

Original Article

Vibrio cholerae O1 Ogawa Strains Carrying the *ctxB7* Allele Caused a Large Cholera Outbreak during 2014 in the Tribal Areas of Odisha, India

Bibhuti Bhusan Pal*, Hemant Kumar Khuntia, Smruti Ranjan Nayak, Anima Mohanty, and Bhagyalaxmi Biswal

Microbiology Division, Regional Medical Research Centre (ICMR) Chandrasekharpur, Bhubaneswar, India

SUMMARY: The large outbreak of cholera reported during July to September 2014 in the Narla block of Kalahandi district, India, was investigated to determine the causative organism. Rectal swabs collected from patients with diarrhea and environmental water samples were cultured following standard techniques. The causative organism was identified as *Vibrio cholerae* O1 Ogawa biotype El Tor, and analysis by double mismatch mutation assay PCR confirmed that all strains were the *ctxB7* variant of Haitian *V. cholerae* O1. The environmental water samples were negative for *V. cholerae*. The *V. cholerae* O1 strains were sensitive to tetracycline, ciprofloxacin, norfloxacin, ofloxacin, doxycycline, and azithromycin, but were resistant to erythromycin, gentamicin, chloramphenicol, furazolidone, neomycin, cotrimoxazole, nalidixic acid, and ampicillin. In the 2014 cholera outbreak, the early reporting of the pathogen enabled the government authorities to implement adequate control measures in time to curtail the spread of the disease. That was the second large cholera outbreak due to Haitian variants of *V. cholerae* O1 after the 2010 Haiti cholera outbreak reported from Odisha, India, and other locations globally. Active surveillance is required to track the spread of this strain in the Odisha region.

INTRODUCTION

Cholera is an ancient water borne disease that continues to cause devastating outbreaks globally, with a high prevalence in developing countries. Cholera is caused by *Vibrio cholerae*, which has more than 200 reported serogroups, among which the O1 and O139 serogroups have been identified as pathogenic/epidemic strains. The *V. cholerae* O1 serogroup has 2 biotypes, namely classical and El Tor. The classical biotype is believed to be extinct, whereas the El Tor biotype continues to circulate as the 7th pandemic strain of cholera. The devastating cholera epidemic during 2010 in Haiti for a century, placed this ancient scourge at the forefront of the global public health agenda (1). In May 2011, World Health Organization (WHO) documented the reemergence of cholera as a substantial global public health problem and asked for the execution of an integrated and inclusive global approach to control cholera (2). Meanwhile several reports were published regarding the evolution of altered El Tor biotype *V. cholerae* O1 strains that have caused cholera outbreaks or epidemics in Asia, Africa, and several other countries around the world. Several *ctxB* alleles differing by a few non-random point mutations were identified in *V. cholerae* strains of different biotypes and serogroups. *V. cholerae* O1 has 3 *ctxB* alleles (*ctxB1* to *ctxB3*), whereas *V. cholerae* O139 has 3 *ctxB* genotypes (*ctxB4* to *ctxB6*). A new *ctxB* genotype similar to *ctxB1* has been reported in the O1 strain,

which was named as *ctxB7*. The *ctxB* alleles differ each other in their nucleotides and the corresponding amino acid sequences (3). In recent years, novel pathogenic *ctxB* alleles of *V. cholerae* O1 have emerged and been documented worldwide (4–7). Several reports have demonstrated that Haitian cholera toxin (HCT)-producing variants of *V. cholerae* O1 were predominant worldwide, and the importance of these reports was highlighted after the disastrous Haitian cholera outbreaks. The presence of *ctxB* and *tcpA* *ctxB* allele was reported in samples from the cholera outbreaks during 2009 and 2010 in Nigeria (8). The appearance and gradual dissemination of the *ctxB7* variant allele in Kolkata from 2006 onwards was reported by Naha et al., 2012 (6). El Tor variant strains of *V. cholerae* O1 classical CT (CCT) have completely replaced the El Tor CT-producing strain in Kolkata since 1995 (6). El Tor-type *ctxB* was replaced by the classical allele in Bangladesh in 2001 (9), and the presence of a CCT-producing variant El Tor strain at the Gulf coast was reported by Olsvik et al, 1993 (10). Additionally, new *ctxB* genotypes were observed in Zambia and Mexico during 2013, which represent variants of *ctxB7* in HCT-producing *V. cholerae* (11,12). The *ctxB7* variant *V. cholerae* O1 was first reported from the tribal areas of Odisha, India, during 2007 (6,13). These HCT variant strains of *V. cholerae* O1 were also reported from the Maharashtra (14) and Bihar states of India during 2012 (15). The present study was envisaged to document the causative agent of the large cholera outbreak during July to September 2014 in samples of obtained from the Narla block of the Kalahandi district of Odisha.

Received January 11, 2017. Accepted April 10, 2017.

J-STAGE Advance Publication July 1, 2017.

DOI: 10.7883/yoken.JJID.2016.585

*Corresponding author: Mailing address: Microbiology Division, Regional Medical Research Centre (ICMR) Chandrasekharpur, Bhubaneswar 751023, India. Tel: +91-674-2305638, Fax: +91-674-2301351, Email: bbpal_rmrcc@yahoo.co.in

MATERIALS AND METHODS

Study area: The microbiologist and the medical officer of the Narla Community Health Centre (CHC) in Kalahandi district visited the diarrhea-affected villages of Narla block between September 16 and 20, 2014,

to obtain information on the index case, date-wise line listing of all diarrhea cases, and clinical signs and symptoms of the patients with severe diarrhea. The index case was traced back from the hospital record. The source of drinking water, chlorination of drinking water sources, and prevailing sanitary conditions in each villages was also recorded.

Isolation and identification of *V. cholerae*: Informed consent was obtained from all participating patients or their guardians before the collection of samples.

Rectal swabs were collected from the diarrhea patients from the villages and from the Narla CHC, transported in Cary-Blair transport medium, enriched in alkaline peptone water, and streaked on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Sucrose-fermenting yellow colonies were selected and tested by standard biochemical tests. The sucrose fermenting colonies from TCBS agar plates were subcultured in triple-sugar iron agar medium and positivity for *V. cholerae* was defined as an acidic slant/acidic butt reaction without acid or gas production and with oxidase positivity. Serological conformation was performed using *V. cholerae*-specific polyvalent O1 and monovalent Ogawa and Inaba antisera. The sensitivity and resistance patterns of *V. cholerae* O1 strains were tested using antibiotic-impregnated commercial disks (Hi-Media, Mumbai, India) (16).

Double mismatch amplification mutation assay (DMAMA)-PCR assay: DMAMA-PCR assay was performed to detect the type of *ctxB* allele in all *V. cholerae* O1 strains isolated from the studied outbreak along with 30 *V. cholerae* strains isolated between 1999 and 2007 (laboratory stocks), using the primer set (*ctxB*-F3/Rv-cla) for the Haitian *ctxB* allele and the primer set (*ctxB*-F4/Rv-cla) for the classical *ctxB* allele. These allele-specific primers each carry specific nucleotides, namely A and C for the Haitian and classical alleles, respectively, at the 3' end, as described by Naha et al., 2012 (6). Template DNA for the PCR assay was prepared from a *V. cholerae* overnight culture grown in Luria-Bertani broth at 37°C. The PCR mixture (total volume, 25 µl) contained 5 µl of template DNA after extraction by the boiling method. The PCR conditions were as follows: initial denaturation at 96°C for 2 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 30 s, and final extension at 72°C for 2 min. The amplified fragments were detected by agarose gel electrophoresis after staining with ethidium bromide (6).

RESULTS

Narla block is located in Kalahandi district in western Odisha. The block has a population of 127,043, among which the total population affected was 46,236, the number of villages affected was 57, the number of total cases reported was 321, and the number of deaths was 3. The index case occurred on the July 28, 2014 in Bankel village, and the number of cases gradually increased thereafter. As per the hospital records, the index case was a 62-year-old male goat keeper from Bairpada Sahi of Bankel village. The village is situated at the bottom of a mountain adjacent to a water reservoir. He went to the mountain to graze his goats during the early morning

of the July 28, 2014, felt severe thirst, drank water from the water reservoir at 11 am, and returned home in the afternoon. He suffered from severe diarrhea at 7 pm that night. He had profuse rice water stool, vomiting associated with severe dehydration, muscular pain, and abdominal cramping. He was admitted to the hospital on the morning of July 29, 2014 and was treated and cured.

The clothes contaminated with excrement were cleaned in the field adjacent to his house. At that time, there was a continuous heavy rainfall for 7–10 days. The rain water and fecal materials might have mixed and flowed downward to contaminate the small nala and water of Sandul river. Gradually, new cases were reported from the nearby villages located along that river in the downstream direction. The diarrhea cases were reported from the villages of Narla and Sargiguda, which were the worst affected, and the disease also spread to other villages. All severe diarrhea cases were analyzed from the available records of hospitals of the CHC. The index case was reported on July 28, 2014, the highest number of severe diarrhea cases (27 cases) was reported on September 11, 2014, and the last cases were reported on September 17, 2014 (Fig. 1). The dates of occurrence for the first and last diarrhea cases in each village were noted from the hospital record. Considering these dates and the smallest distances between the villages, the probable spread of the disease from village to village was drawn (Fig. 2A). The villages that were worst affected include Narla, Bankel, Asurgada, and Sargiguda. The cholera epidemic continued from July to September 2014, and more cases and villages were affected during the August and September 2014 (Fig. 2B).

Among the 17 rectal swabs that were collected, 11 samples were positive for *V. cholerae* O1 Ogawa biotype El Tor, 5 were positive for *Escherichia coli*, and 1 was negative for any bacterial enteropathogens by the culture method. The *V. cholerae* strains were sensitive to ciprofloxacin, norfloxacin, tetracycline, doxycycline, ofloxacin, and azithromycin, but were resistant to ampicillin, gentamicin, furazolidone, nalidixic acid, erythromycin, neomycin, co-trimoxazole, and

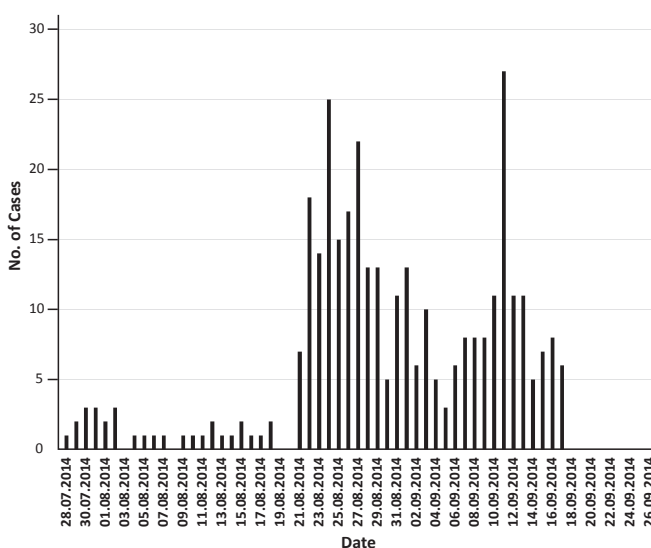


Fig. 1. Date-wise incidence of severe diarrhea cases in Narla block between July 28, 2014 and September 27, 2014.

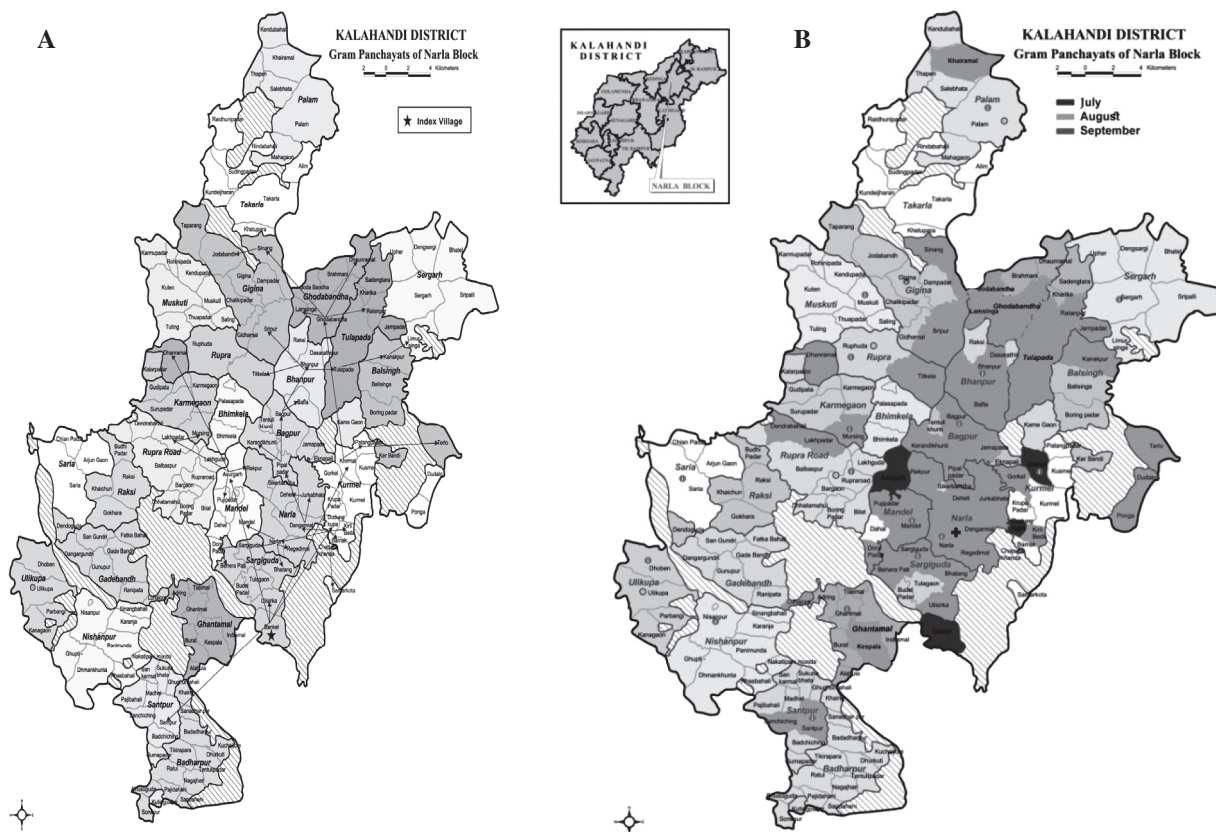


Fig. 2. Date-wise (A) and month-wise (B) spread of cholera cases in Narla block of Kalahandi district of Odisha.

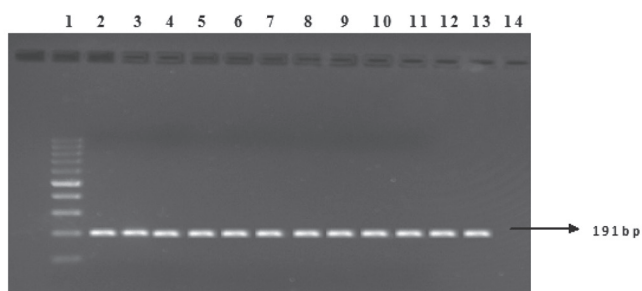


Fig. 3. DMAMA PCR assay showing *ctxB7* of Haitian variants *V. cholerae* O1 Ogawa strains. Lane 1, 0.1-kb ladder; lane 2, positive control Haitian variant; lanes 3–13, *V. cholerae* O1 Ogawa strains; lane 14, negative control.

chloramphenicol. The DMAMA-PCR results revealed that all 11 *V. cholerae* O1 stains were positive for *ctxB7* of the Haitian variant (Fig. 3). Eight of 30 *V. cholerae* strains isolated during 1999–2007 (2 out of 5 in 1999; 3 out of 10 in 2000; 0 out of 5 in 2003; and 3 out of 10 in 2007) were positive for HCT variant *V. cholerae* O1 Ogawa.

DISCUSSION

Group discussions were held among the people in the affected villages during field visits. It was found that some people went to the cotton fields to work and drank water from the nearby water reservoir, took a bath, and returned home in the afternoon. The people reported diarrhea with rice-water stool in the night associated with vomiting and abdominal cramping.

Gradually, the patients developed severe dehydration in the night and were hospitalized on the morning of the next day, treated and cured. This clearly indicated that the source of infection was the large water reservoir. The number of cases gradually increased due to unhygienic conditions in the houses, person to person contact in the families, unsafe disposal of fecal materials, cleaning of excrement-contaminated clothes in nearby water reservoirs, and the migration of people from one village to other villages after attending their relatives who suffered from diarrhea. The infection spread to other families in nearby houses in the same village and to other villages. The older people in the cholera affected villages informed us there had no cholera outbreak in Narla block for the last 30 years. The hospital records of the last 5 years also indicated that there was no diarrheal outbreak reported in Narla block. The contamination of drinking water sources was the main transmission route of cholera. In the rainy season, people mainly go to the paddy fields for farming and drink water from nalas, small water reservoirs, rivers, and streams, and become infected in this way. The water samples collected during the 2007 cholera outbreak in the tribal areas were positive for *V. cholerae* O1 Ogawa (16). The diarrheal patients mostly belonged to scheduled tribes and scheduled caste followed by general caste, and the patients were all above 20 years of age. This group of people were mostly illiterate and had little knowledge about the acquisition and spread of cholera infection.

Although the index case was from Bankel village, the outbreak spread to nearby villages along the Sandul River in the downstream direction. Early reporting enabled the state health authorities to implement adequate

control measures in time, such as chlorinating drinking water sources, placing sandbags mixed with bleaching powder near the upstream source of the water flow near the ghats of the river/nala/water reservoir (every day), safety burying fecal matter and vomit, etc. These control measures curtailed the spread of the disease into unaffected areas of this block.

The *V. cholerae* O1 Ogawa biotype El Tor strains were sensitive to ciprofloxacin, azithromycin, norfloxacin, ofloxacin, doxycycline, and tetracycline, but were resistant to ampicillin, nalidixic acid, neomycin, furazolidone, erythromycin, chloramphenicol, gentamicin, and co-trimoxazole. Similar antibiogram profiles were reported during the 2010 cholera epidemic in Rayagada district, in which the responsible strain became sensitive to tetracycline during the epidemic despite a prevailing background of tetracycline resistance. Similar results were also published from Kolkata and southern India (17,18).

Kumar et al., 2014 (17) reported the isolation of HCT-producing variant *V. cholerae* O1 strain (*ctxB7*). This strain was first reported from Odisha during 2007 and caused a devastating cholera epidemic in Haiti during 2010. Similarly, HCT-producing variants *V. cholerae* O1 Ogawa strains were isolated after a cholera outbreak in Yavatmal district in Maharashtra (14) from southern India during 2012–2014 (18). Several studies indicated that there were certain changes in the *ctxB* gene located in the *ctx* Q element of *V. cholerae* strains. The HCT-producing *ctxB* *V. cholerae* strains carry a mutation at the 58th nucleotide, corresponding to the 20th amino acids (His20 in the classical allele and Asn in the Haitian and El Tor alleles) (8). This mutation has rarely been reported globally and was recently reported in a few outbreaks in India (19). To determine its origin, some representative strains from *V. cholerae* O1 stocks isolated in the previous years were tested using DMAMA-PCR assay. It was found that the HCT-producing variant *V. cholerae* strains originated from the coastal district of Odisha during the aftermath of the cyclone that affected that area in 1999. Further molecular studies are warranted to investigate the spread of this HCT-producing variant *V. cholerae* O1 strains in the coastal and tribal areas of Odisha.

The altered biotype of *V. cholerae* El Tor (CCT) strains has been reported from different geographical regions (19). The hybrid property (CCT in the altered El Tor biotype) enhances the infective potential of the bacterium, and such strains are associated with more fluid loss and a higher fatality rate. These strains have been reported as the causative pathogens of outbreaks in several regions of India, including Odisha, Chennai, Hyderabad, Solapur, and Assam (20–22). The HCT-producing variants of *V. cholerae* were reported for the first time from the cholera outbreak in Odisha during 2007, later from Haiti during 2010, and subsequently from Asia and Africa (15,16). A few reports have described the isolation of HCT-producing variant *V. cholerae* O1 strain or the identification of this strain as the causative pathogen of small outbreaks from West Bengal, Bihar, and southern India (6,13,14). There have been similar reports on the isolation of HCT-producing *V. cholerae* from Zambia (11) and from Mexico during the 2013 cholera outbreak (12). The present investigation provides the first re-

port of a multidrug-resistant HCT-producing variant *V. cholerae* O1 strains as the causative pathogen of a large cholera outbreak in the tribal areas of Odisha, India. This may be the second large cholera epidemic caused by a HCT-producing variant of *V. cholerae* O1 reported worldwide since the cholera epidemic in Haiti during 2010. It is critical to perform continuous surveillance for tracing the origin and spread of HCT-producing variant *V. cholerae* strains among the various regions of Odisha, because there is a significant potential for such strains to cause future outbreaks or epidemics.

Acknowledgments The authors are thankful to the Director, RMRC, Bhubaneswar providing funds for this work. The medical officers and staffs of Narla CHC; Chief District Medical Officer (CDMO), Kalahandi; are highly acknowledged for their support and help during the field visits and sample collection.

Conflict of interest None to declare.

REFERENCES

1. Piarroux R, Barrais R, Faucher B, et al. Understanding the cholera epidemic, Haiti. *Emerg Infect Dis.* 2011;17:1161-7.
2. World Health Organization (WHO). Cholera, 2011. *Wkly Epidemiol Rec.* 2010;86:325-40.
3. Safa A, Nair GB, Kong RY. Evolution of new variants of *Vibrio cholerae* O1. *Trends Microbiol.* 2010;18:46-54.
4. Nair GB, Faruque SM, Bhuiyan NA, et al. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J Clin Microbiol.* 2002;40:3296-9.
5. Raychoudhuri A, Patra T, Ghosh K, et al. Classical *ctxB* in *Vibrio cholerae* O1, Kolkata, India. *Emerg Infect Dis.* 2009;15:131-2.
6. Naha A, Pazhani GP, Ganguly M, et al. Development and evaluation of a PCR assay for tracking the emergence and dissemination of Haitian variant *ctxB* in *Vibrio cholerae* O1 strains isolated from Kolkata, India. *J Clin Microbiol.* 2012;50:1733-6.
7. Okada K, Roobthaisong A, Nakagawa I, et al. Genotypic and PFGE/MLVA analysis of *Vibrio cholerae* O1: geographical spread and temporal changes during the 2007–2010 cholera outbreaks in Thailand. *PLoS One.* 2012;7:e30863.
8. Marin MA, Thompson CC, Freitas FS, et al. Cholera outbreaks in Nigeria are associated with multidrug resistant atypical El Tor and non-O1/non-O139 *Vibrio cholerae*. *PLoS Negl Trop Dis.* 2013;7:e2049.
9. Nair GB, Qadri F, Holmgren J, et al. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. *J Clin Microbiol.* 2006;44:4211-3.
10. Olsvik O, Wahlberg J, Petterson B, et al. Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *J Clin Microbiol.* 1993;31:22-5.
11. Marin MA, Vicente ACP. Variants of *Vibrio cholerae* O1 El Tor from Zambia showed new genotypes of *ctxB*. *Epidemiol Infect.* 2012;140:1386-7.
12. Díaz-Quiñonez A, Hernández-Monroy I, Montes-Colima N, et al. Outbreak of *Vibrio cholerae* serogroup O1, serotype Ogawa, biotype El Tor strain—La Huasteca Region, Mexico, 2013. *MMWR Morb Mortal Wkly Rep.* 2014;63:552-3.
13. Kutar BMRNS, Rajpara N, Upadhyay H, et al. Clinical isolates of *Vibrio cholerae* O1 El Tor Ogawa of 2009 from Kolkata, India: preponderance of SXT element and presence of Haitian *ctxB* variant. *PLoS One.* 2013;8:e56477.
14. Kumar P, Mishra DK, Deshmukh DG, et al. Haitian variant *ctxB* producing *Vibrio cholerae* O1 with reduced susceptibility to ciprofloxacin is persistent in Yavatmal, Maharashtra, India, after causing a cholera outbreak. *Clin Microbiol Infect.* 2014;20:O292-3.
15. Koley H, Ray N, Chowdhury G, et al. An outbreak of cholera caused by a single clone of *Vibrio cholerae* O1 El Tor variant strain in Bihar, India. *Jpn J Infect Dis.* 2014;67:221-6.
16. Pal BB, Khuntia HK, Samal SK, et al. Epidemics of severe cholera caused by El Tor *Vibrio cholerae* O1 Ogawa possessing the *ctxB* gene of the classical biotype in Orissa, India. *Int J Infect Dis.*

Haitian Variant *V. cholerae* in Odisha

- 2010;14:384-9.
17. Kumar P, Mishra DK, Deshmukh DG, et al. *Vibrio cholerae* O1 Ogawa El Tor strains with the *ctxB7* allele driving cholera outbreaks in south-western India in 2012. *Infect Genet Evol.* 2014;25:93-6.
 18. Bhattacharya D, Dey S, Pazhani GP, et al. *Vibrio cholerae* O1 El Tor variant and emergence of Haitian *ctxB* variant in the strains isolated from South India. *Med Microbiol Immunol.* 2016;205:195-200.
 19. Ghosh P, Naha A, Pazhani GP, et al. Genetic traits of *Vibrio cholerae* O1 Haitian isolates that are absent in contemporary strains from Kolkata, India. *PLoS One.* 2014;9:e112973.
 20. Jain M, Kushwah KS, Kumar P, et al. Molecular characterization of *Vibrio cholerae* O1 reveals continuous evolution of its new variants in India. *Indian J Microbiol.* 2013;53:137-41.
 21. Borkakoty B, Biswas D, Devi U, et al. Emergence of classical *ctxB* genotype 1 and tetracycline resistant strains of *Vibrio cholerae* O1 El Tor in Assam, India. *Trans R Soc Trop Med Hyg.* 2012;106:382-6.
 22. Goel AK, Jain M, Kumar P, et al. Molecular characterization reveals involvement of altered El Tor biotype *Vibrio cholerae* O1 strains in cholera outbreak at Hyderabad, India. *J Microbiol.* 2011;49:280-4.