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RESEARCH ARTICLE OPEN ACCESS

*Ctx*B7 Genotypes of *Vibrio cholerae* O1 Causing the 2022 Cholera Epidemic in Tribal Areas of Odisha, India

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Abstract

Cholera in Odisha's tribal and coastal areas has been a persistent problem due to *Vibrio cholerae* O1/O139 for the past 30 years. This study investigates diarrheal outbreaks reported between July to November 2022 from five tribal regions of Odisha; aiming to identify the causative pathogen, its antibiogram profile and virulence genes. Standard techniques were used to culture rectal swabs taken from patients with diarrhea and samples of water to isolate the pathogen, its antibiogram profiles and PCR assays (simplex/multiplex) were used to detect different toxic genes. The heavy rainfall reported in July 2022 led to the spread of the diarrheal disease from Rayagada to neighbouring districts. The *V. cholerae* O1 Ogawa El Tor, characterised by the *ctx*B7 genotype and exhibiting multidrug resistance, were identified. Demographic analysis revealed that people aged 14-40 and over 40 years were the most affected with equal distribution among males and females. A high proportion of strains (ranging from 80% to 100%) tested positive for various virulence and drug-resistance genes. The findings offer important insights into the *ctx*B7 genotypes of *V. cholerae* O1 strains responsible for outbreaks in Odisha, which will aid in the development of improved prevention and control strategies for future cholera outbreaks. The study also suggests that there might be a possible linkage between the strains in Odisha and those from the recent cholera outbreak in Bangladesh, which can be known through whole genome sequencing after collaboration in future.

Keywords: Cholera Outbreak, *Vibrio cholerae* O1, *ctx*B7, Antibiotic Resistance, Tribal Areas, Odisha

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INTRODUCTION

Cholera is a serious gastrointestinal illness resulting from the toxigenic bacterium *Vibrio cholerae*, primarily spread through fecal-oral transmission via contaminated food and water, as well as poor sanitation practices. The infection can rapidly cause severe dehydration, metabolic acidosis, and death if untreated. Every year, between 1.3 and 4.0 million cases of cholera occur worldwide, leading to between 21,000 and 143,000 fatalities.[1] Although there are more than 200 serogroups of *V. cholerae* based on somatic "O" antigens, only O1 and O139 have been connected to significant outbreaks in emerging and impoverished nations. The O1 serogroup is separated into three biotypes: classical, El Tor, and El Tor variants. Genetic changes in the *ctx*B gene, which codes for the cholera toxin B component, result in *ctx*B1 to *ctx*B12 variants, which are referred to as El Tor variants.[2] Cholera outbreaks worldwide have recently been driven by the Haitian form (*ctx*B7) of *V. cholerae* O1.[3] The first outbreak due to the *ctx*B7 genotype was reported in Haiti in 2010 after its emergence from Odisha following the 1999 super cyclone.[4] Subsequently, this *ctx*B7 variant has been associated with outbreaks in several countries, including Afghanistan, Bangladesh, Nepal, Yemen, Nigeria, Ethiopia, and others. The 2022 cholera outbreak in Bangladesh was the second-largest since 2000, with 42,000 cases reported between March and April. Similarly, Pakistan reported over 2000 diarrhea cases and 290 confirmed cholera cases in April-May 2022.[5] In Odisha, cholera outbreaks occur nearly every three years. Over the last decade, *Vibrio cholerae* O1 with the *ctx*B7 genotype and resistance to multiple drugs has caused outbreaks in 2007, 2010, 2012, 2014, 2016, and 2019, particularly in the tribal areas of Odisha.[6] This study reports and investigates the diarrheal outbreaks in five tribal districts (Rayagada, Koraput, Nuapada, Subarnapur, and Nabarangpur) of Odisha from July to November 2022, focusing on the causative pathogen, its antibiogram profile, and virulence genes.

MATERIALS AND METHODS

Study area

A group from the ICMR-Regional Medical Research Centre's Microbiology Department in Bhubaneswar, conducted a comprehensive study on the diarrheal outbreak reported in five tribal districts of Odisha, i.e. Rayagada, Koraput, Nabarangpur, Subarnapur, and Nuapada between July and November 2022. The team gathered crucial information on the outbreak, including index cases and details of every diarrhea patient, including their symptoms and medical histories. The spread of infections across different blocks was analysed, with index cases traced through discussions with villagers and hospital records. The study also examined the outbreak's cause, sources of potable water, chlorination details, and the hygienic conditions in affected villages.

Sample collection

Swabs from diarrhea patients were collected at Community Health Centres (CHCs) and Primary Health Centres (PHCs) in affected villages across five districts (Rayagada, Nuapada, Nabarangpur, Kalahandi, and Koraput) after obtaining informed consent and before administering antibiotics (Figure 1). The swabs were transported in Cary-Blair medium to laboratory for further analysis. On the basis of age, patients were classified into age groups: 0-5 years, over 5 to 14 years, over 15 to 40 years, and above 40 years. Additionally, samples of environmental water were gathered from streams, rivers, tube wells, and household stored water sources used for bathing, cooking, drinking, and cleaning.

Sample processing

Swabs collected from patients and water samples collected from different environmental sources were enriched in APW (alkaline peptone water) and then streaked on TCBS agar, MacConkey agar, and Hektoen enteric agar (BD, USA). Plates were incubated at 37 °C for overnight. Moist yellow colonies were further confirmed serologically by slide agglutination using specific antisera for *V. cholerae* serogroups (polyvalent O1) and serotypes

(monovalent Ogawa/Inaba; BD, USA). Genomic DNA was extracted by the snap chill method for molecular analysis. Pathogen isolation and identification followed established laboratory practices consistent with WHO guidelines.[4,7]

Antibiotic susceptibility assay

The antibiotic sensitivity test of *V. cholerae* O1 was assessed on MHA (Mueller-Hinton Agar) using the disc diffusion method, following CLSI guidelines and our lab practices.[8,9] Fifteen antibiotics were tested: chloramphenicol (C), ciprofloxacin (CIP), azithromycin (AZM), gentamicin (GEN), ampicillin (AMP), tetracycline (TE), streptomycin (S), nalidixic acid (Na), erythromycin (E), ofloxacin (OF), doxycycline (DOX), norfloxacin (NX), cotrimoxazole (COT), furazolidone (FR), and polymyxin B (PB) (BD, USA). Susceptibility was interpreted using standard guidelines and breakpoints.

PCR (Polymerase Chain Reaction) assays

Phenotypically identified *V. cholerae* strains underwent simplex and multiplex PCR for confirmation and virulence gene detection. Two multiplex PCRs (mPCRs) were performed: mPCRI confirmed *V. cholerae* using primers for toxin coregulated pilus (*tcp*A), O1 somatic antigen (*rfb*O1), zonula occuludens toxin (*zot*), and outer membrane protein (*omp*W). mPCRII detected additional virulence genes for accessory cholera enterotoxin (*ace*), hemolysin (*hly*A), repeats-in-toxin protein (*rtx*), toxin regulator (*tox*R), and outer membrane protein (*omp*U).[10] The strains were also evaluated for antibiotic resistance genes encoding trimethoprim (*dfr*A1), sulfamethoxazole (*sul*II), the SXT element and streptomycin (*str*B).[11] Following separation on a 1.8% agarose gel, PCR products were stained with ethidium bromide and examined using a gel documentation system (Bio-Rad, USA).

Mismatch amplification mutation assay (MAMA), Double mismatch amplification mutation assay (DMAMA) PCR & detection of *tcp*A genes

Using particular primers, MAMA and DMAMA PCR tests were utilised to distinguish between the Haitian (*ctx*B7), El Tor (*ctx*B3), and classical (*ctx*B1) genotypes in all *V. cholerae* isolates.[12,13] Targeting the Classical, El Tor, and Haitian *tcp*A gene variants, PCR was used to validate biotype-specific characterisation. Two distinct forward primers for each *tcp*A variant and a common reverse primer for Haitian and El Tor *tcp*A were employed.[14]

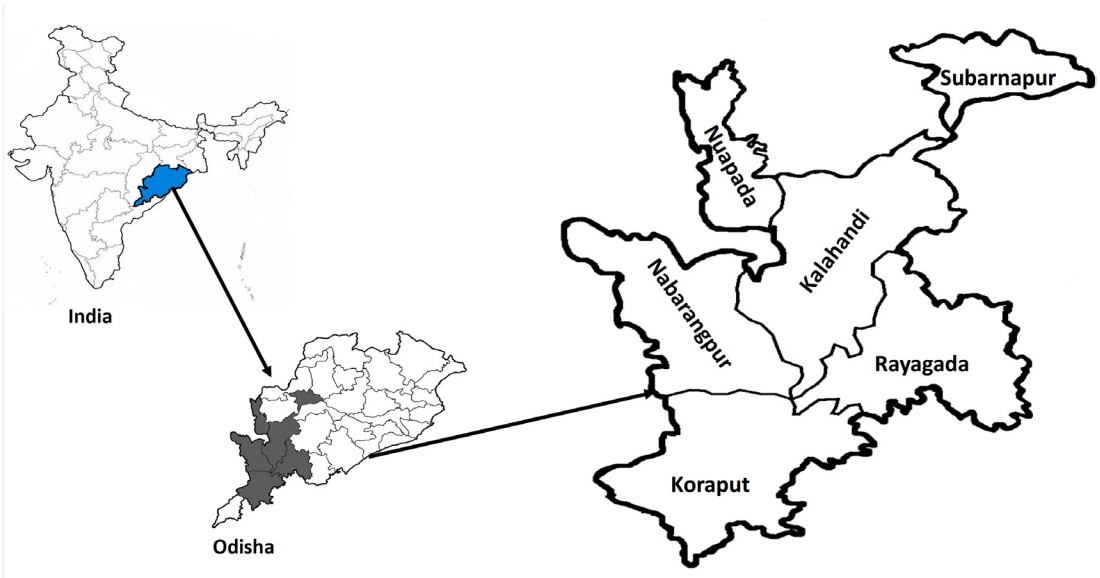

Figure 1. Map of Odisha showing the diarrhea affected districts

RESULTS

In the Rayagada district, Kashipur CHC and Tikiri PHC and reported an unexpected spike in diarrhoea cases in July 2022. The first severe case, reported on 7th July in Dudukabahal village, involved a patient with cholera symptoms who was treated and discharged but succumbed on 9th July after symptoms reappeared. Following persistent heavy rainfall in early July, which likely led to water contamination in downstream, more cases emerged in nearby villages like Tikiri and Kakudipadar. The outbreak eventually spread to other blocks, including Kalyansinghpur, Kolnara, and Rayagada Urban. Between July 7, 2022, and November 3, 2022, 414 cases and 10 fatalities were documented; no more cases were reported after that date.

A diarrheal outbreak was documented in Dasmantapur (74 cases from 12th to 27th July 2022) and Laxmipur block of Koraput district (complete line listing data was not available).

Table. Bacteriological analysis of rectal swabs and water samples

Rectal Swabs	Total Rectal Swabs tested	414
	Culture Positive samples	341 (82.4%)
	E. coli	225 (54.34%)
	V. cholerae O1 Ogawa	114 (27.53%)
	Salmonella spp.	0 (%)
	Shigella spp.	2 (0.48%)
	Culture Negative Samples	73 (17.63%)
Water Samples	Total Water Samples tested	77
	No. positive for *V. cholerae* O1	5 (6.5%) [River, supply water from the hill top, tube well and house-hold water]

Field investigations indicated that individuals from Rayagada district contributed to the spread of the outbreak after migrating to these areas.

Additionally, a 70-year-old woman from Nandahandi village, Nabarangpur district, developed diarrhea after attending to a patient in Dasmantampur. A total of 88 cases and one death were reported in Nabarangpur district between July and August 2022, with 44 cases in Khadiaguda, Pitakumili, and other nearby villages. *V. cholerae* O1 was isolated from the river water in Tulasipadar village, suggesting it as a possible infection source.

In Jatgad village, Nuapada district, another outbreak occurred. The index case, a 70-year-old woman, passed away on July 26, 2022. Diarrhea cases were reported in Majhipada and Harijanpada villages, totaling 84 cases and 1 death between July 25 and September 22, 2022. Tubewell water in Jatgad tested positive for *V. cholerae* O1 Ogawa.

In Subarnapur district, there was another diarrhea outbreak in Digsira village (index case reported on July 23, 2022) and Suryamunda village (index case reported on July 25, 2022). Contaminated water sources were likely the cause of outbreak. A total of 64 diarrhea cases occurred between July 23 and July 31, 2022 (Figure 2).

Bacteriological analysis of rectal swabs and water samples

V. cholerae O1 Ogawa was found to be the main pathogen in 77 water samples and 414 rectal swabs that were subjected to bacterial investigation. Antisera testing verified that 114 (27.53%) of the rectal swabs were positive. Positive water samples included three from Rayagada (river, household, hilltop), one from Nabarangpur (river), and one from Nuapada (tube well) (Table).

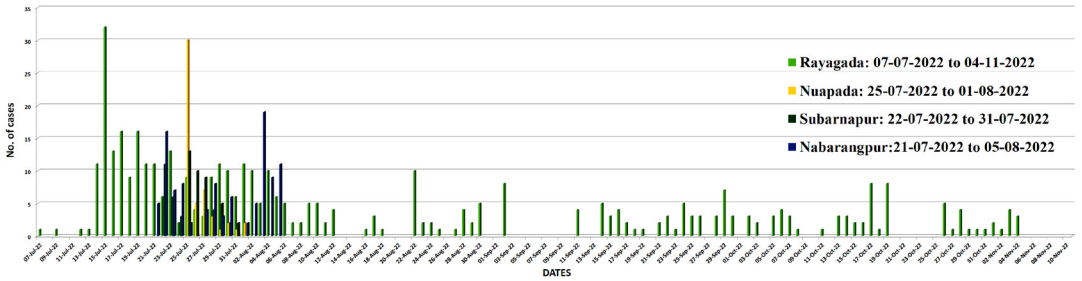

Figure 2. Incidence of severe diarrhea cases in 5 tribal districts of Odisha, during July 07, 2022- November 04, 2022

Antimicrobial susceptibility patterns

The antibiotic resistance profiles of all *V. cholerae* O1 strains were tested against 15 different antibiotics using the disc diffusion method, and interpretation was based on the observed zone of inhibition sizes. All *V. cholerae* isolates showed resistance to ampicillin (100%), furazolidone (100%), and nalidixic acid (100%). Additionally, high levels of resistance were observed for streptomycin (98%), cotrimoxazole (94%), erythromycin (91%), chloramphenicol (79%), and polymyxin B (69%). The *V. cholerae* O1 isolates from the five cholera affected districts exhibited common sensitivity to ciprofloxacin (70%), norfloxacin (90%), gentamicin (85%), tetracycline (90%), ofloxacin (90%), doxycycline (85%) and azithromycin (82%).

Demographic analysis

Three cholera-affected areas (apart from Koraput) had their diarrhoeal patients demographically analysed. Four age categories of patients were identified: 0-5, 5-14, 14-40, and above 40. Most impacted were those aged 14 to 40, then those over age group 40. The infection rates were lower in the youngest groups 0-5 and 5-14. In every age category, the ratio of male to female patients was about equal (1:1.03).

Prevalence of virulence associated genes

Multiplex PCR experiments were used to identify the genes linked to virulence in isolates of *V. cholerae* O1. Every isolate of *V. cholerae* tested positive for the *omp*W and *rfb*O1 genes, confirming the species and O1 serogroup. From these five areas, nearly all of the clinical *V. cholerae* isolates tested positive for every virulence and regulatory gene. All of the *V. cholerae* isolates had the repeat in toxin *rtx*C (99.17%), haemolysin *hly*A (100%) and cholera toxin *ctx*AB (100%) present. However, the *V. cholerae* isolates were 98.35% positive for the accessory cholera enterotoxin (*ace*), 95.87% positive for the zonula occuludens toxin (*zot*), 100% positive for toxin corregulated pilus (*tcp*), *tox*R 97.52% and *omp*U 93.39% positive gene respectively (Figure 3).

Profiling of antibiotic resistant genes

The majority of the strains tested positive for antibiotic resistance genes using the PCR assay, which amplified a 278 bp fragment of *dfr*A1, 515 bp of *str*B, 626 bp of *sul*II, and 1035 bp of SXT element. All of these genes may have contributed to the resistance of *V. cholerae* O1 strains to nalidixic acid, ampicillin, furazolidone, streptomycin, neomycin and cotrimoxazole. 95% of *V. cholerae* O1 strains had positive results for all of the aforementioned genes (Figure 3).

MAMA and DMAMA, PCR assays & detection of *tcp*A genes

The MAMA and DMAMA PCR assays were conducted for all the *V. cholerae* O1 isolates. Out of 119 strains 60 strains were randomly tested for the detection of *ctx*B alleles. All were positive for *ctx*B7 allele of Haitian variant. Additionally, the existence of the Haitian type *tcp*A allele was confirmed by the results of the PCR assay for the identification of the *tcp*A gene (167 bp).

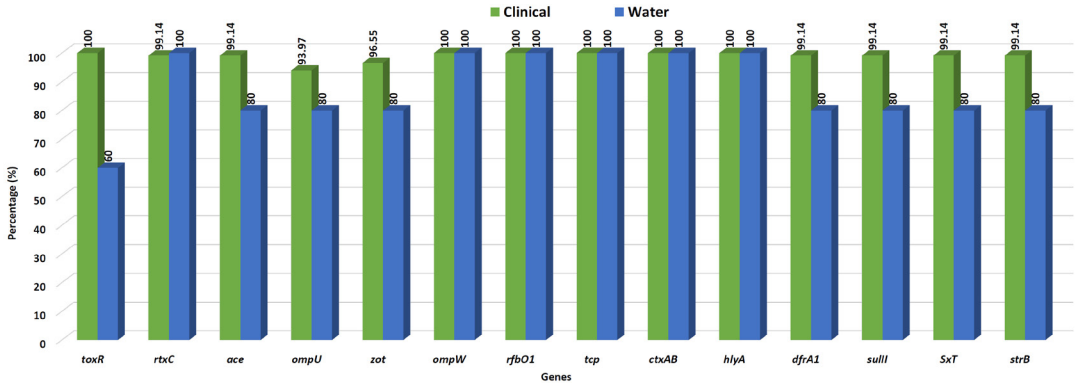

Figure 3. Percentage of virulence and drug-resistance genes in clinical and water isolates of *V. cholerae* O1 strains isolated from different tribal districts

DISCUSSION

Cholera is still a major global public health concern that draws attention to disparities in social progress. Cholera epidemics in underdeveloped nations are frequently associated with a lack of access to clean drinking water and basic sanitation.[15] The WHO estimates that implementing these measures could reduce diarrheal cases by 35%. The absence of safe drinking water and proper sanitation facilities are primary causes of cholera in affected villages. The Indian subcontinent accounts for 78% of reported cholera cases, with India experiencing multiple outbreaks as reported by WHO in 2007.[16] In Odisha, cholera has been a persistent challenge, particularly in rural areas where water from unprotected wells and bore wells, prone to contamination, contributes to the spread of waterborne diseases. Acute diarrhoea epidemics and outbreaks are common in rural regions of tropical developing nations where water is obtained from bore wells, chua (shallow pits in rice fields or riverbeds), and unprotected open wells. These sources are vulnerable to groundwater pollution following rains. The water source contamination through open wells and tube wells contributes to the transmission of waterborne diseases.[17]

This epidemic serves as a compelling example of cholera transmission through both contaminated water sources including poor sanitation practices, inadequate hygiene conditions, and the migration of individuals from outbreak areas—such as patients migrated from Rayagada district to previously unaffected regions like Dasmantapur in Koraput and then to Nabarangpur. The initial detection of the outbreak in the Kashipur block, situated at a higher altitude of Rayagada district highlights the vulnerability of such areas. Subsequently, cholera cases were reported in other blocks of Rayagada district which are located at lower altitudes and downstream of the river originating in the Kashipur block. The geographical gradient with higher-altitude areas being affected first spreading to lower altitude suggests a potential link between altitude and the spread of the disease. Furthermore, an analysis of the index cases in the affected districts reveals a common pattern: individuals visiting neighbouring cholera-affected districts. During the cholera outbreak of 2010 in this region, similar reports were also recorded, which affected Kashipur, Kalyansinghpur, Bissam Cuttack of Rayagada and Mohana of Gajapati district, i.e. affecting from higher altitude to lower altitude.[18] These finding underscores the role of human movement and spread of the diarrhea in this region also. It is interesting to note that the incidence of severe diarrhea cases in Rayagada revealed that cases were persistent from July, to November 2022 covering five months which was contrast to the cholera outbreaks of 2007, 2010, 2012 in the same district. It indicated that *V. cholerae* was existing whether in the environment or in the community for a longer period.

In the present study, the analysis of age groups revealed that the most affected group was aged 14-40 years, followed by those above 40 years. However, in the present study, there was no significant difference between males and females, with a ratio of approximately 1:1.03. This is surprising, as our earlier studies from these tribal areas have shown that females tend to be more affected than males. These findings are consistent with a study conducted in Kathmandu, Nepal, which reported a similar pattern of higher susceptibility among the adult age group.[19] Similar results were also observed from Pakistan.[20] However, contrasting results were reported from PGIMER, Chandigarh, where the age groups of 5-14 and 14-40 years were found to be most infected, and the male-to-female ratio was 120:71.[21] Similarly, contradicting studies were reported from Nepal and Nigeria.[22,23]

Resistance to ampicillin, furazolidone, nalidixic acid, streptomycin, co-trimoxazole, erythromycin, chloramphenicol, and polymyxin B was shown by the *V. cholerae* O1 strains that were identified during this cholera outbreak. But those showed susceptibility to azithromycin, doxycycline, ciprofloxacin, gentamicin, norfloxacin, tetracycline, and ofloxacin. A recent study conducted in Kerala in 2021 also reported sensitivity of *V. cholerae* strains to ciprofloxacin, gentamicin, ofloxacin, and norfloxacin.[24] In contrast, a study from Chandigarh reported a resistance rate of 12.72% towards ciprofloxacin.[25] The reduced sensitivity to ciprofloxacin observed in our study might be attributed due to its indiscriminate use during the COVID-19 pandemic. Similar findings regarding

decreased susceptibility to fluoroquinolones (ciprofloxacin and norfloxacin) have been reported since 1996.[26-28] Comparable resistance profiles have also been reported from Solapur,[29] Maharashtra,[30] Nepal[28] and Mozambique.[31,32] Previous studies conducted in Odisha have also documented reduced sensitivity towards fluoroquinolone antibiotics.[33] The current antibiogram profile of resistance is consistent with previous cholera outbreaks reported in different years in Odisha.[4] The resistance of *V. cholerae* O1 strains to various antibiotics, such as co-trimoxazole, streptomycin and trimethoprim indicates the acquisition of SXT into the chromosome which lead to multidrug-resistance.[34] The indiscriminate use of antibiotics in various sectors, including agriculture has contributed to the emergence of multidrug-resistant strains, posing significant challenges for antibiotic therapy worldwide. The majority of the *V. cholerae* O1 isolates had antibiotic resistance genes, which were subsequently verified by the multiplex PCR experiment.

The pathogenesis of cholera in *V. cholerae* involves the coordinated action of multiple genes. To assess the toxigenicity and pathogenicity of the *V. cholerae* isolates in this study, two sets of multiplex PCR (mPCR) were performed. The mPCR II showed that all isolates tested positive for the *rtx*C, *omp*U, *ace* and *tox*R genes, whereas the mPCR I verified the presence of the *omp*W, *ctx*AB, *rfb*O1 and *tcp*A genes. The bacteria were identified as *V. cholerae* by the presence of the *omp*W gene, and their serogroup was verified as O1 by the presence of the *rfb*O1 gene. The presence of cholera toxin (CT), a critical marker among the many toxins generated by *V. cholerae* that were found in this investigation, was indicated by the detection of the *tox*R gene.[34] Gram-negative bacteria are frequently distinguished from other types of bacteria using the RTX toxin, a crucial pathogenicity component. The presumptive cytotoxin (*rtx*A) and an acetyltransferase (*rtx*C) are encoded by the RTX toxin gene in *V. cholerae*. It is also linked to the ATP binding cassette transporter system, which is physically connected to the core element of *V. cholerae* genome.[35,36] Other genes, including *tox*R and *ace*, were also examined as part of this epidemic study; the results showed that all *V. cholerae* O1 isolates had the core toxin region. Several studies from Kerala,[30] Chandigarh,[21] and Kenya have revealed similar results.[37]

The *ctx*B gene in *V. cholerae* O1 exhibits considerable genetic diversity due to point mutations in the nucleotide sequences, resulting in different amino acid variations. There are twelve distinct genotypes of *ctx*B associated with various serogroups of *V. cholerae*.[2] Among these genotypes, *ctx*B7 differs from *ctx*B1 due to a point mutation at nucleotide position 58 (C to A), leading to a change in the 20th amino acid from Histidine to Aspartic acid.[13] In the present study, all isolated strains were confirmed to have *ctx*B7 genotypes which have also been previously isolated from Odisha during the years 1999, 2007 to 2010, 2012, 2014, 2016, and 2019.[6] Similar findings have been reported by other researchers in West Bengal,[14] Bihar and Southern India.[28] Furthermore, *V. cholerae* O1 strains with *ctx*B7 genotypes have been detected in other regions of India, including Chennai, Hyderabad, Solapur and Assam.[38-40] It is noteworthy that the same Haitian variant of *V. cholerae* O1, which caused the devastating cholera epidemic in Haiti in 2010, is believed to have first originated in Odisha in 1999 and was later reported in Nepal.[2,4] The *ctx*B7 genotypes strain of *V. cholerae* O1 has subsequently might have spread to Africa, Yemen (2015-2017) and most recently reported from Bangladesh in 2022.[5]

CONCLUSION

The present study established that the cholera epidemic in the five districts of Odisha were caused by *V. cholerae* O1 Ogawa with *ctx*B7 genotype; which might be similar to the strains that caused large outbreak in Bangladesh and Pakistan in April, 2022. It is apprehended that this *ctx*B7 genotype might have linkage with the recent epidemic of cholera outbreak which will know through whole genome sequencing. Furthermore, the study made it clear that the primary source of *V. cholerae* that caused cholera outbreaks in Odisha's tribal communities was environmental water bodies. Effective surveillance and response mechanisms, which are frequently subpar in poor nations, are necessary to control cholera epidemics. The necessity of an efficient active monitoring system with capacity building

to identify and contain cholera outbreaks at the right time and location is therefore highlighted by this study. To avoid and control future diarrhoeal epidemics in Odisha's tribal districts, public cleanliness and the provision of safe drinking water should be given top priority.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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