Original Article

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Emergence of Multidrug Resistant *Vibrio cholerae* O139 in Acute Diarrhoea Patients Attending a Tertiary Care Hospital, West Bengal, India

Microbiology Section

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ABSTRACT

Introduction: India is a developing country with many poor sanitation areas. Cholera, a water borne disease is rampant in areas of poor sanitation and is mainly due to *Vibrio cholera* of O1 and O139 serogroups causing acute cases of rice watery diarrhoea and high mortality. Infection due to Multidrug Resistant (MDR) Vibrio is on the rise. It is endemic in over 50 countries including India leading to a number of epidemics and pandemics. Till date, about seven pandemics have been identified due to cholera infection.

Aim: To isolate *Vibrio cholerae* from acute diarrhoea cases with their antibiotic susceptibility and identify MDR strains, if any.

Materials and Methods: A hospital-based, cross-sectional study was conducted in the Department of Microbiology, Burdwan Medical College, Purba Burdwan, West Bengal, India, from January 2021 to December 2021. Rectal swabs/faeces collected in Cary-Blair Transport Media were brought to the laboratory and incubated at 37°C for six hours; a loopful was inoculated in nutrient agar, MacConkey agar and selective media Thiosulphate-Citrate-Bile Salts-Sucrose agar (TCBS). Gram stain, motility, colony characteristics, oxidase test, cholera-red reaction and slide agglutination test was done. Diagnosis was confirmed; antibiotic susceptibility done and interpreted as per Clinical and Laboratory Standard Institute (CLSI) guidelines. The data was collected and entered into Microsoft Excel software and presented as frequency and percentages.

Results: Total 60 samples were collected and tested, out of which 24 (40%) were positive for *Vibrio cholerae* and nine in 24 (37.5%) samples were MDR strains. Twelve samples were from females and 12 were from males, majority {5 (41.67%) females, 9 (75%) males} belonged to the age group of 0-5 years. Serotyping revealed that *Vibrio cholerae* O1 serogroup was identified in 8 (33.33%) cases and O-139 serotype in 16 (66.67%) samples. Rest were *Escherichia coli* (n=10, 25%), *Klebsiella pneumoniae* (n=5, 12.5%) and *Staphylococcus aureus* (n=1, 2.5%).

Conclusion: There is a rise of infections due to MDR strains of Vibrio O139 sero group in the community which needs early diagnosis and treatment for control. *Vibrio cholerae* strains were more resistant to fluoroquinolones, macrolides, tetracycline and Ampicillin.

Keywords: Antibiotic resistance, Gram negative bacteria, MacConkey agar, Selective media

INTRODUCTION

Diarrhoea is defined as frequent passage of excessive watery stool for 3-7 days [1]. The different aetitiological agents causing diarrhoea are viral, bacterial or parasitic [2]. Among the different bacteria responsible for the condition, *Vibrio cholerae* is an important agent. *Vibrio cholerae* of the family Vibrionaceae are slender, comma shaped, water loving, motile, aerobic gram negative bacilli with polar flagella [3]. The bacilli enters the body via contaminated water and unhygienic practices and being very sensitive to pH below 6, most of them are killed by the acidic pH of the stomach [4,5]. The bacilli secretes a very potent enterotoxin, the cholera toxin which binds the bacilli to the plasma membrane of the intestinal epithelial cells and leads to increase in the intracellular Cyclic Adenosine Monophosphate (cAMP). This leads to massive secretion of water and electrolytes into the intestinal lumen leading to the development of cholera [5].

Though cholera outbreaks are quite frequent in monsoons, occasional sporadic cases are also reported throughout the year in different seasons from areas with no proper sanitation [6,7]. The disease, cholera is endemic in India and the whole of the gangetic plains including the delta of the river Ganges is called 'the homeland of Cholera' [8,9]. Worldwide the disease has led to the development of several epidemics and since 1817, about seven pandemics has been recorded and it has become a great public health issue [7,10,11].

About 200 serogroups of Vibrio has been identified belonging either to the classical biotype or ElTor biotype and the two most common infectious strains being either *V.cholerae* O1 *or O139*. The disease spreads via ingestion of food and contaminated water mainly by *Vibrio cholera of O1 and* O139: classical *or ElTor serotype* and it has three sero sub types namely Ogawa, Inaba and Hikojima [12,13]. *Vibrio cholerae* O139 shows similar characteristics like the El Tor serotype and showing difference from *Vibrio cholerae* O1 in the polysaccharide nature of its surface antigen [14,15]. The infection can become severe and the patients develops profuse watery diarrhoea with/without vomiting, muscle cramps, fever, weak pulse, loss of skin tugorsity, scaphoid abdomen and severe dehydration [3]. As a result, the patient develops hypovolumic shock which if not diagnosed early, may lead to death of the patient [16,17].

An early rapid diagnosis is required to save the patient and for this, a wet mount of the liquid watery stool is examined microscopically to find darting, motile *Vibrio cholerae* [18]. Worldwide every year, about 1.3-4.0 million cases of cholera, occur and 21,000-143,000 deaths have been recorded by World Health Organisation (WHO) [19]. Prompt treatment of the condition is started with rehydration therapy-oral/parenteral (IV) with/without antibiotics. WHO recommends the use of antibiotics as they have been found to decrease the bacterial shedding, reduces the intensity and duration of the diarrhoea and hence severity of the infection [20,21].

Tetracycline, doxycycline, furazolidone, erythromycin, trimethoprim-sulphamethoxazole, and chloramphenicol were the drugs used to treat cholera very effectively but the resistant strains were also being isolated in recent years. Improper and unrestricted use of different antibiotics has led to the development of Multidrug Resistant (MDR) strains of *Vibrio cholerae* which has become a source of great concern amongst all. Since no comprehensive data regarding the prevalence and antimicrobial sensitivity pattern of any prevailing MDR *Vibrio cholerae* in Purba Burdwan and its adjoining districts is available. Therefore, the aim of the current study was to identify the presence of the bacteria along with their antibiotic sensitivity pattern together with identification of any MDR strains.

MATERIALS AND METHODS

A hospital-based, cross-sectional study was conducted in the Department of Microbiology in Burdwan Medical College, Purba Burdwan, West Bengal, India, from January 2021 to December 2021 following approval by the Institutional Ethics committee (Memo No: IEC/302 dated 25/04/22).

Inclusion criteria: All the patients presenting with acute watery diarrhoea for three to seven days were included in the study [22].

Exclusion criteria: All the patients having loose watery stool for less than three days or more than seven days or having dysentery (stool with mucus) were excluded from the study.

Study Procedure

A total of 60 stool samples/rectal swabs were sent to laboratory from patients suffering from acute diarrhoea in Cary-Blair Transport medium. About 2 mL of sample was inoculated in 20 mL of Alkaline Peptone Water (APW) (1:10 ratio) and incubated for six hours at 37°C for enrichment. Subculture from Enrichment media was done on MacConkey agar, nutrient agar and selective media Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) agar and checked for colony characteristics. In APW, uniform turbidity with surface pellicles was identified; in Nutrient agar, glistening, translucent colonies were seen. In MacConkey agar, pale, translucent, lactose-non fermenting colonies were seen while in TCBS, yellow button shaped sucrose fermenting colonies were identified [Table/Fig-1]. Hanging drop preparation showed typical darting motility of Vibrio. Further confirmation was done by array of biochemical tests such as glucose, sucrose fermentation, indole, methyl red, oxidase test, catalase test, urease, citrate, cholera-red reaction, Lysine and Ornithine decarboxylation but not of Arginine and in triple sugar iron [23].

Serologic confirmation of *Vibrio cholerae (V.cholerae)* was done by slide agglutination test with specific antisera against *V.cholerae* O1 and O139 [Table/Fig-2]. Agglutination tests for *V.cholerae* somatic O antigens were carried out on a clean glass slide. Few colonies were taken in an inoculating loop from MacConkey agar medium and emulsified in a small drop of normal saline and mixed thoroughly by tilting back and forth for about 30 seconds. A smooth suspension was prepared. A small drop of antiserum O1 (polyvalent O1-*naba, Ogawa and Hikojima* type antisera) was added to the suspension. The same process was repeated with anti-sera O-139. One drop of Normal Saline and the growth emulsion was used as a negative control to observe for auto agglutination [24].

[Table/Fig-1]: *Vibrio cholerae* on Thiosulphate-Citrate-Bile Salts-Sucrose agar (TCBS) media plate. **[Table/Fig-2]:** Positive agglutination test shown by *Vibrio cholerae* isolates. (Images from left to right)

The dehydration which developed due to acute diarrhoea due to *V.cholerae* was divided into mild, moderate and severe types. Patients who passed stool for maximum five times, had normal urine output, moist tongue, normal blood pressure and normal pulse and on pinching the skin it immediately recoiled back were classified as mild dehydration [25,26]. Patients who passed stool for 6-15 times, were conscious but irritable, slightly dry tongue, had sunken eyes, with normal urine output, normal blood pressure and pulse but slight tachycardia, and on pinching the skin tugorsity showed delayed recoil were classified as having moderate dehydration [25,26]. Patients who passed stool for more than 15 times, were in shock/decreased consciousness, deeply sunken eyes, no tears, very dry tongue, urine output very much reduced, hypotensive, with thread pulse and on pinching the skin showed very slow recoil were classified as having severe dehydration [25,26].

Antibiotic Susceptibility Test

The antimicrobial susceptibility testing of *V.cholerae* was performed by Kirby Bauer's disc diffusion method on Mueller Hinton agar [27]. Antibiotic discs used were doxycycline (30 µg/disc), ciprofloxacin (5 µg/disc), cotrimoxazole (25 µg/disc), amikacin (30 µg/disc), ceftriaxone (30 µg/disc), cefoperazone/sulbactam (75 µg/30 µg /disc), norfloxacin (5 µg/disc), chloramphenicol (30 µg/disc), azithromycin (15 µg/disc), ampicillin (30 µg/disc) and erythromycin (10 µg/disc). The interpretation was done based on the guidelines of CLSI [28]. *Escherichia Coli* (E.coli) ATCC®25922 and *Staphylococcus aureus* ATCC®29213 were the controls used by us antibiotic susceptibility tests [29].

Detection of Multidrug Resistant (MDR) Strains

The isolates which were resistant to at least one anti-microbial drug in three or more antimicrobial categories were considered as MDR strain [30]. The groups of drugs tested were: Penicillin (ampicillin), cephalosporins (ceftriaxone), aminoglycosides (amikacin), macrolides (erythromycin, azithromycin), fluoroquinolones (ciprofloxacin, norfloxacin), β-lactam/β-lactam inhibitors (cefoperazone-sulbactam) [31].

All the patients were followed-up till their discharge from the hospital and all necessary information regarding age, sex, symptomatology was collected from the admission files of the patients. Most of the patients having acute diarrhoea were not admitted and were treated in the Outpatient Department (OPD) of the health centres. Only the very severe ones with/without complications were admitted in the hospital.

STATISTICAL ANALYSIS

The data collected from the above test was analysed by the Microsoft Excel software and presented as frequency and percentages for all the variables like age, symptoms, history of hospitalisation, outcome, laboratory results and death, if any.

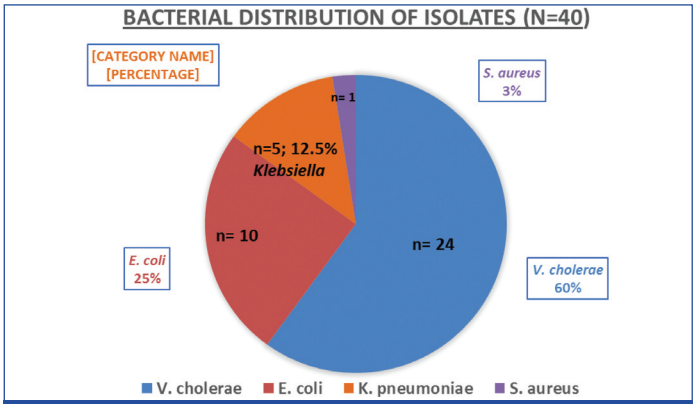
RESULTS

Out of total 60 stool/rectal swab samples received in the laboratory during the study period, 24 samples showed growth of *Vibrio cholerae.* An age-sex proportion of cholera cases were drawn up. The highest proportion of cholera was observed in the paediatric age group (0-5 years) and maximum number of cases was in males (n=9, 75%) followed by in females (n=5, 41.67%) [Table/Fig-3].

Age group (years)	Females (n=12) n (%)	Males (n=12) n (%)
0-5	5 (41.67)	9 (75)
6-20	1 (8.33)	3 (25)
21-30	3 (25)	0
31-40	2 (16.67)	0
≥41	1 (8.33)	0
Total	12 (100)	12 (100)

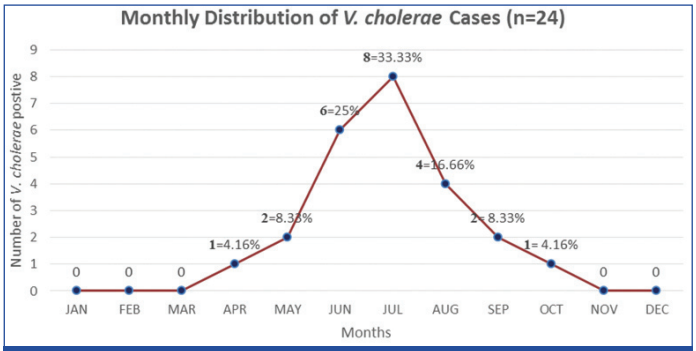
[Table/Fig-3]: Age and gender wise distribution of *Vibrio cholerae* isolates (N=24).

Of the 60 stool/rectal swab samples, 40 (66.67%) samples showed growth of pathogenic organisms. *Vibrio cholerae* was isolated in 24 stool samples. Serotyping, however, showed that *V.cholerae* O1 serogroup was identified in eight (33.33%) cases and *V.cholerae* O-139 serotype in 16 (66.67%) samples. Rest were *E.coli* (n=10, 25%), *KLebsiella pneumoniae* (n=5, 12.5%) and Staphylococcus aureus (n=1, 2.5%) [Table/Fig-4].



[Table/Fig-4]: Bacterial distribution of isolates (N=40).

Vibrio cholerae induced infections are very rampant in the monsoon seasons. We found an intriguing seasonal variation in our study, though 18 (75%) cases were reported during monsoons, 12.5% were found during summer and 12.5% in the winter. The highest number of cholera cases was obtained during months of June, July and August with sharp decline in December and January [Table/Fig-5].



[Table/Fig-5]: Monthly distribution of *Vibrio cholerae* cases (n=24).

High frequency of passage of stool in first 24 hours was noted in 24 patients with positive *V.cholerae* culture; most of the cases (13 cases; 54.16%) passed stool 6-10 times while 14 (58.33%) patients presented with moderate dehydration [Table/Fig-6].

Clinical presentation		*V. cholerae* culture-positive (n=24) n (%)
Dehydration	Mild	08 (33.33)
	Moderate	14 (58.33)
	Severe	2 (8.33)
Frequency of stool (times/24 hours)	3-5 times	8 (33.33)
	6-10 times	13 (54.16)
	11-15 times	1 (4.17)
	>15 times	2 (8.33)
Other clinical presentation	Vomiting	15 (62.5)
	Shock	11 (45.83)
	Convulsion	2 (8.33)
	Fever	8 (33.33)
	Pain abdomen	3 (12.5)

[Table/Fig-6]: Presenting symptoms in the study participants.

The isolates were least susceptible to ampicillin (3-12.5%), ciprofloxacin (3-12.5%) and erythromycin (5-20.83%). they showed moderate susceptibility to doxycycline (10-41.67%), cotrimoxazole (12-50%), amikacin (15-62.5%), norfloxacin (15-62.5%) and azithromycin (16-66.67%). The isolates were most susceptible to cefoperazone/sulbactam (23-96%), chloramphenicol (22-91.67%) and ceftriaxone (20-83.33%) [Table/Fig-7].

Group of antibiotics	Antibiotics n (%)	Sensitivity n (%)
Penicillin	Ampicillin	3 (12.5)
Fluoroquinolones	Ciprofloxacin	3 (12.5)
	Norfloxacin	15 (62.5)
Aminoglycosides	Amikacin	15 (62.5)
Cephalosporin	Ceftriaxone	20 (83.33)
Chloramphenicol	Chloramphenicol	22 (91.67)
Combination	Cefoperazone/sulbactam	23 (96)
Macrolides	Azithromycin	16 (66.66)
	Erythromycin	5 (20.83)
Cotrimoxazole	Cotrimoxazole	12 (50)
Tetracycline	Doxycycline	10 (41.67)

[Table/Fig-7]: Antibiotic sensitivity pattern of *Vibrio cholera* (N=24).

Out of 24 *V.cholerae* isolates, nine (37.5%) samples were found to be MDR with all of these strains showing maximum resistance (66.66%) to Ampicillin, Cefoperazone/Sulbactam, Amikacin and Ciprofloxacin [Table/Fig-8].

Antibiotics	Resistant n (%)	Susceptible n (%)
Ceftriaxone	4 (44.44)	5 (55.55)
Chloramphenicol	4 (44.44)	5 (55.55)
Norfloxacin	5 (55.55)	4 (44.44)
Ampicillin	4 (44.44)	5 (55.55)
Erythromycin	4 (44.44)	5 (55.55)
Azithromycin	5 (55.55)	4 (44.44)
Cefoperazone-Sulbactam	6 (66.66)	3 (33.33)
Amikacin	6 (66.66)	3 (33.33)
Doxycycline	5 (55.55)	4 (44.44)
Ciprofloxacin	6 (66.66)	3 (33.33)

[Table/Fig-8]: Antibiotic susceptibility pattern of Multidrug Resistant (MDR) *Vibrio cholera* strains (n=9).

DISCUSSION

Cholera is a major public health problem in most of the developing countries of the world including India. In India, it is endemic and has led to a number of epidemics and pandemics. Of the 60 rectal swabs/faeces samples from the clinically suspected patients of acute diarrhoeal diseases, 24 (66.67%) of total samples, revealed the growth of 0-139 serotype of *Vibrio cholera.* This was unlike with different studies where *Vibrio cholera O1* is the predominant strains being isolated. For example, Parvin I et al., in Bangladesh, an endemic region like India, conducted a study on diarrhoea stools from 2000-2018. They found that there was a gradual fall of *Vibrio cholera O1* cases from 2006. In 2016, the number of cases due to it was only 6% but thereafter, there was an increase in the number of cases reaching 12% in 2018 [32]. A study conducted by Kumar A and Oberoi A in Punjab in 2013 on 1063 acute diarrhoea stool found 41 *Vibrio cholera* positive cases and all were *Vibrio cholera O1* [23]. Another study conducted by Maharjan S et al., in Nepal from June 2014 to December 2014, found that of 650 stool samples, 50 showed growth of enteric bacterial pathogens; of these, 21 (3%) were *V.cholerae* serogroup O1 and rest were *Shigella* [27].

Cholera being a water-borne disease, contamination of water due to heavy rains and floods in the monsoons is one of the main reasons for its spread. In the present study, 62.5% cases occurred during monsoons from April peaking during the monsoons similar to a study conducted by Kumar A and Oberoi A who found 39/41 (95%) *Vibrio cholerae O1* cases during the monsoons. The cases

were observed by Kumar A and Oberoi A to occur from May to June till August [23]. A prevalence study conducted by Sharma A et al., in Assam from 2003-2013, *Vibrio cholera O1* was isolated in 70 out of 1779 stools. Out of the 1779 stools, 733 stool samples were received in the monsoons (July-October) and *Vibrio cholera O1* was in 50/733 (6.8%) stools in the monsoons [9]. Thakur NH et al., found that diarrhoea due to Vibrio species was present uniformly throughout the year with slight peaks in rainy, winter and summer seasons [3].

In this study, children below five years of age (41.67% in females and 75% in male children) were most susceptible to the infection like Sharma A et al., study in Assam. They observed that *Vibrio cholerae* was maximum cases in children between 0-10 years age group with 11.5% cases in 0-05 years age group [9]. Similar observations were found by Thakur NH et al., who also reported that children below 5yrs of age were the most vulnerable age group to Vibrio infection [3]. Garbern SC et al., however found that the infection due to *Vibrio cholerae* was more common in older children and adults in urban Bangladesh [4].

In the present study, authors found that majority of *V.cholerae* cases (22, 91.67%) presented with moderate and mild dehydration. Thida Oo NA et al., like present study found 02/35 (5.7%) patients presented with no/mild dehydration, some dehydration in 20/34 (57.2%) patients and severe dehydration in 13/34 (37.1%) patients [33]. This was unlike the study conducted in Yangon, Myanmar who found that out of the 24 *Vibrio cholera O1* isolates, 16 patients presented with severe dehydration [34]. Garbern SC et al., also found that 76 (12.2%) patients had no/mild dehydration, 226 (36.3%) had some dehydration while 318 (51%) had severe dehydration [4].

Regarding the frequency of stool, in our study, we found 13 (54.16%) passed stool 6-10 times in 24 hours which was similar to the study by Thida Oo NA et al., who reported that most of the cases 22/35 cases (62.9%) had stool frequency for 6-10 times [33]. Current study showed that the isolated strains were very much susceptible to combination drug-Cefoperazone/sulbactam-23, and to Chloramphenicol-22 and Ceftriaxone-20. This was in concordance with study by Kumar A and Oberoi A who found that all the 41 isolated *Vibrio cholera O1* isolates all were susceptible to Chloramphenicol and Ceftriaxone [23].

Increasing resistance to Ciprofloxacin was also noted in our study-only three strains were sensitive to it (03/24; 12.5%) while the rest were resistant. Emergence of Ciprofloxacin resistance was also observed by Sharma A et al., from 2008-2012 which ranged from 15.3% to 40% in 2012 [9]. Kumar A and Oberoi A however, reported that only 01/41 (2.4%) strain was resistant to Ciprofloxacin [23]. In this study, nine *Vibrio cholerae* MDR strains were detected, which is similar to the study conducted by Afum T et al., in patients from Ghana complaining of acute diarrhoea. They isolated 28 *Vibrio* species, of these there was 4/28 (14.28%) were MDR-being resistant to fluoroquinolones, cephalosporins and aminoglycosides [10].

Antibiotics with rehydration therapy was found to be better in relieving the symptoms faster than rehydration treatment alone similar to the findings of Kitaoka M et al., [35]. But with the emergence of MDR strains of *Vibrio cholerae* rational use of these antibiotics is required. To decrease the incidence of infections due to *Vibrio cholerae*, potable drinking water along with proper hygienic practices-WaSH issues (drinking Water, Sanitation, Hygiene) along with periodic quality check of different water sources should be implemented [8].

Limitation(s)

Molecular characterisation of isolates was not studied and the sample size was small. The study sample size was small because it consisted of the occasional outbreak samples sent by the Chief Medical officer of Health of Purba Burdwan and adjoining districts and thus is a representative sample of the general population.

CONCLUSION(S)

A resistance pattern was observed, wherein the *Vibrio cholerae* strains were more resistant to fluoroquinolones, macrolides, tetracycline and ampicillin as against the pattern in the past decade where the highest sensitivity was reported to ampicillin followed by furazolidone, tetracycline and ciprofloxacin. Together with this, we have observed the emergence of multidrug resistance strains of *V.cholera.*

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