

Original Article

Incidence of Bacterial Enteropathogens among Diarrhea Patients from Tribal Areas of Odisha

Smruti Ranjan Nayak, Ashish Kumar Nayak, Bhagya Laxmi Biswal, Rudra Prasad Jena, Surya Kanta Samal, and Bibhuti Bhusan Pal*

Microbiology Division, ICMR-Regional Medical Research Centre, Bhubaneswar, Odisha, India

SUMMARY: Infectious diarrheal diseases remain a leading cause of morbidity and mortality in developing and underdeveloped countries. The present study documented the etiology of bacterial enteropathogens in three tribal districts of Odisha from July 2010 to September 2013. A total of 1427 rectal swabs were collected and bacteriologically analyzed by following standard procedure. Among the 930 (65.2%) culture positive samples, *Escherichia coli* (*E. coli*) constituted 636 (44.6%); *Vibrio cholerae* (*V. cholerae*) O1, 146 (10.2%); *Salmonella* species (spp.), 10 (0.7%); *Shigella* spp., 79 (5.5%); and *Aeromonas* spp., 59 (4.1%). Of the 729 environmental water samples taken from river, open well, Nala (a small stream), and Chua (a shallow pit on a river bed), 14 (1.9%) contained non-O1/non-O139 *V. cholerae* and 13 (1.8%) had *V. cholerae* O1 strains. An analysis of the demographics showed that people in the 14 to 40-year age group were highly susceptible to diarrhea caused by *V. cholerae* which occurred mainly during the rainy and post-rainy seasons. All enteropathogens were multidrug-resistant and found throughout the study period. The *V. cholerae* strains isolated were El Tor variants carrying the classical, El Tor, and Haitian cholera toxin subunit B (*ctxB*) genes. The classical *ctxB* was the dominant allele, and the prevalence of the Haitian *ctxB* allele increased during the test period. These findings indicate that active surveillance is needed to monitor the changing antibiotic resistance patterns of *V. cholerae* serogroups and biotypes present in this region.

INTRODUCTION

Diarrheal illness presents a significant threat in developing countries where sanitation and public hygiene systems are poorly developed. Acute diarrhea is a devastating disease that affects people worldwide and has a significant impact on public health. Diarrheal diseases cause three million deaths annually, mainly children below five years of age, with 80% of these deaths in children above two years of age (1,2). More than 20 types of viral, bacterial, and parasitic enteropathogens are currently associated with acute diarrhea. Among the viral and bacterial enteropathogens, rotavirus and *Escherichia coli* (*E. coli*) are the main cause of acute diarrhea in children. Infections caused by *Shigella*, *Salmonella*, *Campylobacter jejuni*, *Vibrio cholerae* (*V. cholerae*), *Aeromonas*, *Plesiomonas*, other protozoans, and helminths commonly occur among the lower economic groups, in areas where sanitation, public hygiene, and potable drinking water

is significantly poor (3). Cholera is an acute diarrheal disease caused by infection with the O1 and O139 toxigenic *V. cholerae* serogroups and is associated with a rapid loss of fluid, leading to death if left untreated (4). The state of Odisha, situated on the eastern coast of India, has a long coastal line and is mostly populated by tribal people, including a number of primitive tribes. The state usually experiences annual cyclones and floods, which are followed by diarrheal outbreaks resulting in high morbidity and mortality. Several reports have already been published on diarrheal outbreaks in Odisha caused by the *V. cholerae* O1 and O139 serogroups (5,6). Similarly, the incidence of other bacterial pathogens isolated from hospitalized diarrheal patients in Odisha has also been studied (7). However, reports on the bacterial etiology of acute diarrhea among hospitalized patients in these tribal areas are lacking. Hence, the present study was envisaged to document the incidence of different bacterial pathogens in four blocks of three tribal districts of Odisha from July 2010 to September 2013.

MATERIALS AND METHODS

Specimen collection: Sample acquisition was conducted from July 2010 to September 2013 from diarrheal patients in four blocks of three tribal districts of Odisha (Koraput: Dasamantapur and Laxmipur; Rayagada: Kashipur; Gajapati: Mohana). A total of

Received September 27 2019. Accepted January 20 2020.

J-STAGE Advance Publication February 28 2020.

DOI: 10.7883/yoken.JJID.2019.407

*Corresponding author: Mailing address: Microbiology Division, ICMR-Regional Medical Research Centre, Chandrasekharapur, Bhubaneswar 751023, India. Tel: +91-674-2305638, Fax: +91-674-2301351.

E-mail: bbpal_rmrc@yahoo.co.in

1,427 rectal swabs were collected from diarrheal patients of all age groups before the administration of antibiotics. These samples were transported in Cary-Blair transport medium to the laboratory for further analysis.

Bacteriology: The rectal swabs were sub-cultured on thiosulphate-citrate-bile salt sucrose (TCBS) agar (Becton and Dickinson Co. (BD), USA). For selective isolation of *V. cholerae* and *E. coli*, bacterial colonies were cultured on MacConkey agar (BD, USA). Hektoen enteric agar (BD, USA) was used to isolate *Salmonella* and *Shigella* species (spp.), and *Aeromonas* isolation agar (Hi-Media, India) was used for the isolation of *Aeromonas* spp. Environmental water samples taken from river, open well, Nala (a small stream), and Chua (a shallow pit in river bed) that were suspected of being contaminated, were enriched with alkaline peptone water (APW) and then incubated for 24hr. Samples were then streaked onto TCBS agar for the isolation of *V. cholerae* serogroups. The isolation of the pathogens was carried out according to standard laboratory practices and World Health Organization (WHO) guideline (8). Suspected colonies were isolated for further biochemical and serological tests using specific antisera (BD, USA) to confirm *V. cholerae* and *Shigella* spp.

Antibiotic susceptibility test: Antibiotic susceptibility testing of the isolated pathogens was carried out by the disk diffusion method using commercially available disks as per our lab practice and Clinical & Laboratory Standards Institute (CLSI) guidelines (9, 10). The following antibiotics were used: ampicillin (AMP, 10 µg), co-trimoxazole (COT, 25 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), furazolidone (FR, 50 µg), gentamicin (GEN, 10 µg), nalidixic acid (NA, 30 µg), norfloxacin (NX, 10 µg), streptomycin (S, 10 µg), tetracycline (TE, 30 µg), neomycin (N, 30 µg), azithromycin (AZM, 15 µg), ofloxacin (OF, 5 µg), doxycycline (DO, 30 µg), erythromycin (E, 15 µg) and polymyxin B (PB, 50 U).

Molecular assays for *V. cholerae* and *E. coli*: A multiplex polymerase chain reaction (PCR) assay was used to differentiate between the *V. cholerae* classical and El Tor biotypes by detecting genes specific to each: cholera toxin subunit A (*ctxA*) and the subunit of toxin co-regulatory pilus (*tcpA*) (11). Further, detection of *rfb* O1/O139 was done for serogroup identification of *V. cholerae* along with top regulatory protein (*toxR*) and cholera toxin subunit B (*ctxB*) gene of El Tor variant (12). A MAMA (mismatch amplification mutation assay) PCR assay was conducted to distinguish between classical and El Tor *ctxB*. Whereas a DMAMA (double mismatch amplification mutation assay) PCR assay was performed to differentiate between classical and Haitian *ctxB* (13,14).

E. coli strains were screened for the detection of different virulent genes such as *elt*, a heat-labile toxin for the identification of enterotoxigenic *E. coli* (ETEC); *eae* (enterocyte attachment and effacement gene) for enteropathogenic *E. coli* (EPEC); and *ast*, a heat-stable toxin to identify enteroaggregative *E. coli* (EAaggEC). Primers for different genes were used as per the available protocol (7).

RESULTS

Bacteriological analysis: Out of the 1427 rectal swabs obtained from hospitalized diarrheal patients, 930 (65.2%) samples had positive bacterial cultures. Among these, 636 (44.6%) were identified to contain *E. coli*, 146 (10.2%) had *V. cholerae* O1, 10 (0.7%) contained *Salmonella* spp., 79 (5.5%) were of *Shigella* spp., and 59 (4.1%) comprised of *Aeromonas* spp. There were 497 (34.8%) samples that did not grow any bacterial colonies (Table 1). Among the *Shigella* spp., *S. flexneri* were highest in number followed by *S. dysenteriae*, *S. sonnei*, and *S. boydii*, respectively. Within the same period, 729 water samples were tested for the presence of *V. cholerae*, of which 14 (1.9%) were non-O1/non-O139 (NAV) and 13 (1.8%) were *V. cholerae* O1.

PCR analysis: Out of the 636 *E. coli* strains, 200 were randomly selected for simplex PCR assay to differentiate between the ETEC, EPEC, and EAaggEC strains. It was observed that EPEC constituted 5.1%; ETEC, 7.0%; and EAaggEC strains made up 4.2% of the total samples.

Twenty representative strains of *V. cholerae* were selected for quadruplex PCR assay to detect different toxic genes. All strains were positive for *ctxA*, *tcpA* (El Tor), *rfb* (O1), and *toxR* genes. While conducting MAMA and DMAMA PCR assays, it was observed that the El Tor *ctxB* was the dominant allele found in *V. cholerae* O1 strains in 2010 – 2011. This was followed by the classical and Haitian *ctxB*. Whereas, from 2011 to 2013, the classical *ctxB* was the dominant allele, followed by the Haitian *ctxB*. *V. cholerae* O1 strains carrying the El Tor *ctxB* allele were completely absent during these years (Table 2).

Demographic analysis: The patients were subdivided into four different age groups: 0 - 5 years, 5 - 14 years, 14 - 40 years, and > 40 years of age. Diarrheal disorders affected both males and females equally in all age groups. However, the age group most affected by diarrheal diseases was 14 - 40 years, followed by > 40 years, and then 5 - 14 years. Those aged between 0 - 5 years were the least affected.

Seasonal analysis: Seasonal analysis of different pathogens indicated that *E. coli* was predominantly found throughout the years. *V. cholerae* O1 strains were isolated mainly from samples taken during the rainy seasons, followed by the winter seasons. Whereas, in our previous study of the Puri district, we found that it was present throughout the year (unpublished; Fig. 1). *Shigella* spp. occurrence was highest during winter, followed by summer. Furthermore, *Salmonella* was the least common spp. isolated during this study period. Lastly, *Aeromonas* spp. were commonly found in samples taken during the rainy and summer seasons (Fig. 2).

Antibiotic susceptibility patterns: An antibiotic susceptibility test was conducted against all pathogens isolated. Except for the outbreak strains, all *V. cholerae* O1 isolates were uniformly sensitive to tetracycline, chloramphenicol, azithromycin, neomycin, gentamicin, norfloxacin, ciprofloxacin, ofloxacin, and doxycycline; and resistant to ampicillin, nalidixic acid, furazolidone, streptomycin, erythromycin, and co-trimoxazole. The outbreak strains were also resistant to tetracycline.

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Table 1. Bacteriological analysis of enteropathogens isolated from diarrhea patients (July 2010-September 2013)

Year	2010-2011 (%)	2011-2012 (%)	2012-2013 (%)	Total (%)
Total samples	608	666	153	1,427
Culture negative	278 (45.7)	179 (26.8)	40 (26.1)	497 (34.8)
Culture positive	330 (54.3)	487 (73.1)	113 (73.9)	930 (65.2)
<i>E.coli</i>	249 (41.0)	321 (48.2)	66 (43.1)	636 (44.6)
<i>V.cholerae</i>	62 (10.2)	60 (9.0)	24 (15.7)	146 (10.2)
<i>Shigella</i> spp.	15 (2.5)	51 (7.7)	13 (8.5)	79 (5.5)
<i>Salmonella</i> spp.	4 (0.7)	6 (0.9)	0 (0)	10 (0.7)
<i>Aeromonas</i>	0 (0)	49 (7.4)	10 (6.5)	59 (4.1)

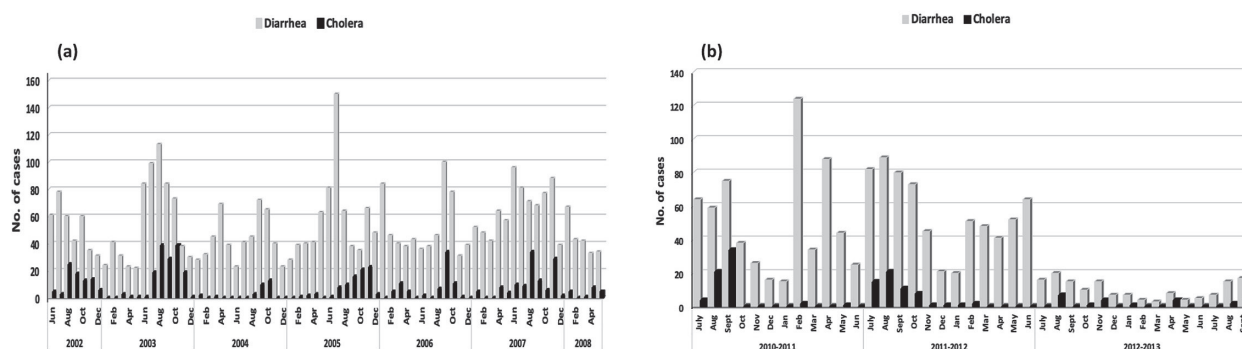


Fig. 1. (a) Month-wise distribution of diarrhea and cholera cases in Puri (June 2002-May 2008); (b) Month-wise distribution of diarrhea and cholera cases in tribal areas (July 2010-September 2013).

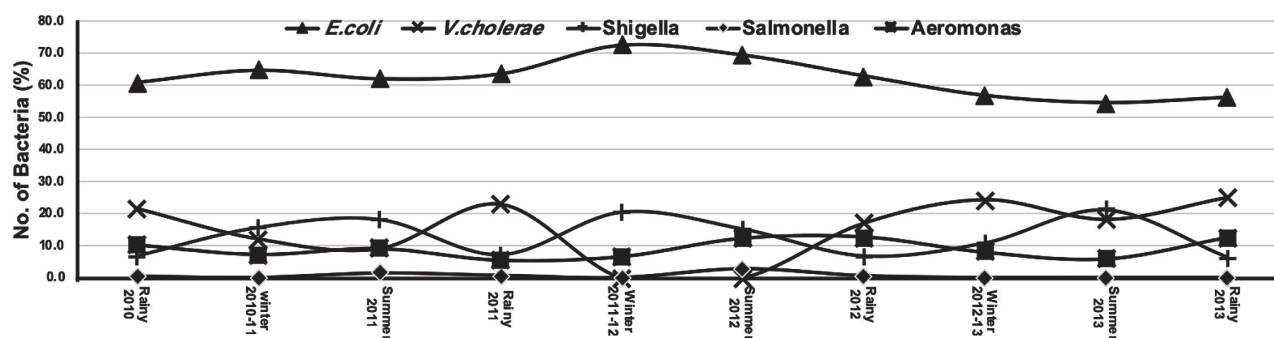


Fig. 2. Different bacterial enteropathogens (%) during different seasons in the tribal areas (July 2010-September 2013).

Table 2. Year wise *ctxB* genotype (El Tor, Classical and Haitian *ctxB*) of *V. cholerae* O1 strains (July 2010-September 2013)

Sl. No	Year	El Tor <i>ctxB</i> (%)	Classical <i>ctxB</i> (%)	Haitian <i>ctxB</i> (%)
1	2010-11 (n=20)	80.00	15.00	5.00
2	2011-12 (n=20)	0.00	80.00	20.00
3	2012-13 (n=20)	0.00	75.00	25.00

The *Shigella* spp. were resistant to ampicillin, chloramphenicol, nalidixic acid, furazolidone, co-trimoxazole, erythromycin, and norfloxacin; but were sensitive to tetracycline, azithromycin, streptomycin, neomycin, gentamicin, ciprofloxacin, and ofloxacin. The *Aeromonas* spp. were resistant to ampicillin, nalidixic acid, furazolidone, erythromycin, co-trimoxazole,

gentamicin, and ciprofloxacin; and were sensitive to tetracycline, chloramphenicol, azithromycin, streptomycin, neomycin, norfloxacin, and ofloxacin.

DISCUSSION

Unhygienic living practices such as open defecation

and poor access to safe drinking water are major causes of diarrhea in the tribal areas of the Odisha state. Diarrhea is a waterborne disease. The use of contaminated water from traditional water sources such as Nala, Chua, streams, and rivers makes the population vulnerable to these diseases, in particular during the rainy seasons. Reports from the 2002, 2007, and 2010 cholera outbreaks in these areas drew the same conclusions (6,15). Surveillance of the diarrhea incidence in Puri district (2004 - 2006) indicated that *E. coli* was the most common bacteria (75.5%), followed by *V. cholerae* (17.3%), whereas diarrhea incidences caused by other bacterial strains such as *Shigella* spp. (4.5%), *Salmonella* spp. (0.7%), and *Aeromonas* spp. (2.0%) (7) were comparable to the current study. Diarrhea from cholera infections is mostly seasonal in North India, such as in Chandigarh, Delhi, or its periphery and occurs mainly during the rainy seasons (16, 17). In the current study, rainy seasons had the highest incidences of cholera cases caused by *V. cholerae* O1 infections. This was followed by incidences reported during winter. A surveillance report from the Puri district indicated that the incidence of cholera cases from *V. cholerae* O1 infection was highest during the rainy seasons (Fig. 1). Although cholera is seasonal in the tribal areas, it is endemic to the Puri district.

In the present study, the most affected age group was 14 - 40 years, followed by the > 40 years. This was in contrast to a report from Chandigarh, where children younger than 5 years were the most affected group (18). A 1992 investigation from Kolkata reported that diarrhea cases in all age groups had a male to female ratio of 1.4:1, which was also comparable to that reported in a study of Delhi and its periphery that showed this ratio at 1.5:1 (16). Both reports are in agreement with the current findings where males are more susceptible to secretory diarrhea than females. A possible explanation for this is that men have greater exposure to contaminated water sources during their day-to-day life.

In the current study, a low prevalence of EPEC (5.1%), ETEC (7.0%), and EAaggEC (4.2%) were found among hospitalized patients. A higher prevalence of pathogenic *E. coli* was reported from diarrheal patients in other districts and countries e.g., from North India, 23.5% ETEC (19); Kuala Lumpur, Malaysia, 14% EPEC (20); Jakarta in Indonesia, 18% ETEC and 1% EAaggEC (21), and Sweden, 8.0% ETEC, 2.0% EAaggEC, and <1% EPEC (22).

All *V. cholerae* O1 strains were El Tor biotypes as detected by quadruplex PCR for the *tcpA* (El Tor-specific) gene. Since 2007, the *V. cholerae* O1 El Tor variant has been found in different regions of Odisha (6, 23). Furthermore, it was established that the prevalence of El Tor *ctxB* had declined, while that of the classical *ctxB*, and the Haitian *ctxB* to a lesser extent, had increased, which corroborates with the findings of a similar report on Kolkata from 1989 to 2005 (24). Another report of Kolkata from 2004 to 2011 showed that the prevalence of the Haitian *ctxB* had increased more than that of the classical *ctxB* (14), which was also comparable to the current study where Haitian *ctxB* increased from 5% to 25% over 3 years.

Diarrhea is primarily treated by supportive intravenous rehydration therapy. Further antimicrobial therapy can be used to reduce the duration of illness and the volume of stool. Tetracycline, along with chloramphenicol, furazolidone, and co-trimoxazole, are the reported drugs of choice for this treatment. However, in the current investigation, it is evident that *V. cholerae* has developed resistance to multiple antimicrobial drugs. Similarly, multidrug-resistant *Shigella* and *Aeromonas* spp. were isolated from the tribal areas of Odisha. Fluctuations in the antibiotics resistance pattern were observed over the year showing changes in resistance to ampicillin, nalidixic acid, furazolidone, streptomycin, erythromycin, and co-trimoxazole. Similar findings were reported from Odisha during the 2004 to 2013 period (25), Pune in 2015 (26), North Karnataka in 2012 (27), various regions of Kenya during 2007 to 2010 (28), and Cameroon (29) and Mozambique from 2012 to 2015 (30).

V. cholerae O1 containing the Haitian *ctxB* gene was isolated for the first time from Odisha in 1999 (31). The present study reported that 25% of all samples acquired during 2012 - 2013 were made up of the Haitian *ctxB* variant. Subsequently, this strain caused a second cholera epidemic in 2014 in the Kalahandi district of Odisha (31), after the Haiti cholera epidemic of 2010.

Diarrhea, in particular, that which was caused by cholera, is a significant health threat in tribal areas, which leads to high morbidity and mortality. This study clearly indicates that active surveillance and control strategies are needed to reduce the number of diarrheal cases in this region. It is also important to monitor the antibiotic sensitivity patterns of the isolated pathogens and to stop the indiscriminate use of antibiotics. Furthermore, the continuous surveillance of diarrheal diseases is highly necessary to monitor the changing patterns of *V. cholerae* that cause cholera outbreaks and epidemics, because the Bay of Bengal region is the cradle of every new strain of *V. cholerae*.

Acknowledgments The present work was supported from the grant of tribal diarrhea project from the tribal task force, Indian Council of Medical research, New Delhi. The authors are very much thankful to the Director, Directorate of Health Services, Govt. of Odisha, Chief district medical officers (CDMOs) of Koraput, Rayagada and Gajapati districts for their cooperation and help during the investigation.

Conflict of interest None to declare.

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