O139 strains are ubiquitous in aquatic environments andform part of the normal flora of aquatic ecosystems. They have been recognized as causative agents of sporadic and localized outbreaks, and the diarrhea caused by these strains is sometimes characterized by blood and mucous (Bagachi et al., 1993; Daisgaard et al., 1995). However, these strains continue to be considered of negligible significance, since they have been associated with illness only in a low percentage of patients hospitalized with secretory diarrhea (Mukopadhyay et al., 1996). Among 206 serogroups of *V. cholerae*, serogroups O1 and O139 are responsible for cholera outbreaks and are associated with cholera epidemics (Longini et al., 2002) and pandemics and they can produce cholera toxin encoded by the *ctx* gene.Singh et al., (2001) have reported that in the absence of cholera toxin, NAG-specific heat-stable toxin, and/ or TCP and OMP, *V. cholerae* O1, O139, and non-O1, non-O139 strains that have clinical or environmental origins have the ability to cause diarrhoea by a mechanism entirely different from that of the toxigenic *V. cholerae.* The other virulent and regulatory genes of pathogenic *V. cholerae* include toxin co-regulated pilus (*tcp*A), zonula occluden toxin (*zot*), accessory cholera toxin (*ace*), outer membrane protein (*omp*U), heat stable enterotoxin (*sto*), hemolysin (*hly*A) and ToxR regulatory protein (Rivera et al., 2001). The non-O1/non-O139 *V. cholerae* strains can cause gastro-intestinal or extra-intestinal infections (Deshayes et al., 2015; Chowdhury et al., 2016) and some of them harbor various virulence genes leading to the emergence of new pathogenic variants of *V. cholerae* (Sharma et al., 1998) and are potential receptors of virulence factors from toxigenic *V. cholerae* O1 (Faruque et al., 1998) arising through lateral gene transfer of the CTXÕ. *Since 2000, an average of around 40 cases of the non-O1 and non-O139* were reported to the Centers for Disease Control and prevention (CDC) each year(CDC, 2015).

The emergence of multidrug resistant *V. cholerae* and the changing patterns of antibiotic resistance in *V. cholerae* are increasing in every year and they can undermine the success of antimicrobial therapy in

Received 20 March 2020; Revised 02 April 2020; Accepted 20 April 2020

**E-mail: reethyramadas@gmail.com*

cholera epidemics. Most of the studies revealed the multidrug resistance of O1 and O139 *V. cholerae strains but,* few reports are available on multidrug resistance of environmental strains of non-O1/non-O139 *V. cholerae (Kumar & Lalitha, 2013).*

In previous studies that determined epidemiological characteristics and genetic relatedness among *V. cholerae* strains for tracing its origin and geographical distribution, ERIC and BOX PCR methods have been successfully used (Singh et al., 2001; Rao et al., 2010; Bakshi B, 2016). Though there have been no reports of localized outbreaks of cholera caused by *V. cholerae* non-O1, non-O139 from Kerala state, this study was initiated to understand the toxigenic potential of these environmental isolates. In addition, we also studied the antimicrobial resistance pattern and genetic diversity among the isolates of *V. cholerae* by ERIC PCR and BOX PCR.

Materials and Methods

A total of 56 sediment samples were collected from aquatic farms located at Ernakulum (n=10), Trissur (n=29), Palakkad (n=4), Kozhikode (n=2) and Alappuzha (n=11) districts of Kerala India. Details of samples are given in the Table: 1.

For preparation of sediment samples for analysis, 56 samples were added to 100ml of distilled water and allowed to settle. An 8-10-ml amount of the slurry was centrifuged at 2,000 rpm for 8 min to remove particulate matter and 1ml of slurry was added to 10 ml of APW (pH 8.5) for enrichment at 37°C for 6-8hrs, streaked onto TCBS agar plates. Incubation continued again for 16-24 hours at 37°c.Typical colonies (2-3 mm diameter, smooth, yellow, slightly flattened with opaque centers and translucent borders) on Thiosulphate Citrate Bile Salt Agar plates (TCBS) (Oxoid, UK) were picked and purified on Tryptic Soy Agar (TSA).

Table 1. Details of samples collected for isolation of *Vibrio cholerae* non-01/non-0139 strain

Sl. No.	Sampling area		No. of Sediment tested Samples	No.of *V. cholerae* Positive sediment samples	No. of positive isolates for *V. cholerae* non-O1/non O139
	Districts	location			
1.	Ernakulam (Farms)	Perumbavoor	3		Nil
		Puthuvype	3		Nil
		Kumbalangi	2	2	9
		Puthiyakavu	2	1	4
2.	Thrissur (Farms)	Mathilakam	2		Nil
		Kaipamangalam	2		Nil
		Padiyur	3		Nil
		Pullut	3		Nil
		S.N.Puram	3		Nil
		Kodakara	2		Nil
		Kodugallur	12	4	14
		Poyya	2		Nil
3.	Palakkad (Farms)	Kannadi	2		Nil
		Pallassena	2		Nil
4.	Alappuzha (Farms)	Ponnemvely	2		Nil
		Arookutty	4		Nil
		Ezhupunna	3	3	22
		Pattanakkad	2		Nil
5	Kozhikode (Farm)	Manassery	2		Nil
	Total		56	10	49

After purification, cultures were screened for the biochemical reactions of *V. cholerae*according to the BAM, USFDA (Kaysner and Depaola, A.Jr, 2005) and Bergey's Manual of Systematic Bacteriology (Brenner and Farmer III, 2005). All dehydrated media used in this study were purchased from BD Difco (Maryland, USA). Cultures were reconfirmed using the Analytical Profile Index (API) 20E kits (BioMerieux SA, France). After biochemical identification, purified and presumptively confirmed *V.cholerae* cultures were preserved as glycerol stock and a paraffin stock for further tests.

Biochemically confirmed isolates were tested for *V. cholerae* poly O1 antiserum (BD Difco, Maryland, USA), if the isolates have shown no agglutination reaction, were further tested for the presence of the *V.cholerae* O139 serogroup using 'Bengal' antiserum (Denka Seiken, Japan) as per manufacturer's instructions.

Antibiogram of *V. cholerae* isolates to antimicrobial agents was assessed by the disc diffusion (Kirby-Bauer) technique as described by the National Committee for Clinical Laboratory Standards (presently called the Clinical and Laboratory Standards Institute 2006; 2015) using standard antibiotic discs (Himedia, India) with an array of twenty antibiotics. *Escherichia coli* American Type Culture Collection (ATCC; Manassas, VA, USA) 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcusaureus* ATCC 29213 were used for the standardization of the Kirby-Bauer test for correct interpretation of the zone diameters. Multidrug resistance (MDR), defined as isolates resistant to ≥3 drugs.

Geneotypic characterization

Molecular detection of *V. cholerae* was performed by the presence of *ompW*gene. The isolates were characterized for the presence of *V. cholerae* virulence

Table 2. Primer Sequences, Annealing temperature, and amplicon size used for molecular characterization by PCR assay

Gene	Primer Sequence	Annealing Temperature (°C)	Amplicon size (bp)	Reference
OmpW	CACCAAGAAGGTGACTTTATTGT GAACTTATAACCACCCGCG	64	588	Nandi et al., 2000
*ctx*A	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	60	564	Keasler & Hall, 1993
*tox*R	TGTTCGGATTAGGACAC TACTCACACACTTTGATGGC	60	883	Rivera et al. 2001
*tcp*A (Eltor)	AAGAAGTTTGTAAAAGAAGAACAC GAAAGGACCTTCTTTCACGTTG	60	471	Keasler & Hall, 1993
*tcp*A (Classical)	CACGATAGGAAAACCGGTCAAGAG ACCAAATGCAACGCCGAATCGAG	60	617	Keasler & Hall, 1993
zot	TCGCTTAACGATGGCGCGTTTT AACCCCGTTTCACTTCTACCCA	60	243	Colombo et al. 1994
ace	GCTTATGATGGACACCCTTTA TTTGCCCTGCGAGCGTTAAAC	55	284	Colombo et al. 1994
rfb- O1	GTTTCACTGAACAGATGGG GGTCATCTGTAAGTACAAC	55	192	Keasler & Hall, 1993
rfb- O139	AGCCTCTTTATTACGGGTGG GTCAAACCCGATCGTAAAGG GGCAAACAGCGAAACAAATACC	55	449	Keasler & Hall, 1993
hlyA	CTCAGCGGGCTAATACGGTTTA GATACACATAATAGAATTAAGG	60	481	Hall et al. 1990
ctxb	GGTTGCTTCTCATCATCGAACCA	60	460	Olsvik et. al 1993

genes *ctxA, tcpA,hlyA, ace,zot* and serogroup specific *rfb* O1 and *rfb* O139.PCR reaction mixture contained 0.4 pmol *m*l^{-1} concentration of each primer, 200 *m*M concentration of each dNTP (Finnzymes, Finland), 1X PCR buffer (20 mmol l^{-1} Tris-HCl [pH 8.2], 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$), 1U of Taq polymerase (Dynazyme II, Finland), and 1 *m*l of sample DNA (~ 50 ng). The PCR amplification reactions were performed in a final volume of 25 μl. The primers used in this study and the reaction conditions are listed in Table 2. All PCR reagents and primers were kept at -20°C refrigerator. *Vibrio cholerae* O1MTCC 3904 was used as control. PCR based assays were performed in thermo cyclers (AB Biosystems, Veriti 96 well thermal cycler) and resulted PCR products were analysed by using 1.5% agarose gel. 50bp and 100 bp molecular size markers (Gene Ruler™, Fermentas, Germany) was used for separation of the amplicons. The amplified PCR products were visualized by ethidium bromide staining and a UV transilluminator and captured a gelpicture using a Gel Documentation system (SYGENE, Biorad, USA).

Isolation of Chromosomal DNA was carried out with slight modifications of Ausubel et al., 1994 method (Kumar et al.2009). The extracted DNA was suspended in TE buffer (pH 8) containing RNAse (20mg/ml). Finally, the concentration of DNA was determined with nano photometer (Implen, Germany) at 260 nm and stored at -20^0C until further use.

Enterobacterial Repetitive Intergenic Consensus sequence PCR (ERIC-PCR) was performed as described as Versalovic et al., 1991, by using two oligonucleotide primers. BOX (Box Element) PCR was performed by using a single nucleotide primer BOX A1R according to Versalovic et al., 1994.

All PCR reactions were carried out in 50 *m*l of reaction buffer and were performed in AB Biosystems, Veriti 96 well thermal cycler. Components of PCR and annealing conditions was used according to respective references. Gel images were recorded using a Gel Documentation system (SYGENE, Biorad). To confirm the reproducibility of fingerprints, the analysis was repeated twice on different days by using freshly prepared DNA template of single sample. A positive control (*V. cholerae* O1 MTCC 3904) was always included in PCR assay.

Amplified PCR products of PCR were run at 6v/cm for 120 min on 1.5% agarose gel containing 0.5 mg ml^{-1} of ethidium bromide. 100 bp plus DNA ladder (Fermentas, Germany) included as a size standard. The amplified PCR products were visualized by ethidium bromide staining and a UV transilluminator and photographed using a Gel Documentation system (SYGENE, Biorad). Dendrogram was constructed based on unweighted pair-group method with arithmetic means (UPGMA) using Gel Compar II software, version 5.1 (Applied-Maths, St-Martens-Latem, Belgium).

Results and Discussion

A total of 260 bacterial cultures from aquaculture sediment samples collected between the years 2013-2015 were screened for the presence of *V.cholerae.* From Ernakulum, Trissur and Alappuzha districts 13, 14 and 22 isolates respectively, of *V. cholerae* were obtained. Subsequently, based on serotyping of biochemically confirmed 49 *V. cholerae*strains, were identified as non-O1 and non-O139.

Among 49 *V. cholerae* non 01 and non 0139 isolates, 8% were sensitive to all 20 various antimicrobials tested, some non-toxigenic strains were resistant to amikacin (26.5%) nalidixic acid (20%), cefpodoxime (26.5%), ceftriaxone (6%), augmentin (26.5%) and ceftazidime (6%). Intermediate resistance was found in some strains to amikacin (36%), nitrofurantion (26.5%), azthreonam (6%) and ceftriazone (6%) and ceftriaxone (6%). All non-O1, non-O139 strains were sensitive towards tobramycin, ofloxacin, norfloxacin, gentamicin, co-trimoxazone, gatifluxacin, cefoxitin, ciprofloxacin, imipenem, levofloxacin, moxifloxacin, colistin, and ceftazidime. (Table: 3). Studies on *V. cholerae* non-O1 and non-O139 isolates from environmental and seafood samples by Kumar et al. (2009) revealed that 10-20% of the strains showed multiple drug resistance to 3-5 classes of antibiotics. In this study, thirteen strains (26.5%) of the *V.cholerae* isolates were shown multi-drug resistance (MDR) against four drugs (amikacin, cefpodoxime, ceftazidime, and azthreonam).

In this study, all 49 *V. cholerae* isolates (100%) carried both species-specific *ompW* gene which gave an amplicon of 588bp, confirming the biochemical identification of suspected strains and *toxR*gene which gave an amplicon of 883bp (Fig. 1a & 1b). They did not harbour the virulence associated genes *ctxA, ctxb, tcpA, ace* and *zot.* However, *hlyA* gene was

present in all the *V.cholerae* non O1 and non O139 isolates and they yielded a single specific amplicon of 481bp size (Figure. 1c). It did not produce the specific amplicon in the PCR using *V. cholerae* serogroup, rfb O1 specific primers and rfb O139 specific, confirmed that they belonged to non 01 and non 0139.

A similar study conducted in Northeastern Brazil, by Menezes et al., 2014 from sediments of rivers, strains of non-O1 and non-O139 *V. cholerae* tested were negative for *ctxAB*, *tcp*, *rfbO1*, *zot*and positive towards *ompW* gene. Our study also confirms the observation of Rivera et al., (2001) as all *V.cholerae* isolates contained both *toxR* and *hlyA* genes.

In the present study, all 49 *V.cholerae* non 01 and non 0139 isolates were ctx negative. However, Rao et al., (2010) isolated *ctx* positive non-O1 and non-O139 *V.cholerae* from shrimp aquaculture Farms in Kerala. Environmental isolates of *V. cholerae* O1 biotype El Tor are nontoxigenic (Rodrigue et al., 1994) and that the hemolysin produced is most likely responsible for the enterotoxic activity (Alm et al., 1991). Hemolysins from non-O1 non-O139 *V. cholerae*have been described to have cytotoxic and cell vacuolating activity on cultured HeLa and Vero cells (Coelho et al., 2000).

Table 3. Antibiotics Resistance Pattern in *V. cholerae*non-O1/non-O139 Isolates

Antibiotics	*V. cholerae* non-O1, non-O139 isolates, % resistance (n=49)
Tobramycin (10 μg)	0
Ofloxacin (5 μg)	0
Norfloxacin (10 μg)	0
Gentamicin (10 μg)	0
Amikacin (30 μg)	13,18*
Nalidixic acid (30 μg)	10
Co-trimoxazole (25 μg)	0
Gatifloxacin (5 μg)	0
Ciprofloxacin (10 μg)	0
Imipenem (10 μg)	0
Levofloxacin (5 μg)	0
Moxifloxacin (5 μg)	0
Ceftriaxone (10 μg)	3,3*
Colistin (10 μg)	0
Cefoxitin (30 mcg)	0
Cefpodoxime (10 μg)	13
Augmentin (30 μg)	13
ceftazidime (30 mcg)	3
Azthreonam (30 mcg)	3*
nitrofurantion (300 mcg)	13*

*Intermediate showed as resistance

All 49 non/non-O139 *V. cholerae* strains tested revealed the genotype *ompW*$^+$ *hlyA*$^+$ *toxR*$^+$ and negative for *ctx,tcp, ace*, and *zot* as per Rivera *et al*. 2001 who observed the genotype *hlyA* ET$^+$ *toxR* $^+$ and negative for *ctxA*, *ompU*, *stn/sto*, *tcpA*ET, and *zot* in 6 of 14 non-O1/non-O139 *V. cholerae* isolates from wastewater. Presence of virulence associated gene *toxR* and virulent gene *hlyA* emphasize their role in pathogenicity. However, it has been reported that non-cholera-toxin-producing *V. cholerae* non-O1, non-O139 strains possess *toxR*, the central regulatory protein gene, can acquire the *tcp* gene from toxigenic *V. cholerae* O1 by horizontal gene transfer (Karaolis et al., 1999). It occurred when they are exposed to the filamentous bacteriophage VPIö. The ctx element can also be acquired by exposure to the toxinoferous phage CTXö (Faruque et al., 1998).Jiang et al., 2018 found that clinical isolates of non-O1/ non-O139 strains contained *toxR,hlyA, ompW*, *rtxA*, *rtxC* and *hapA*genes whereas they lacked *ctxA*, *ctxB*, *tcpA*, *ompU* and *zot* genes.

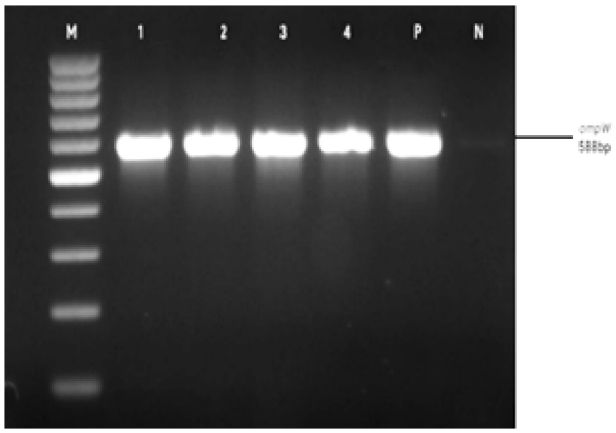

Fig 1a: PCR amplification of *ompW* gene.Lane M: Molecular Weight Marker 50bp, Lane 1-4: Species specific *OmpW*gene positive isolates, Lane P: Positive Control *V.cholerae* O1MTCC 3904, Lane N: Negative control

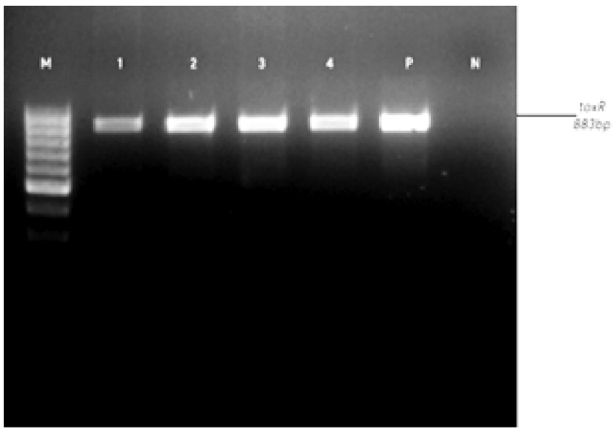

Fig 1b: PCR amplification of *toxR* gene, Lane M: Molecular Weight Marker 50bp,

Lane 1-4: Regulatory gene *toxR*positive *V.cholerae*, Lane P: Positive Control *V.cholerae* O1MTCC 3904, Lane N: Negative control

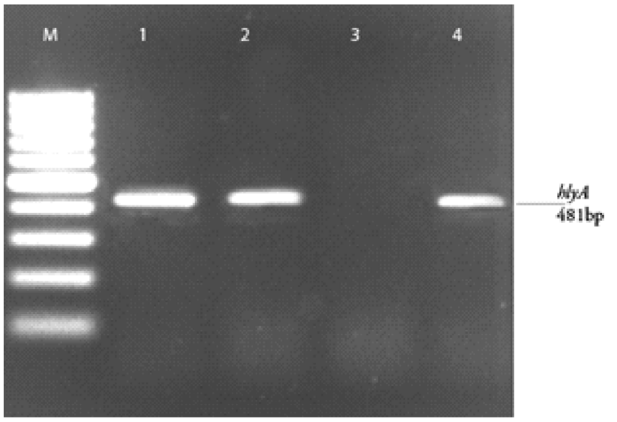

Fig. 1c: PCR amplification of *hlyA*gene.Lane M: Molecular Weight Marker 50bp

Lane 1, 2:*hlyA*gene positive *V.cholerae*, LaneP: Positive Control *V.cholerae* O1MTCC 3904, Lane N: Negative control

ERIC PCR of *V. cholerae* strains resulted in amplification of multiple fragments of DNA in sizes ranging between above 300 bp and 2000 bp, with the number of bands varied from 4 to10 as shown in Fig. 2a. Two bands, ~ 1500 bp were present in all tested *V. cholerae* isolates, except two samples. Amplification bands of 300 bp, 400 bp, 600 bp and 900 bp were present in all of the tested strains. VCEKS7 and VCFKS18 strains isolated from two districts have shown 100% similarity among strains (Fig. 2b). The similarity between the isolates ranged between 50 and 55%.

ERIC-PCR can be used to differentiate toxigenic and nontoxigenic strains of *V. cholerae* serogroup from a variety of sources, including environmental (Rivera et al., 1995). Bakhshi, 2016 found that ERIC PCR of *V. cholerae* isolated from outbreaks in Iran contained 2-5 bands within the rage from 250 bp -1000 bp. Rao et al., 2010 reported the genetic heterogeneity of non-O1and non-O139 *V. cholerae* isolated from shrimp, water and sediment of aquaculture farms by ERIC PCR, were included 1-12 bands within the range between 150 bp-3000 bp. In the present study showed a number of amplification bands 2-10 with size ranging from 200 bp to ~2000 bp.

BOX PCR analysis showed more genetic diversity among *V.cholerae* isolates than ERIC PCR analysis, including with 9 to 14 amplification bands ranged from 200 bp to >3000 bp. Three bands, 500 bp, above 600 bp and 1500 bp were present among all tested *V. cholerae* isolates (Fig. 3a). The amplification bands with molecular size 300 bp, 1200 bp and ~3000 bp were found to be common in all tested *V. cholerae* isolates except two strains. None of the *V. cholerae* strains showed 100% similarity. The similarity between the isolates is 32.23%. In dendrogram

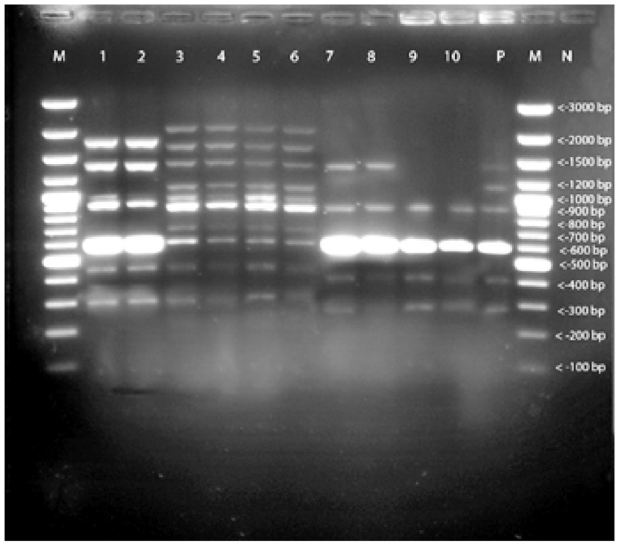

Fig. 2a- DNA Finger print pattern of *V.cholerae* isolates from Aquaculture sediments using Enterobacterial repetitive intergenic consensus –PCR.Lane M-100bp plus marker (Gene Ruler; Fermentas), Lane 1-VC GS5,Lane 2-VC GS4, Lane7-VC AS9,Lane 8-VC AS10 (Trissur district);Lane 3-VC EKS7,Lane 4-VC EKS4 (Alappuzha district); Lane 5-VC FKS18, Lane 6- VC FKS19 ,Lane 9- VC B2S,Lane 10-VC B2S2(Ernakulam District); Lane P-VC MTCCO1,Lane N-Negative control

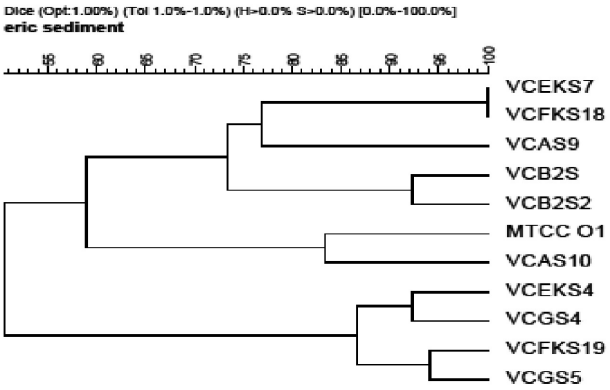

Fig. 2b. ERIC PCR profile of *V.cholerae* strains isolated from aquaculture farm sediments

dendrogram was constructed using strains isolated from aquaculture sediment samples based on unweighted pair-group method with arithmetic means (UPGMA) using Gel Compar II software, version 5.1 (Applied-Maths, St-Martens-Latem, Belgium).

analysis, the strains which have shown multidrug resistance were grouped in same clones (Fig. 3b). The banding pattern were reproducible in ERIC and BOX PCR.

Goel et al., 2010 reported that the BOX PCR of *V. cholerae* genomic DNA, were isolated from clinical samples in Chennai, India, resulted in fragments varying between 0.65 and 6.0 kb. In this study, BOX PCR amplification bands ranged from 200 bp to>3000 bp.

ERIC PCR have shown fewer bands and more homogeneity than BOX PCR. In this study, some of the strains have shown similar fingerprint pattern regardless of different source and different period of time. This may be due to origin of these isolates from same clonal lineages. ERIC and BOX PCR can be applied for the study of molecular ecology of toxigenic and non-toxigenic *V. cholerae*, to prevent origin of epidemics, through source tracking method. This indicates that origin of these strains may be from same source.

Even though in the absence of virulence genes, non-O1 and non-O139 *V. cholerae* can cause diarrhoea (Dutta et al., 2013). It possibly triggered by environmental changes, or by lateral or horizontal transference of virulence genes. So the presence of non O1/nonO139 strains can no longer be ignored.

In conclusion, our study highlights the presence of multidrug resistant non-O1/non-O139 *V. cholerae* which harboured virulence regulator *toxR*

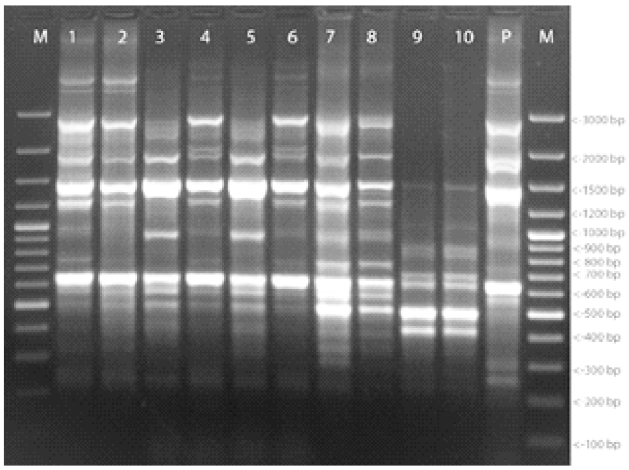

Fig. 3a: DNA Finger print pattern of *V.cholerae* isolates from Aquaculture sediments using Box element PCR. Lane M-100bp plus marker(Gene Ruler; Fermentas), Lane 1-VC EKS7, Lane 2-VC EKS4 (Alappuzha district); Lane 3-VC FKS18,Lane 5-VC FKS 19,Lane 9- VC B2S, Lane 10-VC B2S2 (ErnakulamDisrict);Lane 4-VC GS4,Lane 6- VC GS5,Lane 7-VC A1S9,Lane 8- VC A1S10(Alappuzha district); Lane P-VC MTCCO139,Lane N-Negative control.

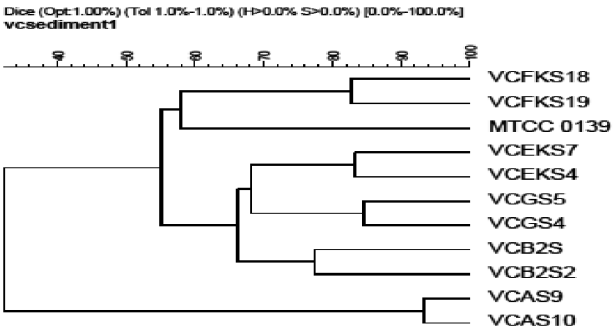

Fig: 3b. BOX PCR profile of *V.cholerae* strains isolated from aquaculture farm sediments.

Dendrogram was constructed using strains isolated from aquaculture sediment samples based on unweighted pair-group method with arithmetic means (UPGMA) using Gel Compar II software, version 5.1 (Applied-Maths, St-Martens-Latem, Belgium).

andvirulent *hlyA* gene, in aquatic sediments may pose severe threat to the entire aquatic community and human health. Therefore, it is highly recommended to pay attention in maintaining good water quality and routine monitoring in aquaculture farms to avoid the health risk problems and rejection of the exported fish and fishery products. This study also attempts to bring new insights into the ecology of non-toxigenic *V. cholerae.*

Acknowledgments

This work was carried out with the financial assistance obtained from National Agricultural Innovation Project (NAIP), The Indian Council of Agricultural Research (ICAR), New Delhi, India. The authors are grateful to the Director, ICAR_Central Institute of Fisheries Technology, Cochin for providing the necessary facilities and kind permission to publish this paper.

References

Alm, R. A., Mayerhofer, G., Kotlarski, I. and Manning, P. A. (1991) Amino-terminal domain of the El Tor hemolysin of *Vibrio cholerae* O1 is expressed in classical strain and is cytotoxic. Vaccine. 9: 588–594

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Scidman, J.G., Smith, J.A. and Struhl, K. (1994) Current Protocols in Molecular Biology. pp 2.0.1–2.14.8. New York: John Wiley and Sons

Bagchi, K., Echeverria, P., Arthur, J.D., Sethabutr, O., Serichantalergs. and Hoge, C.W. (1993) Epidemic of diarrhea caused by Vibrio cholerae non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. J Clin Microbiol. 31: 1315-17

Bakshi, B. (2016) Molecular characterization of *Vibrio cholerae* isolates from Iran 2012 and 2013 Outbreaks. Lett App Microbiol. 62: 466-471

Brenner, D.J. and Farmer, III J.J. (2005) *Enterobacteriaceae*, In: Bergey's Manual of Systematic Bacteriology. 2nd Edition, (D.J. Brenner, N.R. Kreig, and J.T. Staley) pp.587-878. (ed.). Springer, New York, NY, USA

Centers for Disease Controls and Prevention (http://www.cdc.gov/nationalsurveillance/cholera-Vibrio-surveillance.html, accessed 13 December 2015).National cholera and Vibriosis surveillance. Atlanta (GA)

Chowdhury, D., Joshi, S., Battacharya, S., Sekar, U., Birajdar, B., Battacharyya, A., Shinoda, S. and Ramamurthy, T. (2016)Extraintestinal Infections Caused by Non-toxigenic *Vibrio cholerae* non-O1/non-O139. Front Microbiol. 7(144): 1-5

CLSI (2006) Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. CLSI document M45-P, Wayne, PA: Clinical and Laboratory Standards Institute

CLSI (2015) Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. CLSI document M100-S25, Wayne, PA: Clinical and Laboratory Standards Institute

Coelho, A., Andrade, J., Vincente,A. and DiRita,V (2000) Cytotoxic cell vacuolating activity from *Vibrio cholerae* hemolysin. Infect. Immun. 68: 1700-1705

Colombo, M.M., Mastrandrea, S. and Santona, A. (1994) Distribution of the ace, zot, and ctxA toxin genes in the clinical and environmental *Vibrio cholerae*. J Infect Dis 170: 750-751

Dalsgaard, A., Albert, M.J., Taylor, D.N., Shimada,T., Meza, R., Serichantalergs, O. and Echeverria, P (1995) Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. J Clin Microbiol 33: 2715-22

Deshayes, S., Daurel, C., Cattoir, V., Parienti, J. J., Quilici, M. L. and de La Blanchardiére, A. (2015) Non-O1, non-O139 *Vibrio cholerae* bacteraemia: case report and literature Review. Springerplus 4: 1-9

Dutta, D., Chowdhury, G., Pazhani, G.P., Guin, S., Dutta, S., Ghosh, S., Rajendran, K., Nandy, R.K., Mukhopadhyay, A.K., Battacharya, M.K., Mitra, U., Takeda, Y., Nair, G.B. and Ramamurthy, T. (2013) *Vibrio cholerae* Non-O1, Non-O139 Serogroups and Cholera-like Diarrhea, Kolkata, India. Emerg Infect Dis 19: 464-467

Faruque, S.M., Albert, M.J. and Mekalanos J.J. (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 62: 1301-1314

Goel,A.K., Jain,M., Kumar,P. and Jiang,S.C. (2010) Molecular characterization of Vibrio cholerae outbreak strains with altered El Tor biotype from southern India. World J Microbiol Biotechnol 26: 281–287

Hall, R. H. & Drasar, B. S. (1990). *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. Infect Immun. 58(10), 3375-3379

Jiang, F., Bi,R., Deng, L.,Kang, H.,Gu, B. & Ma, P. (2018) Virulence-associated genes and molecular characteristics ofnon-O1/non-O139 Vibrio cholerae isolatedfrom hepatitis B cirrhosis patients in China. Int J Infect Dis. 74: 117-122

Karaolis, D.K.R., Somara, S., Maneval, D.R.JR., Johnson, J.A., Kaper, J.B. (1999) A bacteriophage encoding a pathogenicity island, a type IV pilus and a phage receptor in cholera bacteria. Nature. 399: 375–379

Kaysner, C.A. and DePaola, A.Jr (2005), chapter 9, In: *Bacteriological Analytical Manual*, U.S. Food and Drug Administration, 8thEdition, Revision A, AOAC International, Gaithersburg, Md

Keasler, S.P. and Hall, R.H. (1993) Detecting and biotyping *V. cholerae* O1 with multiplex polymerase chain reaction. Lancet. 341, 1661

Kumar, P.A., Patterson, J. and Karpagam, P. (2009) Multiple antibiotic resistance profiles of *Vibrio cholerae* non-O1 and non-O139. Jpn Infect Dis. 62: 230-232.

Kumar, R. and Lalitha, K.V. (2013)Prevalence and molecular characterization of *Vibrio cholerae* O1, non-O1 and non-O139 in tropical seafood in Cochin, India. Foodborne Pathog Dis. 10: 278-83

Kumar, R., Surendran, P.K. and Thampuran, N. (2009) Distribution and genotypic characterization of *Salmonella* serovars isolated from tropical seafood of Cochin, India. J Appl Microbiol, 106: 1364-5072

Longini, I.M., Longini, I.M. Jr., Yunus, M., Zaman, K., Siddique, A.K., Sack, R.B. and Nizam, A. (2002) Epidemic and endemic cholera trends over a 33-year period in Bangladesh. J. Infect. Dis. 186: 246-251

Menezes, F.G.R.de., Neves, S.Da.S. Sousa, O.V.de., Vilanova, C.M.V.M., Maggioni, R.,Theophilo, G.N.D.T., Hofer, E. and Vieira. R.H.S.d.F. (2014) Detection of virulence genes in environmental strains of *Vibrio cholerae* from estuaries in northeastern Brazil, Rev. Inst. Med. Trop. Sao Paulo 56: 427-432

Mukhopadhyay, A. K., Garg, S., Mitra, R., Basu, A., Dutta, D., Bhattacharya, S. K., Shimada, T., Takeda, T., Takeda, Y. and Nair, G. B. (1996) Temporal shift in traits of *Vibrio cholerae* strains isolated from hospitalized patients in Calcutta: 3 years (1993-1996) analysis. J Clin Microbiol. 34: 2537–2543

Nandi, B., Nandi, R.K., Mukhopadhyay, S., Nair, G.B., Shimada, T. and Ghose, A.C. (2000) Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein *OmpW*, J Clin Microbiol 38: 4145-51

Rao, B. M. and Surendran, P.K. (2010) Genetic heterogeneity of non-O1 and non-O139 *Vibrio cholerae* isolates from shrimp aquaculture system: a comparison of RS, REP- and ERIC-

PCR fingerprinting approaches. Lett Appl Microboil 51: 65-74

Rivera, I.G., Chowdhury, M.A.R., Huq, A., Jacobs, D., Martins, M.T. and Colwell, R.R (1995), Enterobacterial Repetitive Intergenic Consensus Sequences and the PCR to Generate Fingerprints of Genomic DNAs from Vibrio cholerae O1, O139, and Non-O1 Strains, Appl Environ Microbiol. 61: 2898–2904

Rivera, I.N., Chun, J., Huq, A., Sack, R.B. and Colwell, R.R. (2001), Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. Appl Environ Microbiol 67: 2421-2429

Rodrigue, D.C., Popovic,T. and Wachsmuth,K.(1994) Nontoxigenic Vibrio cholerae O1 Infections in the United States. In: *Vibrio cholerae* and cholera: molecular to global Perspectives (Wachsmuth,K.I., Blake,P.A. and Olsvik,O.,Eds.), pp69-76. American Society for Microbiology, Washington, DC

Sharma, C., Thungapathra, M., Ghosh, A., Mukhopadhyay, A., Basu, A., Mitra, R., Basu, I., Bhattacharya, S., Shimada, T. and Ramamurthy, T. (1998) Molecular analysis of non-01, non-0139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. J. Clin. Microbiol. 36: 756-763

Singh, D.V., Matte, M.H., Matte, G.R., Jiang, S., Sabeena, F., Shukla, B.N., Sanyal, S.C., Huq,A. and Colwell,R.R. (2001) Molecular Analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 Strains: Clonal Relationships between Clinical and Environmental Isolates. Appl Environ Microbiol. 67: 910-921

Versalovic,J., Koeuth, T. and Lupski, J.R (1991) Distribution of repetitive DNA sequences in eubacteria and applications to fingerprinting of bacterial genomes. Nucleic Acids. Res., 19: 6823-6831

Versalovic, J., Schneider M, De Brulin FJ, Lupski JR. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol. 1994; 5: 25-40