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Origin and Dissemination of Altered El Tor *Vibrio cholerae* O1 Causing Cholera in Odisha, India: Two and Half Decade's View

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The origin, spread and molecular epidemiology of altered El Tor *Vibrio cholerae* O1 strains isolated from cholera outbreaks/surveillance studies between 1995 and 2019 from different district of Odisha were analyzed. The stock cultures of *V. cholerae* O1 strains from 1995 to 2019 were analyzed through molecular analysis using different PCR assays and pulse field gel electrophoresis (PFGE) analysis. The spread map (month, year and place) was constructed to locate the dissemination of altered El Tor variants of *V. cholerae* O1 in this region. A total of 13 cholera outbreaks were caused by *V. cholerae* O1 Ogawa biotype El Tor carrying *ctx*B1 and *ctx*B7 genotypes. The *ctx*B1 alleles of *V. cholerae* O1 mostly confined to the coastal areas, whereas the *ctx*B7 genotypes, though originating in the coastal region of Odisha, concentrated more in the tribal areas. The positive correlation between virulence-associated genes (VAGs) was found through Pearson's correlation model, indicative of a stronger association between the VAGs. The clonal relationship through PFGE between *ctx*B1 and *ctx*B7 genotypes of *V. cholerae* O1 strains exhibited 80% similarity indicating single- or multi-clonal evolution. It is evident from this study that the spread of multidrug-resistant *V. cholerae* O1-altered El Tor was dominant over the prototype El Tor strains in this region. The origin of altered El Tor variants of *V. cholerae* O1 occurred in the East Coast of Odisha established that the origin of cholera happened in the Gangetic belts of Bay of Bengal where all new variants of *V. cholerae* O1 might have originated from the Asian countries.

Keywords: origin, spread, *V. cholerae* O1, *ctx*B1 and *ctx*B7 genotypes, Odisha

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INTRODUCTION

Cholera is a severe form of watery diarrheal disease, dates back to antiquity, and is caused by the ingestion of food or water contaminated with the pathogenic strains of *Vibrio cholerae* of serogroup O1 or O139 (Pollitzer, 1954). *Vibrio cholerae* is an autochthonous inhabitant of the estuarine aquatic environment of the Bay of Bengal (Alam et al., 2006), where low salinity of rivers or shallow wells (avg. 2.8–8.2 ppt) and temperatures between 26°C and 35°C during the dry season favors the growth and multiplication of *V. cholerae* (Moore et al., 2014). *V. cholerae* has evolved into more pathogenic types due to various genetic assortments and re-assortments in the core toxin region which gives rise to altered and hybrid variants of prototype *V. cholerae* strains (Faruque et al., 2004), causing higher mortality with recorded 21,000–143,000 deaths worldwide (Ramamurthy et al., 2019). The biotype El Tor of *V. cholerae* O1 has been changing the whole disease scenario perpetually due to its better survival capacity in the environment as well as in

the human host and is able to produce significantly higher amounts of cholera toxin (CT) *in vivo* (Ghosh-Banerjee et al., 2010; Grim et al., 2010). Among different El Tor variants, the altered El Tor *V. cholerae* O1 strains have prevailed in different regions of the world, including the US Gulf coast and several countries of Asia and Africa (Nair et al., 2006; Goel et al., 2010; Pal et al., 2017). Odisha, situated at the eastern coast of India, has recorded several cholera outbreaks/epidemics. The cholera epidemics after the disastrous super cyclone in 1999 affected more than 10 million of the population. This might be due to coastal saline-rich aquatic environmental water along the side of Bay of Bengal favoring *V. cholerae* to spread its territory. Cholera has been reported from Odisha over the last two and half decades (Chhotray et al., 2002; Khuntia et al., 2010; Pal et al., 2010, 2017). However, no detailed molecular epidemiological report is available on the origin and dissemination of the altered El Tor *V. cholerae* O1 strains to different parts of Odisha. In this study, we have undertaken a retrospective analysis on *V. cholerae* O1 strains which were isolated during the cholera outbreaks and surveillance studies reported from 1995 to 2019 from different districts of Odisha to illustrate the chronology of the appearance and spread of *V. cholerae* O1 El Tor variant strains to different parts of the state.

TABLE 1 | Year-wise isolation of *Vibrio cholerae* O1 strains from different districts of Odisha: 1995–2019.

Year	No of strains	*V. cholerae* serogroup	Isolation places (district)
1995	16	O1 Ogawa	Cuttack
1996	7	O1 Ogawa	Cuttack
1997	17	O1 Ogawa	Cuttack
1999	65	O1 Ogawa	Cuttack, Jagatsinghpur, Puri, Kendrapara, Jajpur, Balasore, Bhadrak
2000	87	O1 Ogawa	Jagatsinghpur, Puri, Berhampur, Jajpur
2001	58	O1 Ogawa	Puri, Kendrapara, Jajpur, Bhadrak, Balasore, Khordha
2002	82	O1 Ogawa	Puri, Gajapati, Rayagada
2003	160	O1 Ogawa	Puri, Dhenkanal, Keonjhar, Mayurbhanj, Malkangiri
2004	47	O1 Ogawa/Inaba	Puri, Khordha, Dhenkanal
2005	86	O1 Ogawa/Inaba	Cuttack, Puri, Khordha, Kendrapara, Dhenkanal
2006	62	O1 Ogawa/Inaba	Cuttack, Puri, Kendrapara
2007	166	O1 Ogawa/Inaba	Jagatsinghpur, Khordha, Rayagada, Koraput
2008	128	O1 Ogawa/Inaba	Cuttack, Jagatsinghpur, Khordha, Puri, Sundergarh
2009	71	O1 Ogawa	Puri, Khordha, Kendrapara, Mayurbhanj, Rayagada, Kalahandi, Sundergarh
2010	67	O1 Ogawa	Gajapati, Rayagada, Kalahandi
2011	57	O1 Ogawa	Rayagada, Gajapati
2012	153	O1 Ogawa	Rayagada, Kalahandi, Koraput
2013	96	O1 Ogawa	Rayagada, Koraput
2014	11	O1 Ogawa	Kalahandi
2015	1	O1 Ogawa	Nuapada
2016	4	O1 Ogawa	Balasore, Rayagada, Nuapada
2018	5	O1 Ogawa	Bargarh, Rayagada, Kalahandi
2019	31	O1 Ogawa	Rayagada
Total	1492		

MATERIALS AND METHODS

Revival of Strains

A total of 1,492 strains of *V. cholerae* O1 isolated from the coastal and tribal areas of Odisha from 1995 to 2019 during cholera outbreaks and surveillance studies were included in this study (**Table 1**). Thiosulfate–citrate–bile–salt–sucrose (TCBS) agar was used to isolate the *V. cholerae* strains isolated from stool and environmental water samples. Big moist yellow colonies from TCBS agar plates were selected for different biochemical tests (Pal et al., 2010), and serotyping was done by slide agglutination tests with polyvalent O1 and monospecific Ogawa, Inaba antisera (BD, San Jose, CA, United States). In addition, isolated colonies of *V. cholerae* strains from TCBS agar plates were re-streaked on Luria Bertani agar (BD, United States), incubated at 37°C for 24 h and then inoculated in Luria Bertani broth (BD, United States), kept in a shaker incubator at 37°C overnight for bacterial growth, and subsequently used for DNA isolation using the boiling method (Pal et al., 2017).

Multiplex PCR Assays

All phenotypically confirmed *V. cholerae* isolates were further confirmed by multiplex PCR (mPCR) assays by targeting the genes of *V. cholerae*, such as species-specific gene *omp*W, outer membrane protein (*omp*U) gene, identifying genes encoding O1 (*rfb*O1) and O139 (*rfb* O139), and major virulence and toxic genes *ctx*A, *tcp*A, *ace*, *tox*R, and *rtx*C (Kimsey and Waldor, 1998; Nandi et al., 2000; Chow et al., 2001).

Analysis of Virulence Genes

The presence/absence of eight virulence-associated genes in each isolate obtained from 1995 to 2019 was used to define the virulence-associated gene profile. The profile was developed by PCR analysis by calculating the percentage of virulence-associated genes present in each strain of *V. cholerae* O1. The profiles of all isolates were analyzed by hierarchical clustering using a complete linkage method. The dendrogram and a heat map were constructed using the PAST 4.03 software and GraphPad Prism 7 statistical package, respectively. Pearson's correlation coefficient analysis was employed to identify the correlation between the virulence genes (Prajapati et al., 2020).

*ctx*B Genotyping

Mismatch amplification mutation assay (MAMA) and double-mismatch amplification mutation assay (DMAMA) PCR reactions were used to detect the type of *ctx*B in all the laboratory stocks of *V. cholerae* O1 strains isolated during cholera outbreaks and surveillance studies from 1995 to 2019 (Morita et al., 2008; Naha et al., 2012).

TABLE 2 | Antibiotic resistance profiles with *ctx*B genotypes of *Vibrio cholerae* O1 associated with different cholera outbreaks in Odisha: 1999–2019.

Outbreak year	Affected districts	Population affected	Cases reported	Incidence rate (IR)/1,000 individual	Serotypes (%)	Antibiogram resistance profile	Genotypes
1999	Cuttack, Jagatsinghpur, Kendrapara, Jajpur, Puri, Bhadrak, Balasore	8,043,000	97,934	12.18	O1 (Ogawa) = 72.3 O139 = 7.2	AFrCoSNaN	*ctx*B1
2000	Jagatsinghpur, Puri	2,200,000	198	0.09	O1 (Ogawa) = 58.6 O139 = 40.2 Non O1/O139 = 1.2	FrCoNaN	*ctx*B1
2003	Dhenkanal	946	41	43.34	O1 (Ogawa) = 66.7	AFrCoSNa	*ctx*B1
2005	Dhenkanal	2,102	113	53.76	O1 (Ogawa) = 22.2 O1 (Inaba) = 66.7	FrNa	*ctx*B1
2006	Cuttack	10,621	146	13.75	O1 (Ogawa) = 82.6	FrCoSNa	*ctx*B1
2007	Rayagada, Koraput, Kalahandi, Gajapati	123,546	8,206	66.42	O1 (Ogawa) = 94.9 O1 (Inaba) = 2.6	AmFrSNNfNaCo	*ctx*B1
2009	Kendrapara (Rajnagar), Mayurbhanj	108,500	809	7.45	O1 (Ogawa) = 67	AmFrCoCCfSNa	*ctx*B7
2010	Rayagada, Gajapati	113,375	2,152	18.98	O1 (Ogawa) = 51.5	AmTeNaFrSECoNC	*ctx*B1
2012	Rayagada, Kalahandi	735,647	641	0.87	O1 (Ogawa) = 42	AmNaFrSCo	*ctx*B1, *ctx*B7
2014	Kalahandi	46,236	321	6.94	O1 (Ogawa) = 64.7	AmFrNaNECGCo	*ctx*B7
2016	Balasore, Rayagada	59,937	138	2.30	O1 (Ogawa) = 88.9	CFrCoSNaN	*ctx*B7
2018	Bargarh	1,387	55	39.65	O1 (Ogawa) = 13.3	AFrCoSNa	*ctx*B7
2019	Rayagada	500	73	146.00	O1 (Ogawa) = 50 Non O1/O139 = 7.1	AFrCoSNaN	*ctx*B7
	Total	1,145,0199	110,894				

Pulsotyping by Pulsed-Field Gel Electrophoresis

Pulsotyping by pulsed-field gel electrophoresis (PFGE) was performed on some selected strains of *V. cholerae* O1 isolated from 1995 to 2019. The DNA sample of *V. cholerae* O1 in agarose plugs was digested with 50 U of *Not*I (New England Biolabs, Ipswich, MA, United States). The digested DNA was separated through 1% agarose gel (Bio-Rad, Hercules, CA, United States) in 0.5× TBE buffer (pH 8.4) at 14°C in a CHEF Mapper system (Bio-Rad, United States). The dendrograms were constructed on the basis of banding similarity and dissimilarity using the Dice coefficient, and clustering was based on the unweighted pair group method with arithmetic mean (UPGMA) with a band position tolerance of 1.2% (Cooper et al., 2006).

RESULTS

Distribution of *V. cholerae* O1 Strains in Endemic Districts

Odisha, situated at the eastern coast of India, has encountered 13 cholera outbreaks from 1999 to 2019. Details of the epidemiological data of these cholera outbreaks are depicted in **Table 2**. It is evident that mostly coastal districts, namely, Cuttack, Jagatsinghpur, Puri, Kendrapara, Jajpur, Balasore, and Bhadrak, were the most affected districts due to cholera in Odisha. Later, the disease was transmitted to the tribal districts of Odisha, namely, Rayagada, Koraput, Kalahandi, Gajapati, and Keonjhar (**Figure 1**). There were more than 3 million people at risk for cholera in these five tribal districts of Odisha, where cholera has become endemic with repeated waves of outbreaks that occurred in the recent past. The average incidence rate of cholera cases in these endemic areas is 39.89 cases/1,000 population at risk per year.

Genotypic Characteristics of Cholera Strains

A total of 1,492 *V. cholerae* strains isolated from 1995 to 2019 were serologically confirmed to be *V. cholerae* O1. During this study period, *V. cholerae* O1 strains belonging to serotype Ogawa exhibited dominance (92.4%) over serotype Inaba (7.6%) and each strain of *V. cholerae* O1 was positive for the cholera toxin gene, i.e., *ctx*A. In addition, El Tor biotype-specific toxin co-regulated pilus ($tcpA^{ET}$) showed dominance from 1995 to 2006, in contrast to $tcpA^{Haitian}$ type strains that showed dominance from 2007 to 2019. All the *V. cholerae* O1 strains also showed positive results for virulent and accessory genes such as *omp*W, *omp*U, *rtx*C, *tox*R, and *ace* that regulate the toxin production of *V. cholerae*. From the MAMA PCR assay, it was evident that the El Tor *ctx*B3 genotype of *V. cholerae* O1 emerged in 1999 during the super cyclone, became quiescent up to 2004, then increased gradually from 2005 to 2011 (except in 2006) and subsequently disappeared. The DMAMA PCR assay revealed that *V. cholerae* O1 strains isolated from 1995 to 2012 including outbreak strains possessed the *ctx*B1 genotype (classical type CT) except the cholera outbreak in 2009 that happened due to the *ctx*B7 genotype (Haitian type CT). Between 2012–2019, the outbreak strains with the Haitian ctxB7 allele circulated and predominated within the different tribal districts of Odisha (**Table 2**). The hierarchical clustering analyses of *ctx*B alleles isolated in different

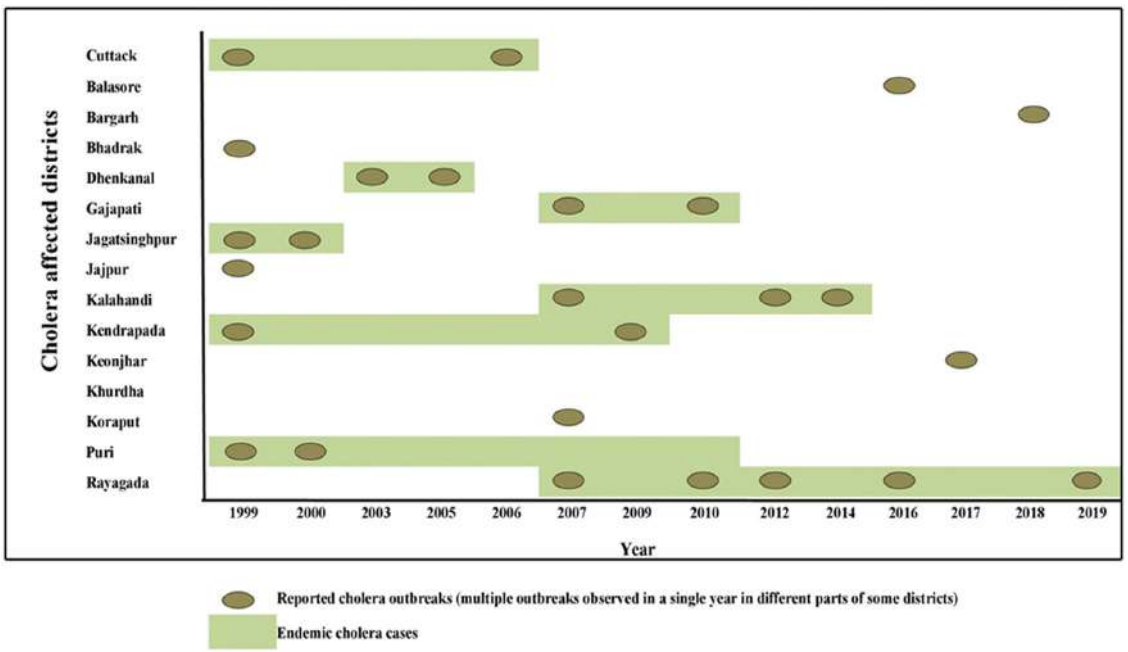


FIGURE 1 | Distribution of cholera cases and outbreaks in Odisha: 1999–2019.

time periods from 1995 to 2019 showed three separate clusters indicative of three different lineages with significant relatedness distributed among the strains of *V. cholerae* O1 (**Figure 2A**).

Profiling of Virulence-Associated Genes

The mPCR assay on virulence-associated genes (VAGs) on 306 randomly selected *V. cholerae* O1 strains showed variation ranging from 50 to 100% as indicated by different color intensities in a heat map. The correlation analysis showed a significant positive correlation between VAGs except for the somatic O-antigen biosynthesis gene (*rfb*O1) which showed a negative correlation with other VAG genes such as outer-membrane protein gene (*omp*W), cholera toxin gene (*ctx*A), toxin co-regulated pilus gene (*tcp*A), outer-membrane protein gene (*omp*U), repeat in toxin protein (*rtx*C), toxin regulator gene (*tox*R), and the accessory cholera enterotoxin gene (*ace*). Pearson's correlation coefficient values of each VAGs are presented in **Table 3**. The scatter plot for correlation values and the heat map are presented in **Figures 3A,B**, respectively. Hierarchical clustering based on VAGs identified in different strains from 1995 to 2019 showed discrete clusters indicative of insignificant relatedness between percentages of VAGs present in different years or their non-uniform distribution among the *V. cholerae* O1 strains (**Figure 2B**).

Spread of Altered El Tor *V. cholerae* O1

Wave of Cholera Toxin Genotype 1 (Classical Cholera Toxin)

The El Tor variant strains of *V. cholerae* O1 carrying classical CT genotype 1 (*ctx*B1) emerged in the coastal regions of Odisha in 1995. The first case was reported from Cuttack in July 1995. Out of 25 *V. cholerae* isolates, 23 were *V. cholerae* O1 Ogawa biotype El Tor carrying classical CT, and 3 were *V. cholerae* O139 serogroup. The gradual dissemination of the *ctx*B1 allele of *V. cholerae* O1 in the coastal districts, namely, Jagatsinghpur, Kendrapara, Puri, Balasore, Bhadrak, and Jajpur, occurred in successive years and propounded into large cholera outbreaks in 1999, 2000, 2003, 2005, 2006, 2007, 2010, and 2012 with 109,431 reported cholera cases (**Table 2**). The El Tor variant strains carrying classical *ctx*B spread to the tribal districts of Odisha in 2007. The first case was reported in August 2007 in the Kashipur block of Rayagada district, subsequently spread to adjacent districts in successive years. The detailed outline of the spread of the *ctx*B1 allele of El Tor variant *V. cholerae* O1 is predicted in **Figure 4A**.

Wave of Cholera Toxin Genotype 7 (Haitian Cholera Toxin)

A new variant of the El Tor biotype of *V. cholerae* O1-producing Haitian cholera toxin (Haitian CT) emerged from the super cyclone of Odisha in October 1999. The first cholera case possessing *ctx*B genotype 7 was reported from the Erasama block of Jagatsinghpur district in November 1999. Later, the Haitian variant strains reemerged from the tribal areas of Rayagada and Koraput districts in August and November 2007 and then spread to coastal areas of Puri in November 2007. The outbreaks continued in the successive years by *ctx*B7 genotypes of *V. cholerae* O1 to neighboring coastal districts, namely, Cuttack, Khordha, and Puri, in July 2008. Later, the *ctx*B7 genotypes of *V. cholerae* O1 spread and caused large cholera outbreaks in Kendrapara and Mayurbhanj districts in 2009 by affecting 104,327 populations with 783 reported cases.

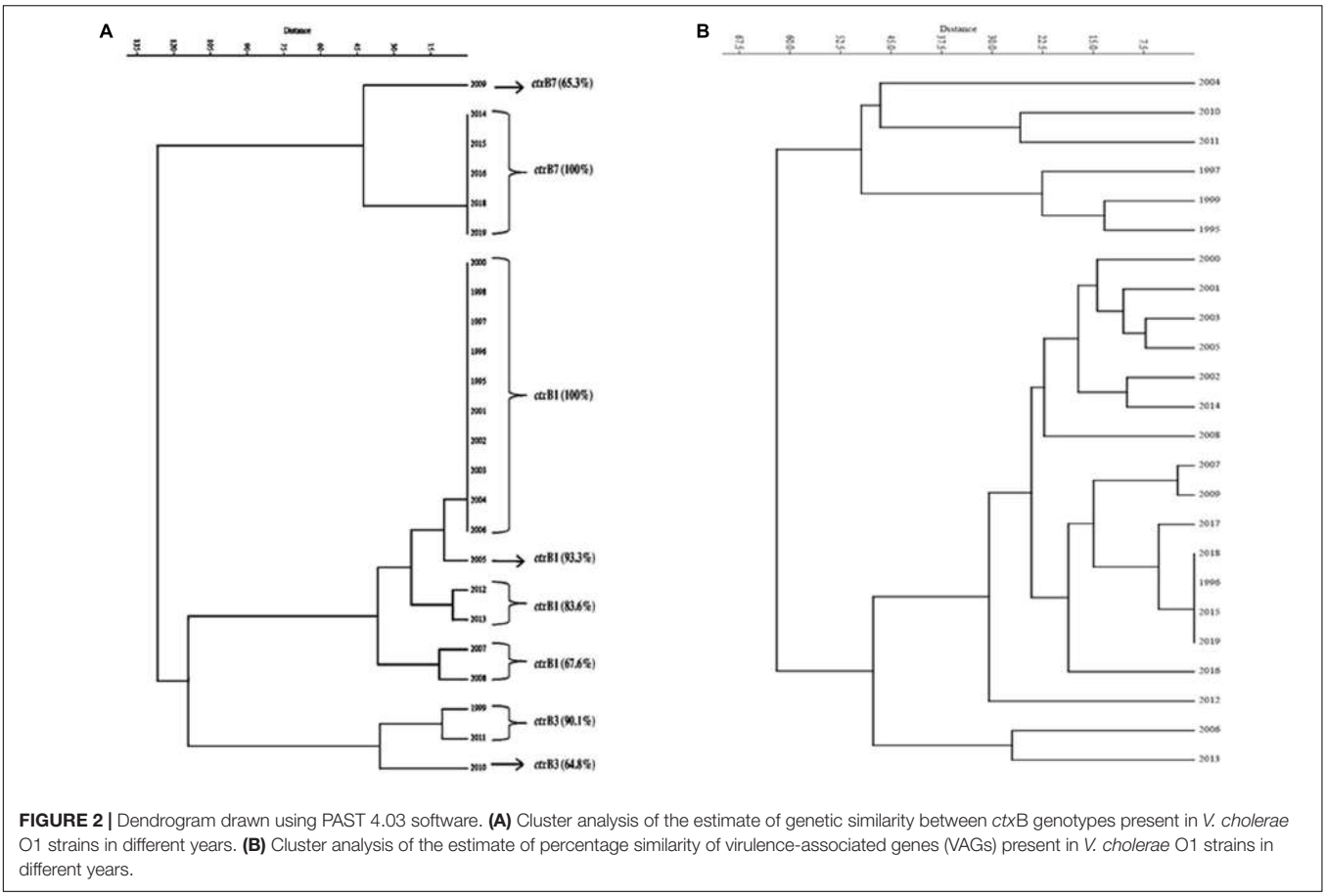


FIGURE 2 | Dendrogram drawn using PAST 4.03 software. **(A)** Cluster analysis of the estimate of genetic similarity between *ctx*B genotypes present in *V. cholerae* O1 strains in different years. **(B)** Cluster analysis of the estimate of percentage similarity of virulence-associated genes (VAGs) present in *V. cholerae* O1 strains in different years.

Subsequently, the transmission of Haitian variant *V. cholerae* O1 strains was confined to the tribal areas of Odisha particularly in Rayagada, Kalahandi, and Koraput districts. The spread map of the *ctx*B7 allele of *V. cholerae* O1 biotype El Tor is shown in **Figure 4B**. This showed that the HCT variant strains of *V. cholerae* O1 have spread predominantly to the eastern and southern parts of Odisha in 2010, 2011, 2012, 2014, 2016, and 2018 and to a lesser extent to western and northern parts of Odisha in 2019. The month- and village-wise spread of cholera cases during the 2010 cholera outbreak in three tribal blocks, i.e., Kashipur, Kalyansinghpur, and Bissam Cuttack of Rayagada district, due to HCT variant *V. cholerae* O1 Ogawa has been described, which is very interesting (**Figure 5**).

Severe Cholera Cases Among Tribal Populations

The spread of altered El Tor strains of *V. cholerae* O1 among the tribal areas has the connecting link with the coastal outbreak strains, evident through the sequential occurrence of cholera cases. In total, 520 *V. cholerae* O1 strains were collected from the tribal areas of Odisha from 2002 to 2019 from Rayagada, Kalahandi, Koraput, Gajapati, Keonjhar, Sundergarh, Mayurbhanj, Nuapada, and Boudh (**Table 4**). The first appearance of hybrid El Tor strains carrying classical CT was reported from the Mohana block of Gajapati district in 2002. This hybrid strain of *V. cholerae* O1 emerged as an epidemic form in August 2007 in the four tribal districts, namely, Rayagada, Gajapati, Kalahandi, and Koraput, by affecting 358 villages with a population at risk of 123,546 (Pal et al., 2010). The altered El Tor strains of *V. cholerae* remained in the environmental reservoirs and reemerged as epidemic strains in 2010 in the tribal areas of Rayagada and Gajapati districts causing cholera outbreaks, accounting for 2,152 cholera cases. Interestingly, all the isolates of *V. cholerae* O1 showed resistance to tetracycline,

TABLE 3 | Correlation between virulence genes of *Vibrio cholerae* O1 strains using Pearson's correlation coefficient model.

	***omp*W**	***ctx*A**	***rfb*O1**	***tcp*A**	***omp*U**	***rtx*C**	***tox*R**	***ace***
*omp*W	1							
*ctx*A	0.09	1						
*rfb*O1	−0.11	−0.08	1					
*tcp*A	0.71	0.58	−0.19	1				
*omp*U	0.28	0.57	−0.13	0.41	1			
*rtx*C	0.42	0.74	−0.17	0.68	0.71	1		
*tox*R	0.19	0.84	−0.12	0.49	0.72	0.65	1	
ace	0.22	0.76	−0.22	0.54	0.77	0.74	0.84	1

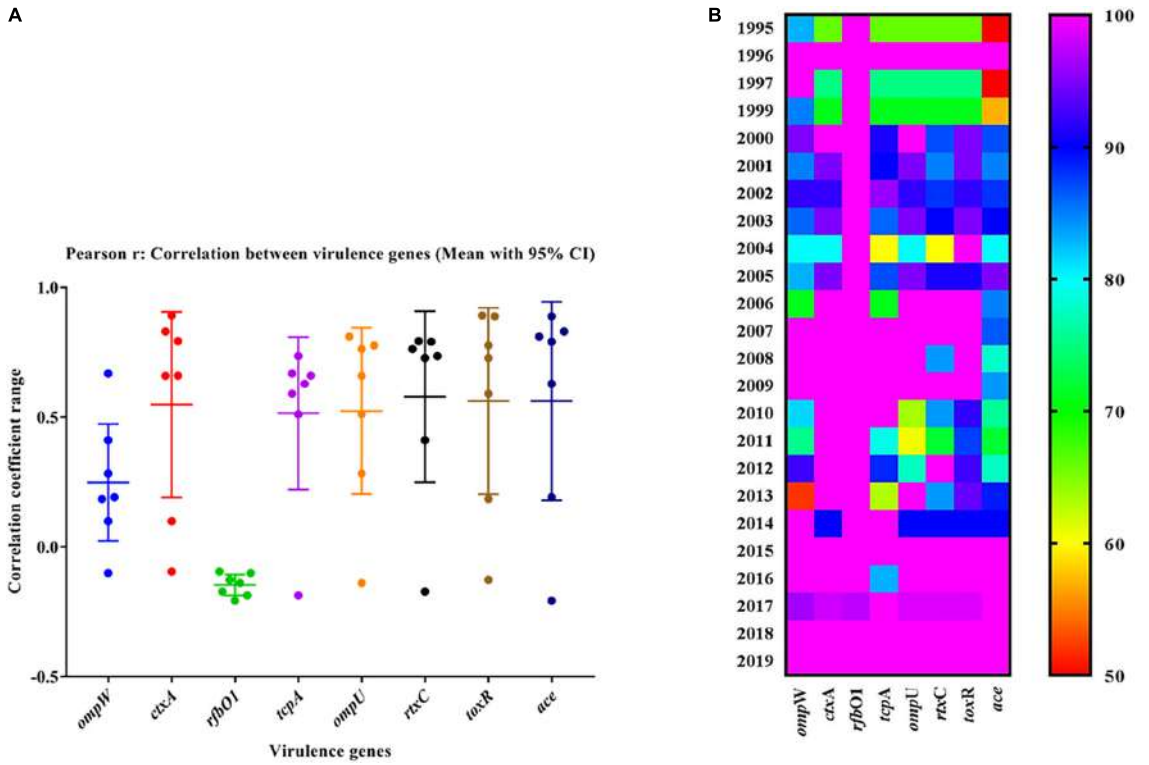


FIGURE 3 | (A) Scatter plot of the correlation between virulence genes of *Vibrio cholerae* O1 strains. The data represent different magnitudes of correlation coefficient (r) ranges between –0.5 and 1.0. A positive correlation indicated a stronger association between the variables and vice versa. The greater the absolute value of the Pearson product–moment correlation coefficient, the stronger the *linear* relationship. **(B)** Heat map showing the percentage variation in different virulence-associated genes distributed among the strains in different years.

a unique feature first reported from cholera outbreaks in the tribal areas of Odisha (Kar et al., 2015). The cholera infection again returned to some parts of Rayagada and Kalahandi in 2012 due to a mixed infection of *ctx*B1 and *ctx*B7 alleles of *V. cholerae* O1 Ogawa biotype El Tor, which accounted for 641 cholera cases. The first case of the Haitian variant (*ctx*B7 genotype) strain of *V. cholerae* O1 from tribal areas was obtained from the cholera outbreak in 2009 from Mayurbhanj district with a total of 94 cases which were reported with one death (CFR—1.06%). Subsequently, the spread of the *ctx*B7 allele of *V. cholerae* O1 in the tribal areas was reported from the Kalahandi district in 2014, then from Rayagada and Balasore districts in 2016, and reappeared in a recent cholera outbreak from Rayagada district in 2019. A total of 575 cholera cases were reported from these cholera outbreaks with an incidence rate of 40.29% and a case fatality rate (CFR) of 10.13%. The common resistance profile of isolated *V. cholerae* O1 strains obtained in these outbreaks was ampicillin, nalidixic acid, furazolidone, streptomycin, neomycin, erythromycin, and co-trimoxazole (Pal et al., 2017, 2021; Nayak et al., 2020).

Pulse Field Gel Electrophoresis Analysis

A PFGE analysis of *Not*I digested genomic DNA of 90 representatives of *V. cholerae* O1 strains isolated from stool and environmental water sources from different regions of Odisha over the past two and half decades were performed. The majority of strains (n = 73) belonged to two major clusters A and B, while the rest of the strains (n = 17) showed discrete patterns and thus belonged to four different banding patterns C, D, E, and F (**Figure 6**). Therefore, it is evident that the spread of El Tor variant strains of *V. cholerae* O1 throughout Odisha might have originated from a single clone or developed from multi-clonal emergence of the El Tor variants of *V. cholerae* O1 in each region of Odisha.

Cluster A

Cluster A includes 46 strains of *V. cholerae* O1 that showed close relatedness among the clinical and environmental samples, as the PFGE banding patterns were highly homogenous (100% similarity). For example, the environmental water isolates of *V. cholerae* O1 from Rayagada (KPW-3/2007) possessing the El Tor variant *ctx*B1 genotype exhibited 100% similarity with Rayagada and Kalahandi outbreak strains of 2012 carrying both *ctx*B1 and *ctx*B7 genotypes and also showed similar banding patterns with Bargarh and Rayagada outbreak strains of 2018 and 2019, respectively, where both *ctx*B7 genotypes were reported (**Figure 6**). It was also interesting to note that typical El Tor strains carrying the *ctx*B3 genotype had a close relationship with El Tor variant strains of *ctx*B1 and *ctx*B7 genotypes. The genetic relatedness linking the coastal

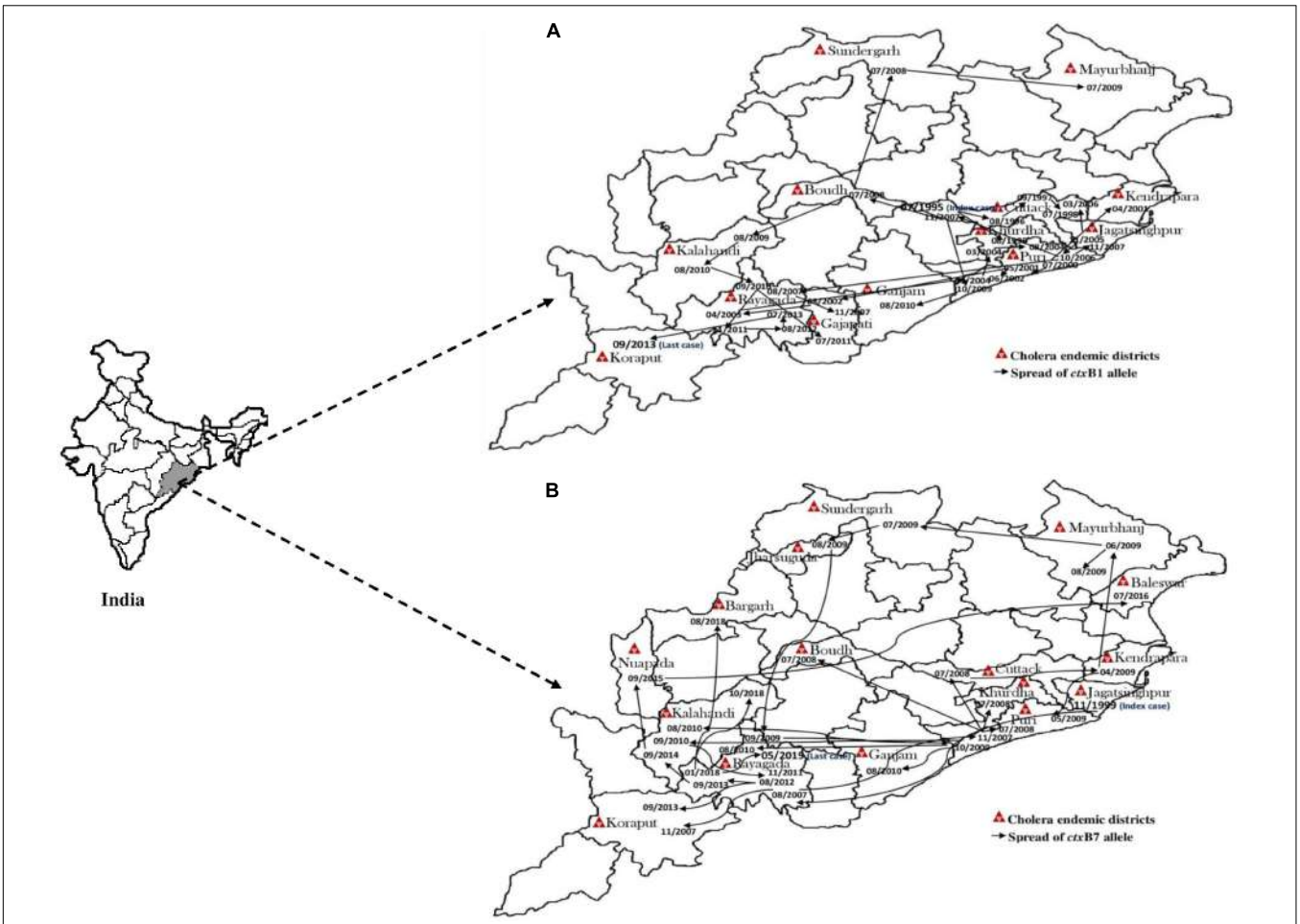


FIGURE 4 | Month- and year-wise spread of El Tor variant *Vibrio cholerae* O1 strains. **(A)** Spread of *ctx*B1 **(B)** spread of *ctx*B7 genotypes in Odisha: 1995–2019.

and tribal strains of *V. cholerae* O1 was also proved by a 95% similarity coefficient obtained between isolates from these districts.

Cluster B

Cluster B includes 27 strains of *V. cholerae* O1 that showed a 90% similarity coefficient with cluster A. This cluster showed an interrelationship between *ctx*B3 with *ctx*B1 and *ctx*B7 genotypes. The *V. cholerae* O1 strains from Puri, Kalahandi, Nuapada, and Gajapati possessed the *ctx*B3 genotype, i.e., prototype El Tor strain of *V. cholerae* O1 characteristic, which exhibited 92% similar banding patterns with *V. cholerae* O1 strains of altered El Tor genotypes isolated from the coastal as well as tribal districts of Odisha from 1995 to 2019. This might indicate that mutation of prototype El Tor strains occurred in due course of time that produced varied genotypes but showed nearly similar banding patterns.

Clusters C and D

The clusters C and D showed discrete banding patterns composed of six different strains of *V. cholerae* O1.

Cluster E

Cluster E showed an interesting result that comprised 10 *V. cholerae* O1 strains of *ctx*B7 genotype isolated from both the coastal and tribal areas of Odisha that showed the first reported Haitian variant strain (CY-44/1999) having a close link with outbreak strains of Balasore and Rayagada (2016) strains and 94% similarity with Narla outbreak strain of Kalahandi district (2014) and Nuapada (2015) strain. The PFGE analysis of El Tor variant *V. cholerae* O1 strains from Odisha proved that each strain has clonality with the other as more than 80% similarity index was obtained between them.

DISCUSSION

Cholera has been a major public health concern in the coastal areas in both Bangladesh and India for centuries (Ali et al., 2015). Odisha being a coastal state of India has faced recurrent cholera outbreaks/epidemics followed by cyclone/flood almost every year causing significant morbidity and mortality since 1995–2019 (Nayak et al., 2021). Transmission of cholera in the coastal communities is getting higher due to increased global

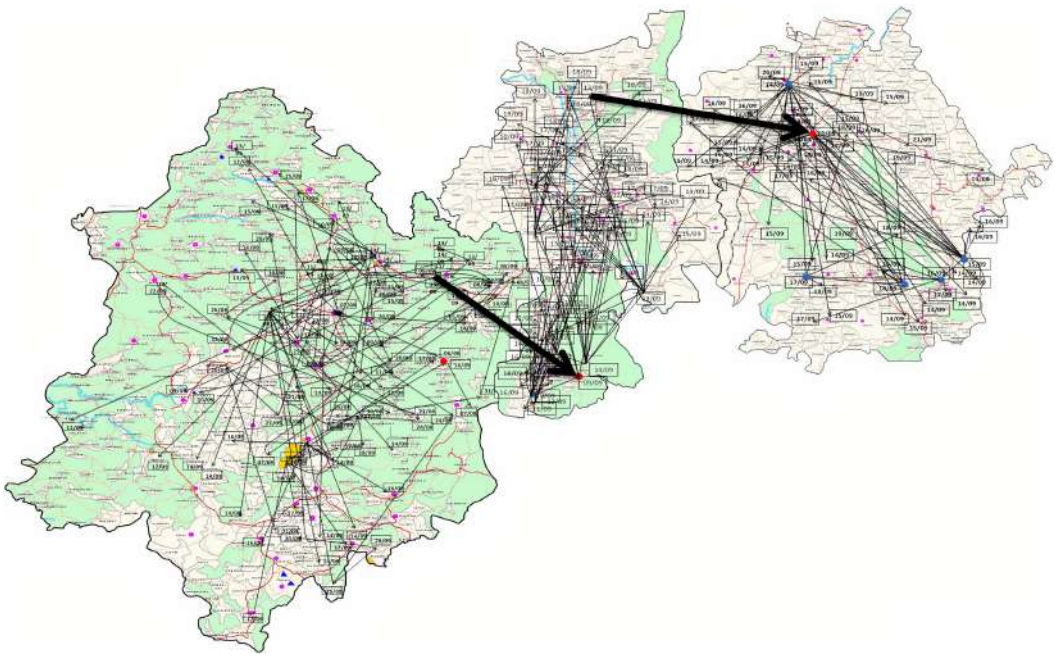


FIGURE 5 | Spread of *V. cholerae* O1 biotype El Tor in three blocks of Rayagada district (Kashipur, Kalyansinghpur, and Bissam Cuttack): August–September, 2010.

temperature and the saline-rich water that favors *V. cholerae* and other pathogenic bacteria to flourish and spread across boundaries (Colwell, 2004). At present, altered El Tor strains of *V. cholerae* O1 that were identified in the isolates from 1995 onward replaced the normal prototypes of El Tor strains causing almost all cholera outbreaks and epidemics throughout Odisha. Our earlier studies proved that *V. cholerae* O1 strains isolated from 1995 to 2006 were El Tor variant strains with a *ctx*B classical genotype that had been circulated in Odisha (Pal et al., 2010). However, hybrid El Tor variant strains of *V. cholerae* O1 were also reported from Odisha in 2008 and 2009 that possessed *ctx*B genes of both classical and El Tor biotypes (Khuntia et al., 2013). The hybrid strains of *V. cholerae* O1 have also been reported from other parts of India and Thailand (Taneja et al., 2009; Na-Ubol et al., 2011).

The altered biotype of *V. cholerae* O1 El Tor (classical CT) strains belonged to the Gulf Coast of the United States, designated as genotype 1 (Olsvik et al., 1993). Later the altered El Tor strains carrying classical CT have been reported from different regions of the globe particularly Bangladesh, Mozambique, Vietnam, Hong Kong, Japan, Zambia, India, Sri Lanka, Africa, Nigeria, and Haiti (Nair et al., 2002; Raychoudhuri et al., 2009; Safa et al., 2010; Marin et al., 2013). In Odisha, these strains were first reported in 1995 from Cuttack and later found from other coastal districts, namely, Jagatsinghpur, Kendrapara, Jajpur, Puri, Bhadrak, and Balasore, during the 1999 super cyclone. The data also supported the fact that *V. cholerae* O1 El Tor variant strains were circulating in the tribal districts of Odisha subsequent to the 2007 cholera epidemic (Pal et al., 2010). Previously, changes in the amino acid sequences in *ctx*B of El Tor strains at positions 39 and 68 had been reported and these sequences were similar to those of classical strains (Kumar et al., 2009). However, the emergence of new El Tor variant strains with a modified classical CT due to mutation at amino acid position 20 (histidine–asparagine) has been described as Haitian variant reported elsewhere (Naha et al., 2012). This Haitian *ctx*B strain arose in fame after causing a devastating epidemic in Haiti in 2010. The altered El Tor strains carrying the *ctx*B7 genotype (HCT variant) were first reported from Odisha in 1999 much before the outbreak in Haiti in 2010 (Pal et al., 2017); subsequently reported in Kendrapara district in 2009 and spread to tribal areas of Rayagada and Kalahandi districts in 2012–2019 (Pal et al., 2013, 2017, 2021; Nayak et al., 2020). This strain was also reported from sporadic cholera cases in Kolkata and Yavatmal (Kutar et al., 2013; Kumar et al., 2014); in Africa and Yemen during 2015–2017 (Weill et al., 2019). In Odisha, there is a progressive increasing trend of antibiotic resistance toward commonly used antibiotics such as ampicillin, streptomycin, neomycin, nalidixic acid, co-trimoxazole, and furazolidone, which were used as the first line of treatment for cholera (Nayak et al., 2021). Similar findings have been reported in Ghana and the Democratic Republic of Congo (Miwanda et al., 2015; Abana et al., 2019). Tetracycline-resistant strains of *V. cholerae* O1 were reported in 2010 from the tribal areas of Odisha, but its reversal was observed in successive years (Pal et al., 2018). This phenomenon might be due to the extensive use of tetracycline or due to rapid fluctuations in nature.

In this study, we found that 1,380 isolated *V. cholerae* O1 serotype Ogawa strains had dominance (92.4%) over serotype Inaba (7.6%) and were responsible for all the cholera outbreaks reported from 1999 to 2019. The pathogenesis of cholera is conferred due to synergistic actions of core CTX elements and the TCP pathogenicity island (Faruque et al., 1998). All isolates

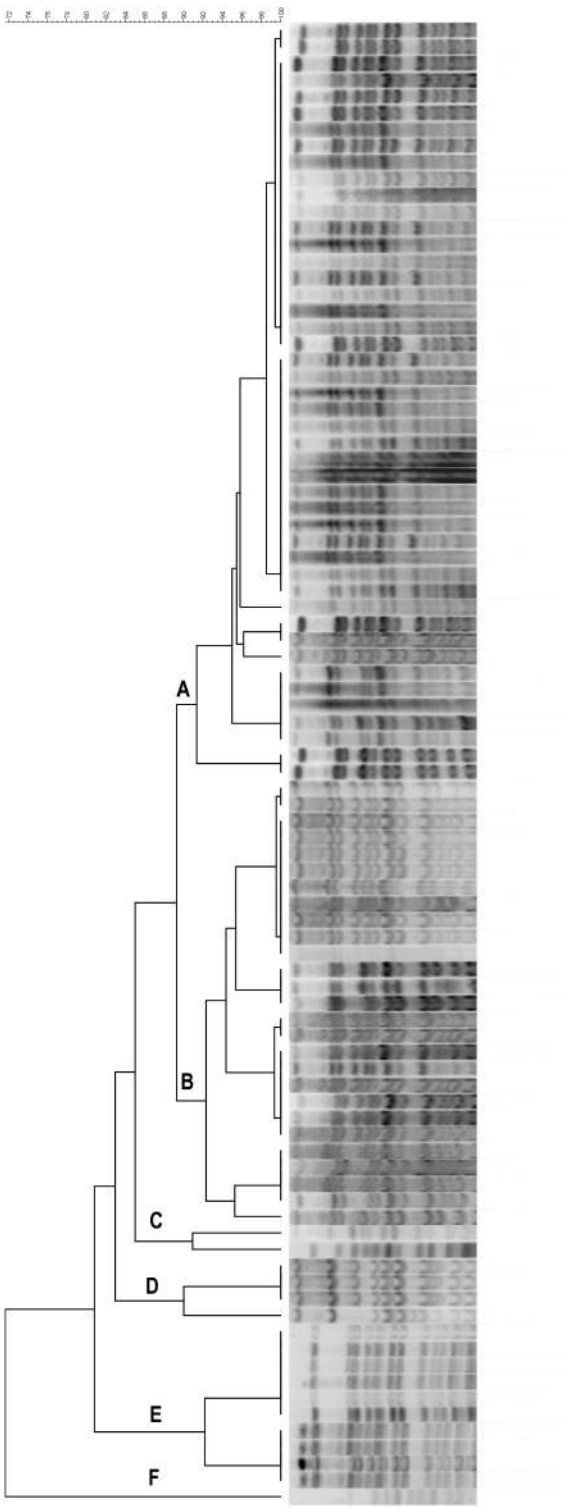
TABLE 4 | Year-wise isolation of *Vibrio cholerae* O1 strains from the tribal areas of Odisha: 2002–2019.

Year	Block/Town	District	Number of strains isolated	Strain type (genotypes)
2002	1. Kashipur 2. Mohana	1. Rayagada 2. Gajapati	21	El Tor variant strains of *V. cholerae* (*ctx*B1)
2007	1. Kashipur 2. Dasamantapur	1. Rayagada 2. Koraput	51	El Tor variant strains of *V. cholerae* (*ctx*B1 and *ctx*B7)
2008	1. Boudh	1. Boudh	12	El Tor variant strains of *V. cholerae* (*ctx*B1 and *ctx*B7)
2009	1. Karanjia, Pandra, Sindurgoura, Badakuldhia 2. Biswanathpur 3. Koira 4. Kashipur	1. Mayurbhanj 2. Kalahandi 3. Sundergarh 4. Rayagada	25	El Tor variant strains of *V. cholerae* (*ctx*B1 and *ctx*B7)
2010	1. Kashipur, Kalyansingpur, Bissam Cuttack 2. Mohana	1. Rayagada 2. Gajapati	66	Typical El Tor strains of *V. cholerae* (*ctx*B3) El Tor variant strains of *V. cholerae* (*ctx*B1)
2011	1. Kolnara, Bissam Cuttack 2. Mohana, R.Udayagiri, Govindpur, Liligad, Adora, Sikulipadar	1. Rayagada 2. Gajapati	54	Typical El Tor strains of *V. cholerae* (*ctx*B3) El Tor variant strains of *V. cholerae* (*ctx*B1)
2012	1. Kashipur, Kalyansingpur, Gudari, Gunupur, Rayagada 2. Kalampur, Jaipatna, Junagarh 3. Laxmipur, Dasamantapur	1. Rayagada 2. Kalahandi 3. Koraput	153	El Tor variant strains of *V. cholerae* (*ctx*B1 and *ctx*B7)
2013	1. Kashipur, Kalyansingpur, Gunupur, Muniguda, Chandili, Kotapeta, Ramnaguda, Seskhal 2. Dasamantapur, Laxmipur	1. Rayagada 2. Koraput	86	El Tor variant strains of *V. cholerae* (*ctx*B1 and *ctx*B7)
2014	1. Narla	1. Kalahandi	11	El Tor variant strains of *V. cholerae* (*ctx*B7)
2015	1. Majhisahi	1. Nuapada	1	El Tor variant strains of *V. cholerae* (*ctx*B7)
2016	1. Kalyansingpur 2. Komna, Kharsel	1. Rayagada 2. Nuapada	4	El Tor variant strains of *V. cholerae* (*ctx*B7)
2018	1. Ekalabya School 2. Thuamul Rampur	1. Rayagada 2. Kalahandi	5	El Tor variant strains of *V. cholerae* (*ctx*B7)
2019	1. Kalyansingpur	1. Rayagada	31	El Tor variant strains of *V. cholerae* (*ctx*B7)
		Total	520	

in this study belonged to the El Tor biotype on the basis of the repeat in toxin gene (*rtxC*). The correlation between the virulence and accessory genes showed positive values except for *rfb*O1. The positive correlation indicated a stronger association between the genes that regulate the action of major toxin production in all the strains. It was noted that VAGS were non-uniformly distributed among *V. cholerae* O1 strains by exhibiting discrete clusters indicative of different virulence patterns shown by these strains.

Analysis of the *Not*I-digested PFGE profiles of *V. cholerae* O1 isolates revealed six different clusters with a similarity matrix of 72%. Among the clusters, clusters A and B comprised 73 strains of *V. cholerae* O1 from the clinical and environmental water sources that shared 92% similarity coefficient among them. This suggests that contamination of the water sources by this pathogen thus might have acted as a reservoir in the transmission of disease (Adewale et al., 2016). It was already published by us that the HCT variant of *V. cholerae* O1 was reported in 2009 (pond water, roadside reservoir water, well water), 2011 (open well), 2012 (stream water), 2016 (open well), and 2019 (Chua water) from both coastal and tribal areas of Odisha. These findings strengthened the fact that the HCT variant of *V. cholerae* O1 had adapted to various environmental water sources to enable viability in different parts of Odisha (Pal et al., 2021). Similar findings were reported from Malaysia in 2009 and also from Bangladesh in 2015–2016, where PFGE results showed clonality among *V. cholerae* strains (Ang et al., 2010; Rahman et al., 2018). Clonality among altered El Tor strains carrying classical *ctx*B1 and Haitian *ctx*B7 genotypes isolated from different geographical regions in Odisha was also proved through PFGE. This finding is similar to the previous reports obtained from Nigeria, Africa, in 2016, where a clonal relationship between classical *ctx*B and Haitian *ctx*B was established through PFGE (Adewale et al., 2016). A pulsotype with 100% similarity index was obtained between the first Haitian variant (*ctx*B7) of *V. cholerae* O1 isolated in 1999 and cholera outbreak strains in 2014–2016 and 2018, respectively, indicative of one clone or lineage of single ancestral origin. Similar findings on the spread of Haitian variant *V. cholerae* O1 strains were reported from South India (Bhattacharya et al., 2015).

In another study, we have detected the environmental reservoirs of *V. cholerae* in the flowing freshwater environs in the tribal areas of Odisha (Pal et al., 2021), where partial stagnant conditions of water at the bank of the river, nala, stream, or temporary storage of water partially encircled by the stones served as the reservoir of *V. cholerae* strains in the flowing aquatic environment which subsequently behaved as the source



Strain Code	Source	Place	Year	ctxB type	Area
KP-6	Stool	Rayagada	08/2007	ctxB3	Tribal
PU-2246	Stool	Puri	07/2008	ctxB7	Coastal
RJ-40	Stool	kendrapara	05/2009	ctxB1	Coastal
RBC-1	Stool	Rayagada	09/2011	ctxB1	Tribal
RP-11	Stool	Rayagada	08/2013	ctxB1	Tribal
LD-587	Stool	Koraput	08/2013	ctxB1	Tribal
RDH-156	Stool	Rayagada	09/2012	ctxB1	Tribal
GM-5	Stool	Ganjam	08/2010	ctxB1	Coastal
KPW-3	Water	Rayagada	11/2007	ctxB1	Tribal
MH-17	Stool	Gajapati	09/2010	ctxB3	Tribal
SW-4	Water	Puri	11/2007	ctxB3	Coastal
SG-16	Stool	Sundaegarh	07/2009	ctxB7	Tribal
KAL-19	Stool	Kalahandi	08/2010	ctxB7	Tribal
KBP-28	Stool	Kalahandi	10/2012	ctxB7	Tribal
RDS-1576	Stool	Rayagada	04/2019	ctxB7	Tribal
KKP-3	Stool	Kalahandi	08/2012	ctxB7	Tribal
KYS-122	Stool	Rayagada	04/2019	ctxB7	Tribal
RP-1	Stool	Rayagada	09/2012	ctxB7	Tribal
KYS-117	Stool	Rayagada	04/2019	ctxB7	Tribal
MYJ-16	Stool	Mayurbhanj	07/2009	ctxB7	Tribal
RJR-1	Stool	Rayagada	08/2012	ctxB1	Tribal
BRD-5	Stool	Bargarh	08/2018	ctxB7	Tribal
RG-9	Stool	Rayagada	09/2012	ctxB1	Tribal
RJRW-57	Water	Rayagada	08/2012	ctxB1	Tribal
RDS-1569	Stool	Rayagada	04/2019	ctxB7	Tribal
KYS-120	Stool	Rayagada	04/2019	ctxB7	Tribal
RGP-20	Stool	Rayagada	08/2013	ctxB7	Tribal
KPD-7	Stool	Rayagada	09/2009	ctxB7	Tribal
KTR-7	Stool	Kalahandi	10/2012	ctxB7	Tribal
KTRW-2	Stool	Kalahandi	08/2012	ctxB7	Tribal
RBCW-9	Water	Rayagada	09/2011	ctxB7	Tribal
KJP-14	Stool	Kalahandi	08/2012	ctxB7	Tribal
RJR-6	Stool	Rayagada	08/2012	ctxB7	Tribal
KYW-699	Water	Rayagada	04/2019	ctxB7	Tribal
BRD-6	Stool	Bargarh	08/2018	ctxB7	Tribal
RDS-1577	Stool	Rayagada	04/2019	ctxB7	Tribal
RG-37	Stool	Rayagada	09/2013	ctxB1	Tribal
RJW-33	Water	Kendrapara	05/2009	ctxB7	Coastal
CHP-1	Stool	Puri	11/2007	ctxB1	Coastal
NJG-3	Stool	Kalahandi	08/2012	ctxB1	Tribal
RKS-41	Stool	Rayagada	10/2012	ctxB1	Tribal
RKSW-40	Water	Rayagada	07/2012	ctxB1	Tribal
KPD-58	Stool	Rayagada	09/2010	ctxB1	Tribal
RKS-10	Stool	Rayagada	07/2012	ctxB1	Tribal
BM-4	Stool	Boudh	07/2008	ctxB1	Tribal
DHS-7	Stool	Khurda	09/2009	ctxB1	Coastal
CH-43	Stool	Khurda	08/1999	ctxB1	Coastal
RDS-1539	Stool	Rayagada	04/2019	ctxB7	Tribal
SC-91	Stool	Cuttack	08/1996	ctxB1	Coastal
PU-760	Stool	Puri	08/2004	ctxB1	Coastal
SC-158	Stool	Cuttack	07/1995	ctxB1	Coastal
M-13	Stool	Rayagada	04/2003	ctxB1	Tribal
POCY-145	Stool	Puri	09/2000	ctxB1	Coastal
PU-2168	Stool	Puri	07/2008	ctxB1	Coastal
PU-1449	Stool	Puri	10/2006	ctxB1	Coastal
PU-1101	Stool	Puri	10/2005	ctxB1	Coastal
POCY-187	Stool	Puri	11/2000	ctxB1	Coastal
PU-1851	Stool	Puri	11/2007	ctxB1	Coastal
BD-2	Stool	Boudh	07/2008	ctxB7	Tribal
PU-2222	Stool	Puri	07/2008	ctxB3	Coastal
MH-91	Stool	Gajapati	07/2011	ctxB3	Tribal
KAL-23	Stool	Kalahandi	08/2010	ctxB3	Tribal
SC-221	Stool	Cuttack	07/1997	ctxB1	Coastal
PU-2511	Stool	Puri	08/2009	ctxB7	Coastal
NU-8	Stool	Nuapada	08/2010	ctxB3	Tribal
RDH-151	Stool	Rayagada	11/2012	ctxB7	Tribal
KP-18	Stool	Rayagada	08/2007	ctxB7	Tribal
RDH-327	Stool	Rayagada	09/2013	ctxB7	Tribal
SG-11	Stool	Sundargarh	07/2008	ctxB1	Tribal
KAL-11	Stool	Kalahandi	08/2009	ctxB1	Tribal
MYJ-12	Stool	Mayurbhanj	06/2009	ctxB1	Tribal
DM-278	Stool	Koraput	09/2013	ctxB7	Tribal
PU-2488	Stool	Puri	07/2009	ctxB3	Coastal
RDH-158	Stool	Rayagada	09/2012	ctxB1	Tribal
JP-3	Stool	Koraput	07/2000	ctxB7	Tribal
SC-37	Stool	cuttack	11/1999	ctxB1	Coastal
SC-63	Stool	Cuttack	07/1995	ctxB1	Coastal
MR-5	Stool	Khurda	07/1998	ctxB1	Coastal
B-1	Stool	Puri	06/2002	ctxB1	Coastal
BALW-4	Water	Balasore	07/2016	ctxB7	Coastal
KSP-5	Stool	Rayagada	07/2016	ctxB7	Tribal
BAL-2	Stool	Balasore	07/2016	ctxB7	Coastal
CY-44	Stool	Jagatsinghpur	11/1999	ctxB7	Coastal
BAL-5	Stool	Balasore	07/2016	ctxB7	Coastal
KSP-4	Stool	Rayagada	07/2016	ctxB7	Tribal
N-17	Stool	Kalahandi	09/2014	ctxB7	Tribal
NU-20	Stool	Nuapada	09/2015	ctxB7	Tribal
RDS-179	Stool	Rayagada	01/2018	ctxB7	Tribal
N-1	Stool	Kalahandi	09/2014	ctxB7	Tribal
KSP-10	Stool	Rayagada	07/2016	ctxB7	Tribal

FIGURE 6 | PFGE patterns of the *Not*I-digested *Vibrio cholerae* O1 strains isolated from different districts of Odisha: 1995–2019. Capital letters means different clusters.

of infection in the tribal areas. So, as the people in the tribal areas depend on the stream, nala, chua, and river water, the stored water in the hilltops supplied to the villages should be chlorinated in different time intervals before and during the monsoon season. People should be aware toward the use of potable water for drinking and cooking. So, the possible outbreak of cholera will

be checked in this region. Previous publications by sequencing and comparing hundreds of bacterial genomes of *V. cholerae* have shown that all the explosive epidemics of cholera in Africa and America in the past-half century arose after the arrival of new strains that had evolved in Asia (Domman et al., 2017; Weill et al., 2017; Pal et al., 2019). The present findings also strengthened the above facts.

CONCLUSION

The spread of multidrug-resistant, *ctx*B1- and *ctx*B7-possessing *V. cholerae* O1 strains from the coastal to tribal areas in Odisha occurred during the past two and a half decades in a sequential manner which might be due to a single clone or due to lineages of a single ancestral origin. This study also provides evidence for clinical and water isolates that shared genetic linkage with a similarity matrix of 100% proved through PFGE analysis indicative of environmental water sources which might have acted as a reservoir for the transmission of this disease from the coastal to tribal areas of Odisha. So, provision of potable water supply should be in places especially in communities residing in the inaccessible areas which mainly depend on chua, nala, and stream water particularly in the tribal areas. From the present findings, it is also evident that the altered El Tor *V. cholerae* O1 carrying *ctx*B1 and *ctx*B7 genotypes originated in the east coast of the Bay of Bengal and gradually spread to the tribal areas, which strengthened the hypothesis that the hometown of cholera was the Gangetic belts of Bay of Bengal.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BP conceptualized and designed the study, interpreted the data, and finalized the manuscript. DB analyzed the data and edited the draft for the manuscript. SN and AN did the molecular works and contributed to the data analysis. All authors reviewed and approved the manuscript.

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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).

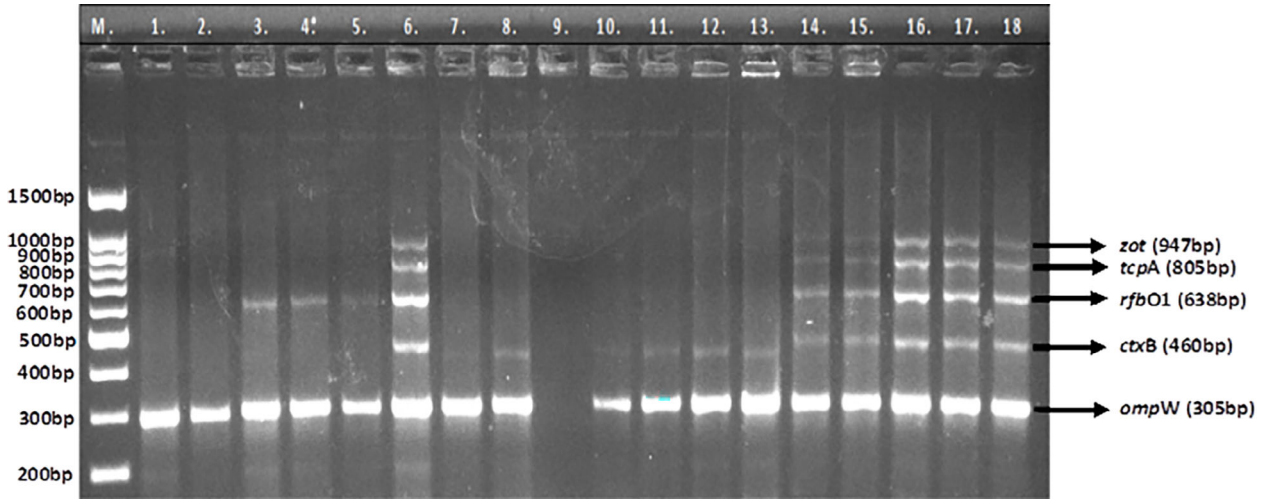

Fig 1. Detection of different toxic genes by multiplex PCR assay (M: Marker; Lanes 1–5: *V. cholerae* non O1 non O139; Lane 6: *V. cholerae* O1 (clinical isolate); Lane 7–13: *V. cholerae* O139 (water isolates 2017); Lanes 14–18: *V. cholerae* O1 (plankton isolates 2018).

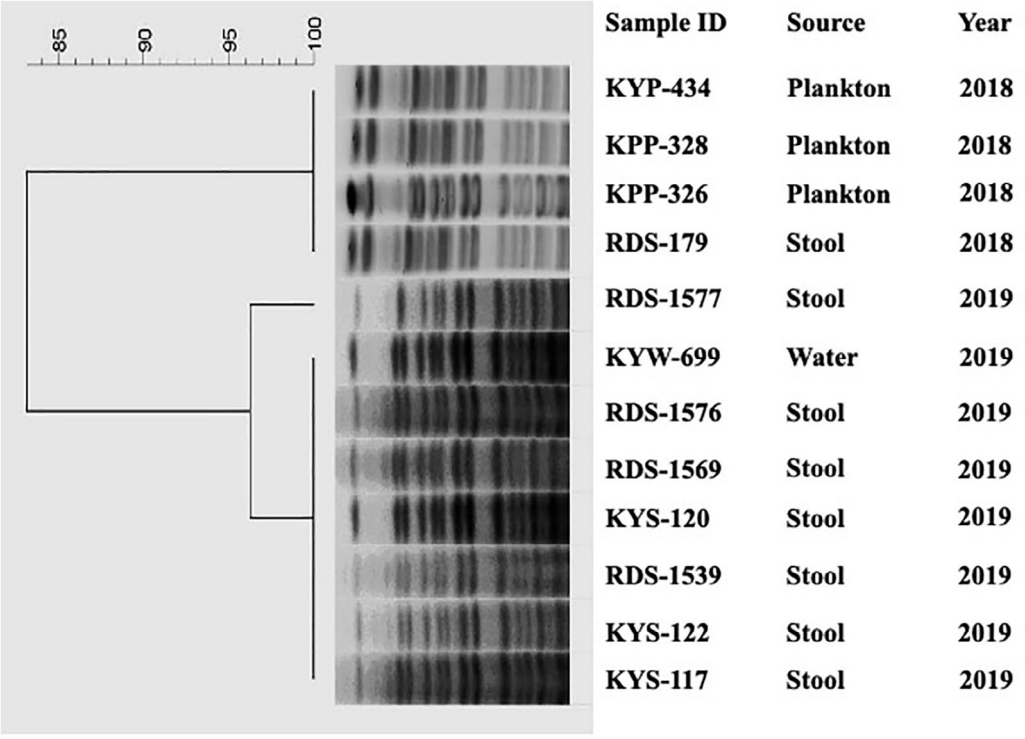

Fig 2. PFGE patterns of the *Not*I digested *V. cholerae* O1 strains isolated from Rayagada district of Odisha with the dendrogram analysis using Bionumerics software (Applied Maths, Sint-Martens-Latem Belgium).

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).

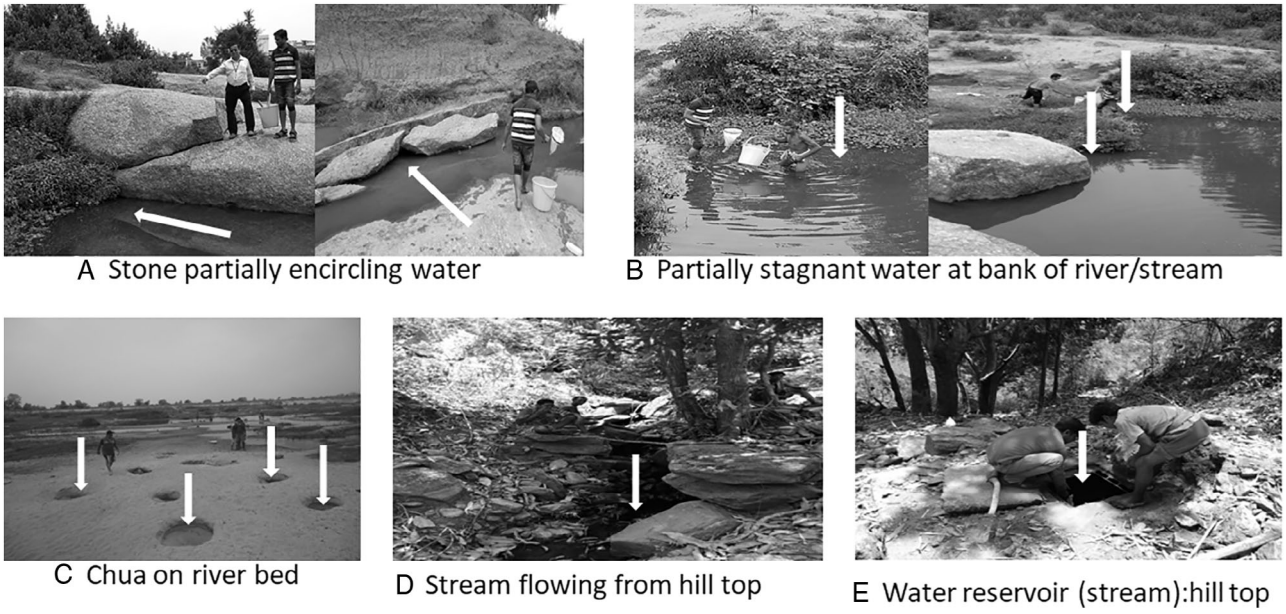

Fig 3. Arrows indicate the sampling sites which were positive for *V .cholerae* serogroups in the river, stream and chua (A–E) from the Rayagada district.

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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