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Epidemiological Report

Outbreak of Cholera Caused by *Vibrio cholerae* O1 El Tor Variant Strain in Bihar, India

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SUMMARY: An outbreak of cholera struck Bihar, an Indian state, in August 2008 following a massive flood. Here we report the phenotypic and genotypic characteristics of *Vibrio cholerae* strains isolated from patients with diarrhea. Rectal swabs were obtained from patients with diarrhea who were admitted to medical camps or the hospital, and the strains were biochemically and serologically characterized. *V. cholerae* was isolated from 21 (65.6%) of 32 rectal swabs. Serological studies revealed that all the 21 isolates belonged to *V. cholerae* O1 Ogawa. Mismatch amplification mutation assay (MAMA)-PCR showed that the isolates belonged to El Tor variant group, and pulsed-field gel electrophoresis (PFGE) proved that these isolates were of a different lineage than the conventional El Tor variant strains. These isolates were resistant to several drugs, including ampicillin, streptomycin, tetracycline, nalidixic acid, and furazolidone. The uniqueness of the current report arises from the fact that records of cholera in Bihar are availiable for the early 1960s but not for the next 4 decades. Moreover, the present study is the first to report a cholera outbreak in Bihar that was caused by an El Tor variant strain.

INTRODUCTION

Diarrhea is among the leading causes of morbidity and mortality among children and adults in the developing countries of the world where access to safe water and adequate sanitation cannot be guaranteed for all; it is responsible for an estimated 3.7–4.6 million deaths annually (1). Cholera, caused by the microorganism *Vibrio cholerae*, has the potential to cause explosive outbreaks, epidemics, and even pandemics (2). The world has already faced 7 cholera pandemics over the past 2 centuries (2), and the Indian subcontinent has been an epicenter for cholera (3). One of the most powerful virulence factors of this organism is the cholera toxin encoded by *ctxAB*, which is located on the CTX prophage (4). *V. cholerae* serogroup O1 is classified into 2 biotypes, classical and El Tor; the El Tor biotype is responsible for the ongoing seventh worldwide pandemic of cholera (2). Recent studies suggest that novel strains of *V. cholerae* O1 are emerging, including hybrid strains as well as altered El Tor or El Tor variant strains (5). Such altered El Tor or El Tor variant strains are of the El Tor biotype but produce classical cholera toxin. This type of *V. cholerae* O1 is spreading across many regions of the world (6), including the US Gulf Coast and several countries of Asia and Africa (7,8).

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The flood of August 2008, which ravaged in Bihar, was one of the worst and disastrous floods in Indian history, and was caused by a breach of the Kosi embankment at Kusha in Nepal near Indo-Nepal border. The mighty river Kosi in north Bihar changed its course eastward, flowing through a channel it had abandoned over 200 years ago, drowning towns and thousands of villages and thereby rendering millions of people homeless (9). During the past few hundred years (since 1731), Kosi, once called the ''Sorrow of Bihar'', has exhibited a unique character, shifting its course from east to west to a distance of 210 miles (10). The direction of the river Kosi is very unstable. It carries huge amounts of silt and sediments from its upper basin (comprising Nepal and Tibet), which are then deposited in its lower basin. This results in elevation of the river bed, which in turn causes obstruction. About 100-million cubic meters of sediment is estimated to be annually deposited by the river (11). *V. cholerae* is normally present in coastal waters worldwide, even in countries where cholera is normally absent. It thrives in brackish water and can also occur in rivers and lakes. These bacteria cling to zooplankton, particularly to tiny crustaceans called copepods and can be indefinitely present in relatively low numbers (12). Thus, outbreaks can occur when a series of environmental factors, including increases in water temperatures, alkalinity, and nutrient levels, conspire to cause zooplankton blooms. These blooms, in turn, would offer the cholera microbe an opportunity to proliferate (13). Ingestion of such contaminated water causes sickness, which further spreads upon contamination of drinking water sources with feces from the infected people (14). We report our findings on the prevalence of di-

arrhea among flood-affected individuals, with an emphasis on the bacteriological characteristics of the strains causing outbreaks of cholera.

METHODS

Region of study: A central team from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata and the National Centre for Disease Control (NCDC), New Delhi visited Madhepura district in the state of Bihar. The district headquarter is situated at a distance of 278 km from Patna, the capital of Bihar. The team members observed the overall situation in relief camps set up to offer shelter to flood-affected people. Daily surveillance data for infectious diseases and rectal swab samples from diarrhea cases were collected for microbiological investigation from 15th to 21st September 2008. Furthermore, diarrhea cases admitted to medical camps and district hospital, logistics, chlorination of drinking water and sanitary conditions prevailing in the relief camps were thoroughly examined by the central team. The team also visited 1 mega (larger) and 3 smaller camps daily. All the 5 mega camps, catering to about 20,000–50,000 affected individuals, were found to have medical facilities with indoor beds and timed outpatient clinics. The 55 small camps, catering to about 2,000–7,000 people, were attended to by doctors through rotating mobile units. The medical facilities set up in these relief camps in Bihar during the flood sought to provide treatment for diarrhea, fever, and other ailments. The central team also visited and stayed at Saharsa district from 24th to 28th September 2008.

Isolation and identification of *V. cholerae*: Rectal swabs were obtained from patients with diarrhea who were admitted to the camps or hospital, and transported in Cary–Blair medium to the laboratory. The samples were then streaked either directly onto thiosulfate-citrate-bile-salts-sucrose (TCBS) agar (Difco, Detroit, MI, USA) or after enrichment in alkaline peptone water (APW, pH 8.0). Sucrose-fermenting yellow-colored colonies resembling *V. cholerae* were selected and tested using standard biochemical methods (15), including sucrose fermentation in triple sugar iron (TSI) medium (A/A reaction) and oxidase positivity. Serological confirmation was obtained using *V. cholerae*-specific polyvalent O1 and monovalent Ogawa and Inaba antisera (3). The samples which were positive for *V. cholerae* were subjected to pulsed-field gel electrophoresis (PFGE), PCR, phage typing, and antibiogram.

(i) PFGE: PFGE was performed for all *V. cholerae*-positive samples according to the PulseNet protocol for *V. cholerae* (16). DNA from *Salmonella enterica* serotype Braenderup strain H9812 was subjected to restriction digestion using 50 U of *Xba*I (Takara, Otsu, Japan) at 37°C for 4 h and employed as the universal size standard. The DNA of test strains was digested with *Not*I and subjected to electrophoresis. The gel was then stained for 30 min using 500 ml of ethidium bromide solution (50 μg/ml), followed by rinsing several times in distilled water. The band pattern was observed under UV illumination (BioRad, Hercules, CA, USA).

The DNA fingerprint patterns of a representative isolated strain SRK6 and the reference strains of *V. cholerae* were analyzed using the computer software package BioNumerics (Applied Maths, Kortrijk, Belgium). Background subtraction and gel normalization were performed; the fingerprint patterns were then subjected to typing on the basis of banding similarity and dissimilarity, using the Dice similarity coefficient and cluster analysis based on the unweighted-pair-group method using average linkages (UPGMA), as recommended by the software manufacturer (17). The results were graphically represented as dendrograms.

(ii) PCR analysis: Genomic DNA of the *V. cholerae* strains was harvested according to the method detailed by Murray and Thompson (18) and used for PCR analysis using primers specific for *ctxA* and allele-specific *tcpA* (19,20).

The strains were also examined by mismatch amplification mutation assay (MAMA)-PCR for detecting the *ctxB* alleles, using a forward primer, FW-Com (5′-AC TATCTTCAGCATATGCACATGG-3′) common for both the alleles, and 2 allele-specific primers, Re-cla (5′-CCTGGTACTTCTACTTGAAACG-3′) and Re-elt (5′-CCTGGTACTTCTACTTGAAACA-3′), for classical and El Tor biotypes, respectively (21,22).

(iii) Phage typing: Phage typing was performed according to the standard methodology routinely employed in our laboratory (23). *V. cholerae* MAK 757 (ATCC 51352) was used as control for each set of experiments because this strain is sensitive to lysis by the O1 group of phages. The reaction was recorded as positive if the number of plaques was 5 or more. The method detailed by Basu and Mukerjee and the new phage-typing scheme were employed for typing these strains (23).

(iv) Antimicrobial susceptibility: Antibiotic susceptibility assay was performed according to the method described by Bauer *et al*. (24) using commercially available antibiotic disks (BD Biosciences, Sparks, MD, USA). The following disks were employed: ampicillin (10 μg), ciprofloxacin (5 μg), sulfamethoxazole (23.75 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), tetracycline (30 μg), and furazolidone (10 μg). *Escherichia coli* ATCC 25922 was used as a quality control strain. The data was interpreted according to the guidelines of Clinical and Laboratory Standards Institute (25).

Ethical approval: We visited flood affected area in Bihar from 14th to 28th September 2008, as per order of Ministry of Health, Central Government, No. D32020/18/2008-EMR/HR, on emergency basis. This special investigation has been made as per Ministry of Health directive and that mean it includes all necessary ethical clearance, obtained by Ministry of Government of India, on our behalf.

RESULTS

Nature of devastation: Kosi had breached its eastern embankment, rushing down as a miles-wide torrent to the river Ganges and crossing over 100 km south. The overflow resulted in disastrous flood in 14 districts of Bihar, which affected over 3.3 million people. Of the 5 badly-affected districts, Madhepura was the worst hit, with1.5 million affected people; 11 blocks in this district

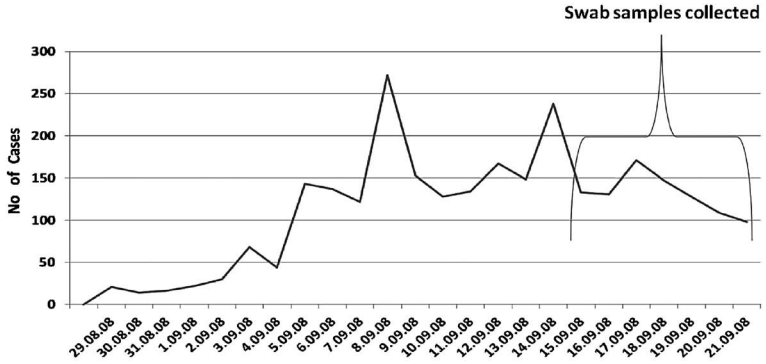

Fig. 1. Epidemic curve with marked time period when samples were collected during outbreak of diarrhea in Madhepura.

Table 1. Observations during relief camp visits with control measure checklist

Checklist item	Findings regarding mega-camp	Findings regarding mini-camp
Visited	4	20
No. of people in camps	20,000–50,000	2,000–7,000
Presence of doctors	5–6 doctors on daily duties in rotation in each of the 4 mega-camps	1–2 doctors attended daily in each of the 5 mini camps and in other 15 mini camps doctors conducted outdoor 2–3 times a week on rotational basis
Immunization against tetanus toxoid for antenatal mothers	Carried out in all 4 camps	Carried out in 17 camps
Primary immunization against BCG, TT, DPT, and measles for children <5 yr + vitamin A supplementation	Carried out in all 4 camps	Carried out in 16 camps
Measles mass immunization	Not conducted	Not conducted
Medicine (+ORS) well stocked	In all 4 camps	In 4 camps (doctors visited the other 16 camps with mobile van containing medicines and ORS packets)
Water source	Tube wells were installed and in addition drinking water was supplied with army tanker in 4 camps	Tube wells installed in all small camps
Chlorination of water done	In all camps	In all camps
Chloroscope availability to check for chlorine level	Not available in any of these 4 camps	Not available in any of these 20 camps
Trained human resource for chlorine testing	In all camps, there were trained individuals	In all camps, there were trained individuals
Residual chlorine level in drinking water stored by the families	None of the samples tested was found positive for residual chlorine	None of the samples tested was found positive for residual chlorine
Presence of sanitary latrine	Inadequate in all 4 camps (about 1 per 1,000 inmates)	Inadequate in all 20 camps (about 1 per 1,000 inmates)
Use of latrine	Latrines were not being used by camp inmates; they rather preferred open air defecation	Latrine were not being used by camp inmates, they rather preferred open air defecation
Garbage disposal by deep burial/burning	Observed in all 4 camps	16 camps
Mosquitogenic condition (checked by entomologist)	Not prevail any of the 4 camps	Not prevail out of the 20 camps

were inaccessible and only 2 blocks were partly accessible by road. The flood water destroyed major infrastructure, including roads, bridges, shelters, and agricultural lands. Relief work was difficult as the water was not calm, unlike during the yearly monsoon flood, but still rising and flowing fast enough to prevent relief workers from reaching the affected people. A few relief workers were also reported dead in a boat capsize. An epidemic curve was plotted to provide a clear picture of the timing of sample collection during the Madhepura outbreak (Fig. 1).

Discoveries in Madhepura: Five mega camps were set up on elevated lands and had road connection with the district headquarter at Madhepura, while 55 smaller camps were set up in nearby villages (Table 1). Logistics were satisfactory in the mega camps; however, a few small camps were not properly maintained or attended to by doctors. Adequate latrine facilities were lacking in most of the camps. Medicines were not in short supply, but occasionally, pediatric preparations were not available. There were enough packets of oral rehydration solution (ORS) and intra venous (IV) fluids. Surveillance

data was incomplete and mortality statistics were unavailable, which was notified to the headquarter at Patna and local health authorities at the district level. The attack rate of diarrhea was 0.66% among the affected population of 1.5 million between 15th and 21st September 2008. Sixty-six cases with severe watery diarrhea were admitted during this period to the mega camps and district hospital in Madhepura. Among these, a 12-year-old boy, who was admitted with symptoms of fever, vomiting, diarrhea, and abdominal pain, died after suffering for 3 days. Estimated diarrhea-associated mortality for the admitted cases was thus calculated as 1.5% (1/66).

There was no dearth of chlorine tablets and bleaching powder, but the state healthcare workers did not have chloroscopes for measuring residual chlorine. We determined, using the chloroscope that we carried, that the drinking water in different relief camps did not test positive for free residual chlorine. The only exception was water samples from army tankers, which tested positive for optimum chlorination.

Isolation and characterization of *V. cholerae*: Twenty-one (65.6%) of the 32 rectal swabs obtained from patients with acute watery diarrhea were found to be positive for *V. cholerae* O1 Ogawa. These samples were further analyzed by PFGE, PCR, phage typing, and drug susceptibility.

(i) PFGE: Twenty-one strains of *V. cholerae* O1 Ogawa formed an indistinguishable, homogenous restriction fragment pattern upon PFGE analysis. Therefore, only 1 strain (SRK6) was selected for dendrogram analysis using BioNumerics (Applied Maths) to examine the relatedness of this strain with other representative *V. cholerae* strains. The results obtained revealed that the outbreak was caused by a strain of *V. cholerae* O1 Ogawa, which formed a separate cluster from the reference *V. cholerae* strains, including El Tor (V-54), Classical (569B), and El Tor variant (B-33), thus indicating a different lineage of these 21 isolates (Fig. 2).

(ii) PCR: The *ctxA–tcpA* multiplex PCR analysis revealed that these strains were positive for *ctxA* and El Tor *tcpA*. In addition, a product was obtained in MAMA-PCR assay using classical *ctxB*-specific primers but not El Tor-specific primers. Taken together, the results of the PFGE and PCR analyses show that the outbreak was caused by *V. cholerae* O1 Ogawa, El Tor variant strain of a different lineage.

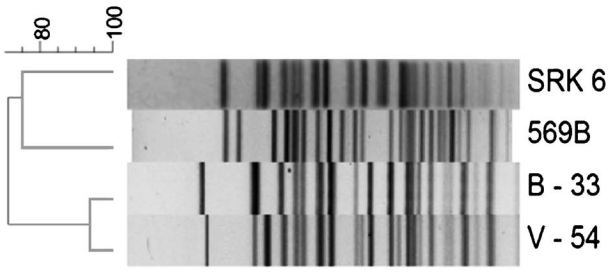

Fig. 2. Dendrogram showing genomic fingerprint pattern of a representative *V. cholerae* O1 biotype strain (SRK6) isolated in Bihar outbreak (2008) and the reference *V. cholerae* O1 strains: Classical (569B), El Tor variant (B-33) and El Tor (V-54). The dendrogram was prepared by Dice similarity coefficient and UPGMA clustering methods by using PFGE images of *Not*I-digested genomic DNA; PFGE gels were run under the same conditions. The scale bar at the top (left) indicates the correlation coefficient (%). The picture shows that the outbreak has been caused by a *V. cholerae* O1 Ogawa strain of a different lineage suggesting a regional signature.

(iii) Phage typing: The strains were found to be typeable by both conventional and New phage-typing schemes; T2 by Basu & Mukerjee typing scheme and as T27 by New phage typing scheme.

(iv) Antimicrobial susceptibility: All 21 isolates (100%) were resistant to ampicillin, streptomycin, tetracycline, nalidixic acid, and furazolidone, while 15 (71.4%) were resistant to sulfamethoxazole. All these isolates (100%) were susceptible to gentamycin, while 18 (85.7%) were susceptible to norfloxacin and chloramphenicol. Nine of these isolates (42.85%) were susceptible to neomycin, and only 6 isolates (28.57%) were found to be susceptible to ciprofloxacin.

DISCUSSION

Exploring the causes of diarrhea and other infectious diseases following natural calamities through rapid situation assessment is necessary for rapidly mounting appropriate responses or modifying control measures. Kosi is a trans boundary river that flows north to south from the Himalayas in Nepal and Tibet through northern Bihar in India to join the river Ganges. It carries huge amounts of silt because of extensive soil erosion and landslides in its upper catchment areas in Tibet and Nepal. On account of steep slopes and narrow gorges in its upper basin, sediments are carried to the plains and deposited in the river bed where the slopes are flatter. This opposes fixed flow, resulting in the river splitting into a number of interlacing channels, which occasionally cause floods. The course of the river thus remains unpredictable and necessitates quick relief support at regular intervals (26). People in flood-affected areas were forced to use contaminated water for drinking and food preparation, diarrhea outbreak therefore became imminent.

In recent years, isolates of *V. cholerae* O1, altered El Tor, and El Tor variant strains have been observed during different cholera outbreaks in various parts of India. However, most of these reports have been generated from southern, south-western and eastern parts of the country (27-30), though the northern states, Punjab and Haryana have also reported cases of cholera caused by new variants of *V. cholerae* O1 El Tor (28). Remarkably, while preparing the current investigation report, we could not locate any such report of cholera outbreaks from Bihar, indicating a gap in disease surveillance and documentation. During surveillance, we noticed that the case definition of diarrhea was not strictly adhered to, and mortality statistics were not described in the surveillance report. The absence of proper surveillance weakens the managerial system and results in challenges for allocating appropriate logistics resources. However, most of the flood hit areas were inaccessible; therefore, damage assessment and approaching the affected population posted difficulties. We also identified gaps pertaining to feedback as well as meeting and intersectoral coordination between the health and other administrative departments of the same district. It is important to note that the isolates of *V. cholerae* recovered

during epidemics in West Bengal, Bihar, Orissa, Uttar Pradesh, and Chennai (formerly called Madras) were used for research on cholera in the early 1960s (29,30). The migration of a large labor population from Patna-Munger regions of Bihar to West Bengal and back to their native region was also implicated in the cholera outbreak in Bihar during this time; this finding was supported by the similarity of the strains isolated in these 2 states. While other states continued to report (albeit incompletely) cases of cholera even after the 1960s (8), data from Bihar ceased from mid-1960s onwards.

During the current investigation, *V. cholerae* O1 Ogawa was isolated from 21 (65.6%) of the 32 specimens examined. Such a high isolation rate warranted the characterization of the situation as cholera outbreak among the flood-affected people of Madhepura. The PFGE patterns of these 21 isolates revealed a homogenous banding pattern. The dendrogram of a single randomly-selected isolate along with *V. cholerae* O1 Ogawa reference strains showed that the outbreak was caused by *V. cholerae* O1 Ogawa strain of a different lineage than the reference strains employed in the study. Moreover, the strains phenotypically resembled El Tor but contained classical type of *ctxB,* as detected by the recently developed MAMA-PCR.

The emergence of multiple antibiotic-resistant strains of *V. cholerae* over the last 2 decades has been a major public health concern (31,32). *V. cholerae* strains resistant to tetracycline, ampicillin, kanamycin, streptomycin, sulphonamides, trimethoprim, and gentamicin have been previously reported from India and neighboring countries (28,31,32). Multi-drug resistance among *V. cholerae* strains was identified in our study as well, which limits the therapeutic potential of many drugs (33,34). Ongoing surveillance with regard to the antibiotic sensitivity of diarrhea-causing organisms, as well as prescription practice of physicians, is therefore of paramount importance, which could help the development of appropriate management guidelines at regular intervals. However, we could not compare the measures taken during the earlier diarrhea outbreak in Bihar with the current one under investigation because of lack of documentation; no published reports were available to carry out such an exercise. The current study, therefore, bridged this gap in information, which was long overdue. It is also to be emphasized that the availability of simple facilities such as sanitary latrines and deputing adequate human resource are central to effective disaster response following floods.

In conclusion, *V. cholerae* O1, Ogawa El Tor, variant strain of a different lineage than the reference strains was isolated as the etiologic agent causing the cholera outbreaks in Madhepura district of Bihar, India in the year 2008. The present study is the first to report the emergence of this strain in this region. The checklist used by us for recording different mitigating measures turned out to be a useful situation assessment tool. We urge the use of such systematic approaches, which would help in quick situation assessment during disaster and in developing appropriate site-specific management guidelines.

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Conflict of interest None to declare.

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