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Brief Report

Environmental reservoirs of *Vibrio cholerae* serogroups in the flowing freshwater environs from the tribal areas of Odisha, Eastern India

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Summary

The environmental reservoirs of different serogroups of *Vibrio cholerae* causing cholera in the flowing freshwater bodies of the tribal areas of Odisha are not known. So the present study was conducted from June 2017 to March 2020 to find out the environmental reservoirs of *V. cholerae* serogroups in the water and plankton samples collected from the river, nala, stream and chua from Rayagada district. Similarly, rectal swabs were collected from diarrhoea patients and correlation was established among the *V. cholerae* strains isolated from diarrhoea patients and environmental *V. cholerae* isolates through routine culture, different multiplex PCR assays and pulse field gel electrophoresis (PFGE) analysis using standard techniques. The multiplex PCR assays on biotypes and different toxic genes exhibited similar correlation between the clinical and water isolates, which was further strengthened by PFGE analysis. The planktonic DNA was positive for *ctx*A gene which established that the environmental water bodies were the reservoirs for virulence genes of *V. cholerae* serogroups. The detection of environmental reservoirs of *V. cholerae* serogroups in temporarily stagnant condition of water; partially encircled by stones, and near the bank of the river, nala and stream were the reservoirs which is a rare report from Odisha, India and Globe.

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Introduction

Vibrio cholerae, the causative agent of cholera is an autochthonous resident of the water bodies survives in association with plankton, crab and shrimps, and so on, whereas human acts as an accidental host for the bacterium. However, it was rarely possible to isolate them during inter-epidemic periods from the same water bodies by culture methods (Huq *et al*., 1983; Colwell and Sipra, 1992; Chatterjee and Chaudhuri, 2004; Alam *et al*., 2006). *Vibrio*s exist as viable but nonculturable state and also as non-O1 and non-O139 serogroups mostly without major virulence factors during inter-epidemic period. Due to the ability to acquire other virulence genes by bacteriophages, environmental *V. cholerae* strains are accepted as precursors of pathogenic strains (Xu *et al*., 1982; Leclerc *et al*., 2002). Investigators from different countries like Bangladesh, Haiti, Uganda and India have tried to establish the source of *V. cholerae* in different water bodies like river, canal, pond, nala (small stream), chua (shallow pit on river/stream bed) and well from time to time (Islam *et al*., 1992, 1993, 1994,1995; Alam *et al*., 2014, 2015; Bwire *et al*., 2018; Pal *et al*., 2010). Researchers around the world identified some common characteristics that illustrated the endemicity of cholera in epidemic areas, i.e., population density, location near water bodies, low land and high humidity (Swaroop, 1951). Depending upon the geographical areas all the features might not be correct, but it explained the role of the environment in the endemicity of cholera in particular regions. Although the exact mechanism is unknown and the stimulation of growth of unculturable *V. cholerae* and its link with the plankton boom; but plankton serves as a reservoir of *V. cholerae* serogroups (Colwell and Sipra, 1992). Cholera is endemic in Odisha and it is seasonal in the tribal areas where regular epidemics and outbreaks of cholera were reported causing high morbidity and mortality (Pal *et al*., 2010; Kar *et al*., 2015). So far, no study has been done to find out the environmental reservoirs of *V. cholerae* different serogroups in the flowing freshwater

bodies from the tribal areas of Odisha. The primary objective of this study was to find out the environmental reservoirs of different serogroups of *V. cholerae* in the flowing water environs like river, stream, nala and chua in the tribal areas of the state. Again to validate these results both phenotypic and genotypic characterizations of *V. cholerae* strains were done which were isolated both from the diarrhoea patients as well as water and plankton samples between June 2017 and March 2020 from the Rayagada district of Odisha.

Results

During the study period, 2801 rectal swabs were collected from indoor diarrhoea patients from different Community Health Centres (CHC) of Kashipur and Kalyansinghpur blocks, and diarrhoea outbreak villages in April-2019 from the Kalyansinghpur block. Out of 2078 culture positive samples *E.coli* were 1881 (67.2%), *Shigella* spp. were 137 (4.9%), *Salmonella* spp. were 26 (0.9%) and *V. cholerae* O1 biotype El Tor were 34 (1.2%) (Table 1). Out of 34 *V. cholerae* O1 strains 31 were isolated during the cholera outbreak from three different villages and the rest three were isolated during the surveillance study. The patients were divided into five different age groups: ≤5 years, >5–10 years, >10–-14 years, >14–40 years and > 40 years. Among all the age groups of diarrhoea patients >14–40 years age group was worst infected followed by >40 years and ≤5 years age groups respectively. The age group >5–14 years was least infected. Both male and female patients were almost equally infected.

Water and plankton samples were collected fortnightly throughout the year. Out of 1889 water samples collected from different study sites and sources, one (0.1%) was *V. cholerae* O1, 6 (0.3%) were *V. cholerae* O139 and 19 (1.0%) were non-O1 non-O139. Similarly out of 1475 plankton samples which were collected through 20 μm plankton net, 6 (0.4%) were *V. cholerae* O1, 1 (0.1%) was *V. cholerae* O139 and 18 (1.2%) were non-O1 non-O139 (Table 2). The *V. cholerae* O1 strain that was isolated from the stream water was the source of cholera outbreak which was reported in April 2019 from Podachuan village of Kalyansinghpur block. This water was used by the people for preparing food during the marriage ceremony which was observed in that village. Further the diarrhoea outbreak spread to other two villages where the people participated in the marriage function.

Molecular characterization

Forty one *V. cholerae* O1 strains isolated from rectal swabs collected from diarrhoea patients, water and plankton samples were positive for all virulence and accessory genes, whereas seven *V cholerae* O139 were positive for only *tox*R, *ctx*B, *omp*W gene and negative for rest of the genes including *rfb*O139. Similarly, in the case of 37 non-O1 non-O139 *V. cholerae*, only *omp*W and *hly* genes were positive in all the isolates; whereas *omp*U and *rtx* were positive in only 8.1% of isolates and *tox*R gene was positive in 94.6% isolates and 35.1% isolates were positive for the *rfb*O1 gene. Out of 1475 direct planktonic DNA extract, *ctx*A was positive only in 0.3% of samples (Table 3 and Fig. 1).

Detection of ctxB and tcpA gene

All the *V. cholerae* O1 strains isolated from stool, water and plankton were analysed for the detection of *ctx*B genotype by DMAMA-PCR assay which revealed that all the strains were El Tor variant harbouring *ctx*B7 (191 bp) genotype irrespective of place and source of isolation. The PCR assay for the detection of *tcp*A gene (167 bp) confirmed the presence of Haitian type *tcp*A allele in all the *V. cholerae* O1 isolates.

Analysis of plankton samples

A total of 28 different genera of phytoplankton and 12 different genera of zooplankton were identified from different water bodies of Kalyansinghpur and Kashipur blocks.

Table 1. Bacteriological analysis of enteropathogens isolated from diarrhoea patients (June 2017 to March, 2020).

Year		2017–2018	2018–2019	2019–2020	Total
Total samples		341	1258	1202	2801
Culture positive	*E.coli*	250 (73.3%)	894 (71.1%)	737 (61.3%)	1881 (67.2%)
	Shigella spp.	29 (8.5%)	48 (3.8%)	60 (5.0%)	137 (4.9%)
	Salmonella spp.	1 (0.3%)	19 (1.5%)	6 (0.5%)	26 (0.9%)
	V. cholerae O1	1 (0.3%)[a]	2 (0.2%)[b]	31 (2.6%)[c]	34 (1.2%)
Culture negative		60 (17.6%)	295 (23.4%)	368 (30.6%)	723 (25.8%)

[a]This *V. cholerae* O1 was isolated in January 2018.
[b]These *V. cholerae* O1 were isolated in August 2018.
[c]These *V. cholerae* O1 were isolated from the cholera outbreak village of Kalyansinghpur block in April 2019.

Table 2. Isolation of different serogroups of *V. cholerae* from environmental water and plankton samples (June 2017 to March 2020).

Sl No.	Year	Source	Total samples	*V. cholerae* O1	*V. cholerae* O139	Non-O1 non-O139 *V. cholerae*
1	2017–2018	Water	760	0 (0%)	6 (0.8%)[a]	11 (1.4%)[d]
		Plankton	426	0 (0%)	1 (0.2%)[a]	14 (3.3%)[d]
2	2018–2019	Water	624	0 (0%)	0 (0%)	6 (1.0%)[e]
		Plankton	594	6 (1.0%)[b]	0 (0%)	1 (0.2%)[e]
3	2019–2020	Water	505	1 (0.2%)[c]	0 (0%)	2 (0.4%)[f]
		Plankton	455	0 (0%)	0 (0%)	3 (0.7%)[f]
4	Total	Water	1889	1 (0.1%)	6 (0.3%)	19 (1.0%)
		Plankton	1475	6 (0.4%)	1 (0.1%)	18 (1.2%)

[a]*Vibrio cholerae* O139 were isolated in August 2017.
[b]*Vibrio cholerae* O1 were isolated in August 2018.
[c]*Vibrio cholerae* O1 was isolated from the stream water during the cholera outbreak in April 2019.
[d]Isolated from June 2017 to December 2017.
[e]Isolated from June-2018 to November 2018.
[f]Isolated from January 2019 to May 2019. All the *V. cholerae* strains were isolated from different sources during different years.

Kashipur block had more diversity in the phytoplankton than Kalyansinghpur; whereas zooplanktons identified from both the areas were completely different.

Pulse field gel electrophoresis

The PFGE analysis on *V. cholerae* O1 strains isolated from the stool, water and plankton samples exhibited 3 different pulsotypes. The overall similarity between the strains was 84%, whereas the *V. cholerae* O1 strains isolated in 2018 from clinical as well as plankton samples were 100% similar. The *V. cholerae* O1 strains isolated in 2019 were almost similar including water and clinical samples except for one clinical strain which had 97% similarity (Fig. 2).

Discussion

Diarrhoea in general, cholera in particular might be one of the causes of high morbidity and mortality in the tribal areas of Odisha which was due to unhygienic living conditions along with poor access to potable drinking water, and so on. Contaminated drinking water from sources like river, stream, nala and chua made the population vulnerable mostly during pre and post-monsoon season which reported during the cholera epidemic in 2007 and also during a large cholera outbreak in 2010 from the Rayagada district of Odisha (Pal *et al*., 2010; Kar *et al*., 2015). Surveillance activities on diarrhoea patients from Koraput, Rayagada and Gajapti district of Odisha (2010–2013) indicated a higher incidence of *E. coli* (44.6%) followed by *V. cholerae* (10.2%); whereas *Shigella* spp was 5.5% and *Salmonella* spp. was 0.7% which was quite similar to the current study (Nayak *et al*., 2020a, 2020b). Cholera was mostly seasonal in North India including Chandigarh, Delhi and its periphery which occurred mostly in the rainy season; which is similar with the present findings (Sharma *et al*., 2007; Devnikar *et al*., 2012). The cholera cases were detected mostly in the pre monsoon and monsoon seasons in the tribal areas. From another study, it was reported that cholera cases were reported throughout the year in the coastal districts showing its endemicity (Nayak *et al*., 2020a, 2020b). The main reason for this was that the environmental water bodies in the Puri (coastal district) area were mostly of stagnant condition of water, whereas flowing water systems of different categories existed in the tribal areas.

Demographic analysis of the infected diarrhoea patients showed that the males and females were almost equally infected, where the age groups >14–40 and > 40 years were worst infected in comparison to ≤5 years, >5–10 years, >10–14 years age groups of patients. A surveillance study on diarrhoea from 2010 to 2013 (Nayak *et al*., 2020a, 2020b); 2004–2013 (Pal *et al*., 2018) and an outbreak study during 2019 from this tribal areas (Nayak *et al*., 2020a, 2020b) revealed similar results. Although the results are similar to the previous studies from this area; contradicting reports were available in other parts of India like Chandigarh, North India, Kolkata, Delhi and its periphery; where the age group <14 years were more infected (Taneja *et al*., 2003, 2020; Basak *et al*., 1992; Sharma *et al*., 2007). The age group >14–40 years and above were more vulnerable to the infection; because this age group of people were more exposed due to farming and day-to-day household activities.

The major focus of this study was to know the viability of *V. cholerae* serogroups during inter-epidemic periods. Previous studies from these areas established that cholera outbreaks occurred every 2–3 years during 2007, 2010, 2012, 2016 and 2019 in this region (Pal *et al*., 2010; Kar *et al*., 2015; Nayak *et al*., 2020a, 2020b). *V. cholerae* O139 were isolated during monsoon season

Table 3. Detection of different *tox*ic genes of *V. cholerae* from water and Plankton samples (June 2017 to February 2020).

Strains	No of Samples	*rfb*O1	*rfb*O139	*ctx*A	*tcp*A	*tox*R	*zot*	*ctx*B	*omp*W	*rtx*	*ace*	*hly*	*omp*U
V. cholerae O1	41	41 (100.0%)	0	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)	41 (100.0%)
V. cholerae O139	7	0	0	0	0	7 (100.0%)	0	7 (100.0%)	7 (100.0%)	0	0	0	0
V. cholerae non O1/O139	37	13 (35.1%)	0	0	0	35 (94.6%)	0	0	37 (100.0%)	3 (8.1%)	0	37 (100.0%)	3 (8.1%)
Direct plankton DNA	1475	0	0	5 (0.3%)[a]	0	0	0	0	0	0	0	0	0

[a]The plankton DNA were tested during June to August 2018.

of 2017 from the flowing water of river/nala and plankton samples from Kalyansinghpur block. Although the *V. cholerae* O139 were isolated by culture methods; those were negative for the *rfb*O139 gene by PCR assay. These strains were positive for few genes like *tox*R, *ctx*B and *omp*W genes. Similar results were also published by other investigators from Bangladesh during 2004 (Alam *et al*., 2006) and Lubumbashi, Congo in 2008 (Page *et al*., 2012). Timely reporting enabled the state health authorities to implement adequate control measures to check the possible cholera outbreak in this region.

During 2018–2020, the *V. cholerae* O1 strains isolated from the stool, water and plankton samples were positive for all the virulence and accessory genes, whereas the *V. cholerae* non-O1/O139 isolates were negative for many virulence as well as accessory genes. Meanwhile, the direct DNA of *V. cholerae* isolated from the plankton after enrichment were negative for all the genes except *ctx*A (0.3%). Similar type of result was reported by other investigators from Bangladesh. The presence of the *ctx*A gene irrespective of the *rfb* gene of *V. cholerae* either serotype indicated a possible reservoir for *ctx*A in the environmental water bodies. Again the presence of the *rfb* gene in the absence of the *ctx* gene in PCR assay indicated the availability of progenitor of strains with the ability to cause an outbreak (Alam *et al*., 2006).

More number of *V. choleare* strains were isolated during 2019, which was due to a diarrheal outbreak in Podachuan village of Kalyansinghpur Block, Rayagada district. The outbreak was due to the use of contaminated stream water by El Tor variant *V. cholerae* O1 Ogawa. The detailed analysis of the *V. cholerae* O1 strains isolated from the cholera outbreak was addressed in an another report (Nayak *et al*., 2020a, 2020b).

The presence of different types of strain in the same environment is a serious concern as *V. cholerae* has the ability to acquire the virulence genes through transduction by phages (Cariri *et al*., 2010). The ability to remain as viable but non-culturable state enables the *V. cholerae* to survive between interepidemic periods without losing its toxigenicity in a freshwater ecosystem (Islam *et al*., 1989). The *V. cholerae* and its association with varieties of plankton were reported from different countries during different times such as Brazil (Goncalves *et al*., 2004), Peru (Tamplin and Parodi, 1991) and Bangladesh (Islam *et al*., 1989). Association of *V. cholerae* has been found with cyanobacteria (*Anabaena*); freshwater green algae (Islam *et al*., 1989) *Nitzschi*a (Seeligmann *et al*., 2008). Similar types of phytoplankton and zooplankton were isolated from this study. The probable explanation for *V. cholerae* being attached to aquatic organisms might be due to their capacity to produce chitinase and mucinase, which provide support for the attachment (Epstein *et al*., 1993).

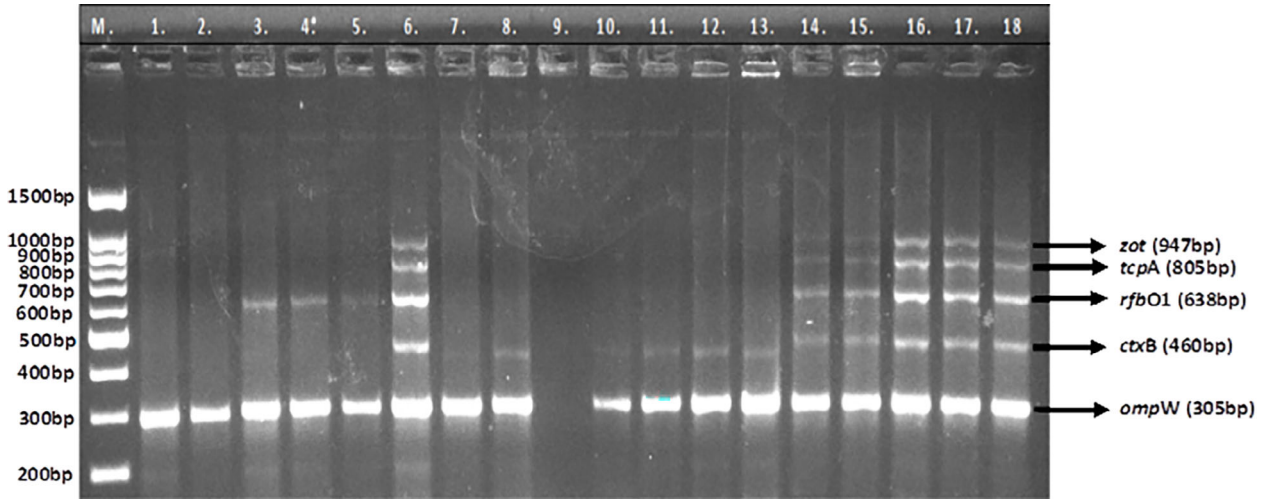

Fig 1. Detection of different toxic genes by multiplex PCR assay (M: Marker; Lanes 1–5: *V. cholerae* non O1 non O139; Lane 6: *V. cholerae* O1 (clinical isolate); Lane 7–13: *V. cholerae* O139 (water isolates 2017); Lanes 14–18: *V. cholerae* O1 (plankton isolates 2018).

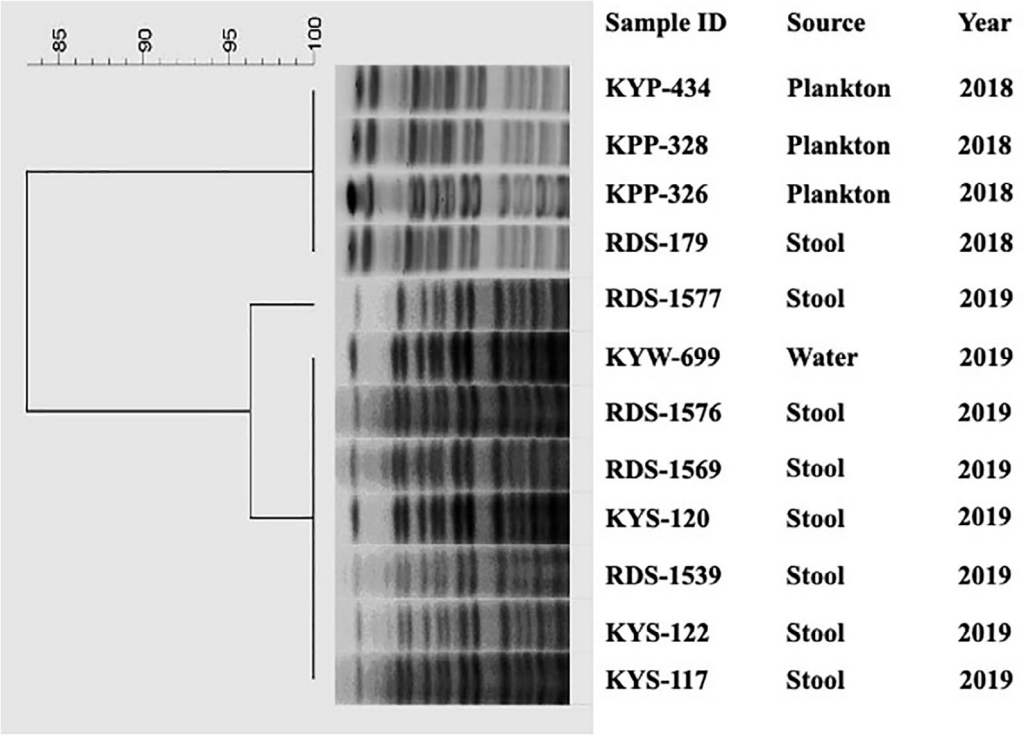

Fig 2. PFGE patterns of the *Not*I digested *V. cholerae* O1 strains isolated from Rayagada district of Odisha with the dendrogram analysis using Bionumerics software (Applied Maths, Sint-Martens-Latem Belgium).

Another possible reason could be the availability of nutrients on the mucilaginous surface of plankton secreted extracellularly which helped the *V. cholerae* to get nutrients during unfavourable conditions (Islam *et al*., 1989).

The PFGE analysis from the above study clearly indicated that the *V. cholerae* O1 strains isolated from stool samples and plankton during 2018 belonged to the same pulsotype. Again the *V. cholerae* O1 strains (clinical and water) isolated from the cholera outbreak villages from the Kalyansinghpur area in 2019 had the same pulsotype. Similar studies were reported from Nepal and Thailand (Chomvarin *et al*., 2013; Dixit *et al*., 2014).

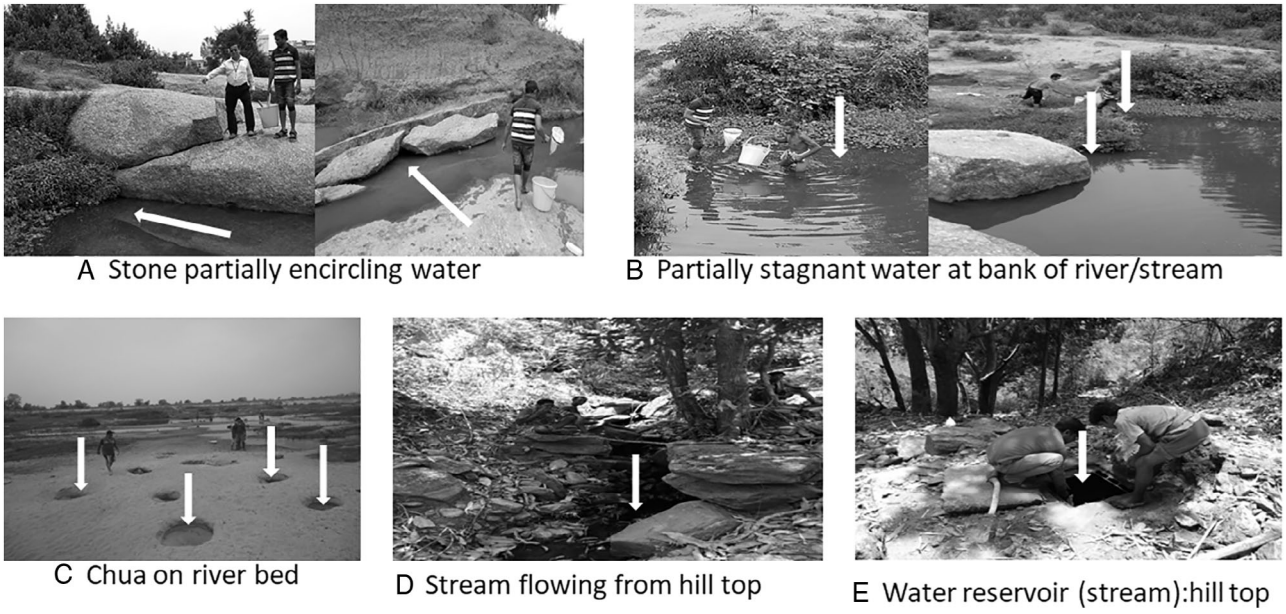

Fig 3. Arrows indicate the sampling sites which were positive for *V .cholerae* serogroups in the river, stream and chua (A–E) from the Rayagada district.

The detection of environmental reservoirs of *V. cholerae* serogroups in the temporary stagnant condition of water; partially encircled by stones, and near the bank of river, nala, stream and chua from the tribal areas is further strengthened by different PCR assays and PFGE analysis, which is a breakthrough of this study. Therefore, this is a rare and novel report to document the environmental reservoirs of *V. cholerae* O1 strains in the flowing freshwater environs from the tribal areas of Odisha, India and Globe.

The present study distinctly established that the flowing water bodies partially encircled by stones, and temporary stagnant condition of water near the bank of river, nala and stream in the tribal areas of the Rayagada district of Odisha were the environmental reservoirs of *V. cholerae* different serogroups (Fig. 3). Different mPCR assays indicated similar results for the presence of different toxic and virulence genes for *V. cholerae* O1 strains isolated from different sources. The *V. cholerae* strains isolated from different sources like diarrhoea patients, water and plankton were also clonal in nature as indicated by PFGE analysis. This is a rare report to document the environmental reservoirs of *V. cholerae* O1 strains in the flowing freshwater environs from the tribal areas of Odisha, India and Globe. Similar studies are warranted to validate these findings in other parts of the tribal areas from this state.

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