

ESTIMATION OF *VIBRIO* SPECIES INCIDENCES AND ANTIBIOTIC RESISTANCE IN DIARRHEA PATIENTS

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ABSTRACT

Objectives: Diarrheal diseases are globally important public health concern due to high morbidity and mortality rates among all age groups. Incidences and antimicrobial resistance patterns of *Vibrio* species are either underreported or overlooked in low-to-middle income countries. The present study is a hospital surveillance conducted to estimate the incidences of *Vibrio* infections in diarrhea.

Methods: A total of 572 diarrheal stool specimens were collected from two major hospitals and investigated using bacteriological tests coupled with molecular assays for characterization of *Vibrio* species. Kirby-Bauer's disk diffusion method was utilized to monitor antibiotic resistance against different antibiotic classes.

Results: Biochemical and molecular analysis of isolates revealed that *Vibrio* species accounted for about 5% of diarrheal incidences. Higher rates of *Vibrio* spp. infections were observed among adolescent and adult age groups than children and elderly. Seasonal distribution analysis showed uniform *Vibrio* spp. infections throughout the year; however, frequency peaks were observed during rainy seasons. *Vibrio* spp. showed sensitivity for most antibiotics classes: Quinolones, aminoglycosides, and carbapenems; however, alarming resistance rates were observed against cephalosporins and nalidixic acid.

Conclusion: The present study provides credible estimates of *Vibrio* incidences and resistance pattern in diarrheal patients. Our findings will help in establishing trends in diarrhea etiology and management of severe diarrhea cases.

Keywords: *Vibrio* spp., Secretory diarrhea, Antimicrobial resistance, Childhood diarrhea, Seasonal variations.

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INTRODUCTION

Diarrhea is a multifactorial disease associated with numerous etiological agents which may cause high disease severity and persistence. A vast array of diarrhea associated pathogens includes bacterial, viral, and parasitic agents of which rotavirus and diarrheagenic *Escherichia coli* have been reported as main diarrheal pathogens among children [1]. Increased diarrhea incidences have been reported from the past decade, which implicated recurrent floods and natural catastrophe as main drivers of the communicable diseases [2]. *Vibrio* species signify as important public threat responsible for watery diarrhea in infected humans and are transmitted by fecal-oral route [3]. Globally, *Vibrio* is known to cause approximately three million cases per annum [4]. However, according to the World Health Organization (WHO), officially reported cases represent only 5–10% of the total incidences [4]. Recent cholera outbreaks in Yemen alleged a very high morbidity and mortality incidences in the country [5]. Due to high morbidity and mortality rates associated with *Vibrio* spp. in epidemics and pandemics, continuous surveillance studies should be accelerated in developing nations. However, the national level surveillance for *Vibrio* species is limited to certain outbreaks in India, and there are no reports from most parts of the country [6].

Vibrio, member of family *Vibrionaceae* is a comma shaped, Gram-negative, aquatic bacterium which colonizes small intestine of human [7]. Infections caused by *Vibrio* are typically categorized into *Vibrio cholerae* and non-cholera *Vibrio* infections. *Vibrio* spp. causes profuse watery diarrhea and are historically known for worldwide epidemics and pandemics [8]. Symptoms of *Vibrio*-mediated gastroenteritis include rice watery stools, severe dehydration, fever, and vomit. *Vibrio* species

express several virulence factors such as toxins (*ctxA*, *stn*, *OmpW*, and *toxR*), colonization factors (lipopolysaccharides, flagellar components, outer membrane proteins, hemagglutinins, and *tcpA*), protease (hemolysins, cytolysins, thermolysins, and metalloproteases), and iron acquisition systems [9]. Cholera toxin encoded by *ctxA* gene has been isolated only from clinical strains and is utilized as an epidemiological marker to access toxigenic potential of *Vibrio* strains [10,11].

Clinical guidelines for the treatment of diarrhea caused by *Vibrio* mainly focus on rehydration therapy and its maintenance. Probiotics and other traditional herbs are also utilized as traditional therapy [12,13]. However, use of antibiotics is mainly known to reduce severity and shedding of the pathogen [14]. Isolation of multidrug resistant *Vibrio* species from clinical sources is a major concern. Recent studies have reported a rise in antimicrobial resistance in *Vibrio* species against major classes of antibiotics (aminoglycosides, tetracyclines, and β -lactams) [14,15].

The National Institute of Cholera and Enteric Diseases works as collaborating body with the WHO and depicted underreporting and negligence in cholera incidence surveillance [6]. Till date, seven cholera pandemics have been reported, and the Bay of Bengal was identified as an epicenter for the most recent one [16,17]. Molecular typing of *Vibrio cholerae* in nearby region of Himachal Pradesh revealed predominant ribotype IV and RIII [18,19]. Our previous study elucidated incidences of viral pathogens among moderate to severe cases [20]. The present research was performed as a part of the study to reveal *Vibrio* incidences and coinfection rates in diarrheal disease. Simultaneously, antibiotic resistance profiles of *Vibrio* were also deciphered to understand the

emergence of multidrug-resistant diarrheal pathogens. Current findings will help in the establishment of key facts for active surveillance and management of diarrheal diseases as well as prioritizing strategies for combating drug resistance.

MATERIALS AND METHODS

Materials

The selective, differential, and routine culture media - thiosulfate citrate bile salt (TCBS) agar, MacConkey agar, glucose phosphate medium, triple sugar iron agar, Simmon's citrate agar, and peptone were purchased from Hi-Media Lab. Ltd., India. Commercially available antibiotic disks were also purchased from Hi-Media, Mumbai, India. Sodium chloride, Tris base, glacial acetic acid, ethylenediaminetetraacetate, ethidium bromide, and other chemicals of analytical grade were purchased from Merck Pvt. Ltd. India. Polymerase chain reaction (PCR) Master Mix was purchased from ClonTech Takara, and DNA ladder of 100 bp and 1 kb were bought from New England Biolabs. The primers used in the study were synthesized and procured from Integrated DNA Technology, India. Sequencing of amplified PCR products was performed commercially at Eurofins Genomics India Pvt Ltd.

Study subject and sample collection

During February 2013–April 2016, a total of 572 fecal specimens were collected from diarrheal patients admitted in Indira Gandhi Medical College, Shimla, and Government Zonal Hospital Solan, Himachal Pradesh. Diarrheal stool specimens were immediately transferred to the laboratory. The research protocols and methodology followed in the study were approved by the Institutional Ethics Committee (IEC/project no-04-2014). Patients enrolled in the study were from different age groups, and written informed consents were obtained before specimen collection. Clinical features such as frequency of diarrheal episodes, dehydration, fever, vomit, and abdominal pain were also documented as a part of clinical history.

Culturing and biochemical characterization

Collected stool specimens were initially screened for different bacterial and viral agents as per the standard microbiological protocols [21]. For *Vibrio* species, specimens were instantly inoculated into alkaline peptone water (pH 8.5) and enriched by incubating at 37°C for 16–18 h. Following incubation, specimens were streaked onto TCBS agar and Mac-Conkey agar and incubated at 37°C for 24–48 h. Yellow golden colonies from TCBS agar were chosen and preserved as glycerol stock at –20°C for further analysis. Presumptive colonies from TCBS agar were subjected to a series of carbohydrate and amino acid utilization assays including indole, methyl red, Voges-Proskauer's, Simmon's citrate, and triple sugar iron agar test.

Extraction of nucleic acid

Biochemically characterized *Vibrio* isolates were further taken for molecular confirmation. DNA from bacterial isolates was extracted by colony boiling method [21]. In brief, 2–3 colonies from TCBS agar plate were suspended in 20 µl nuclease-free water in PCR vial and heated at 95°C for 10 min in a water bath. Suspension was centrifuged at 3000 rpm for 1 min, and the supernatant was utilized as DNA template for PCR assays.

Molecular characterization of *Vibrio* isolates

The molecular characterization of *Vibrio* spp. was performed by PCR amplification of 16S rRNA gene [22]. Primers used for molecular identification of *Vibrio* species included forward primer 5'-CGGTGAATGCGTAGAGAT-3' and reverse primer 5'-TTACTACGGATTCCGAGTTC-3' [22]. 2 µl of DNA extracted by colony heat boiling was amplified in a 15 µl PCR reaction mixture, 7.5 µl of ×2 PCR master mix, 0.25 µl of each primer, and nuclease-free water.

PCR amplification was accomplished using following conditions: 1 cycle of pre-denaturation at 95°C for 5 min, followed by 35 cycles of final denaturation at 95°C for 30 s, annealing at 48°C for 40 s, extension at 72°C for 50 s, and final extension at 72°C for 7 min. A no template

PCR was run as a negative control for the reaction. The amplified PCR products were analyzed with ethidium bromide stained 1.5% agarose gel (in ×1 TAE buffer) and visualized using UV transilluminator. The amplified PCR product of 16SrRNA gene was confirmed by sequencing.

Antibiotic susceptibility tests

Antimicrobial drug susceptibility assays were performed according to the Clinical Laboratory Standard Institute (CLSI) guidelines [23]. Kirby-Bauer's disk diffusion on Mueller-Hinton agar was used to evaluate antibiogram patterns against fifteen antibiotics: Cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), netilmicin (30 µg), amikacin (30 µg), kanamycin (30 µg), tobramycin (10 µg), streptomycin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (10 µg), nalidixic acid (30 µg), and vancomycin (30 µg). Results were interpreted according to manufacturer's instruction for *Enterobacteriaceae* family.

Statistical analysis

Determination of *Vibrio*-specific incidences among particular age group was accessed by Fisher's exact test. The statistical analysis was performed at 95% confidence interval (CI), and odds ratios (OR) were calculated for *Vibrio*-infected population ($n = 29$) and *Vibrio*-negative population ($n = 543$). A $p \leq 0.05$ was considered as the positive level of significance for Fisher's exact test.

RESULTS

Vibrio incidences among population

From February 2013 to April 2016, a total of 572 fecal specimens were collected and screened for rotavirus, diarrheagenic *E. coli* and *Vibrio* species by biochemical and molecular methods to characterize these pathogens in regions of Himachal Pradesh. Collected specimens were from patients aged between 13 days and 85 years and grouped into five categories: Infants (0–2 years), children (3–5 years), adolescent (6–17 years), adult (18–65 years), and elderly (>65 years). Molecular characterization of biochemically confirmed *Vibrio* isolates showed amplification of *Vibrio* genus-specific 16S rRNA gene in 5% ($n = 29/572$) isolates (Fig. 1). Mixed enteric infection rates of *Vibrio* with *E. coli* and with rotavirus were observed to be 1.2% and 0.03%, respectively. The amplified product of *vibrio* genus-specific 16SrRNA gene [663bp] was confirmed by sequencing.

Pattern of *Vibrio* infections among different age groups

Incidences of *Vibrio* were analyzed among all five age groups, i.e., infants (0–2 years), children (3–5 years), adolescents (6–17 years), adults (18–65 years), and elderly (>65 years) (Fig. 2). The highest frequency of *Vibrio* infection was observed in adolescents (7.3%) and adult age (6.6%) groups, while lowest infection rates observed among infants (2.9%), followed by the elderly (3.7%) and children population (4.4%).

Fisher's exact test was calculated to Decipher highest risk age group among *Vibrio*-infected population (Table 1).

Seasonal frequency of *Vibrio* incidences

Screening of *Vibrio* species was carried out throughout the year to analyze season-wise distribution (Fig. 3). Season-wise classification of

Table 1: Identification of high-risk age group among diarrheal patients by Fisher exact test

Age groups	p value at 95% CI	OR	Significance
0–2 years	0.5896	0.7959	NS
3–5 years	1.0000	1.2	NS
6–17 years	0.9259	2.053	NS
18–65 years	1.0000	1.842	NS
>65 years	Reference category		

NS: Not significant, CR: Confidence interval, OR: Odd ratio

months was performed according to Indian seasons: Summer (March to May), rainy (June to September), autumn (October to November), and winter (December to February). Highest incidences of *Vibrio* were observed for rainy season (34.3%), followed by winter (27.5%) and summer seasons (23.9%). Among different age groups, infants were highly susceptible to *Vibrio* species during winter and summer seasons while no case was reported among infants in rainy season. For adolescent and adults, being the most active and productive group, highest infection was observed during rainy season, followed by winter and summer. Overall, seasonality peaks observed to have a rise in summer, reached at a maximum in rainy and a final decline during autumn.

Antimicrobial resistance patterns of *Vibrio* species

According to the Indian Council of Medical Research (ICMR) different

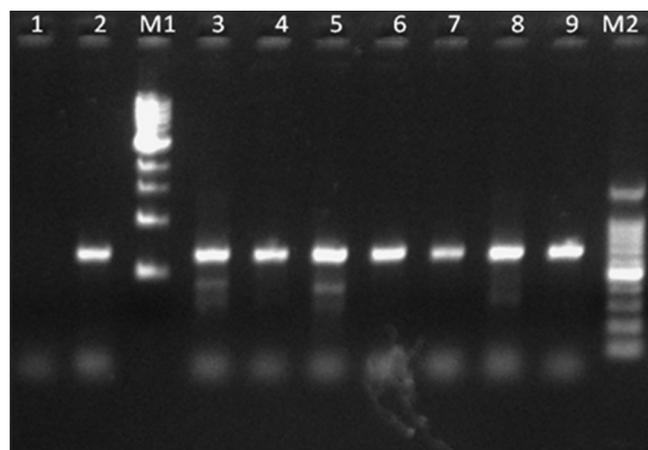


Fig. 1: Polymerase chain reaction (PCR) assay for characterization of *Vibrio* spp. through amplification of 16S rRNA gene. Lane 1 shows a no template control. Lane 2 and lanes 3–9 show amplified PCR product for *Vibrio* genus-specific 16S rRNA gene (663bp). Lanes M1 and M2 show 1Kb and 100 bp DNA ladder (NEB), respectively

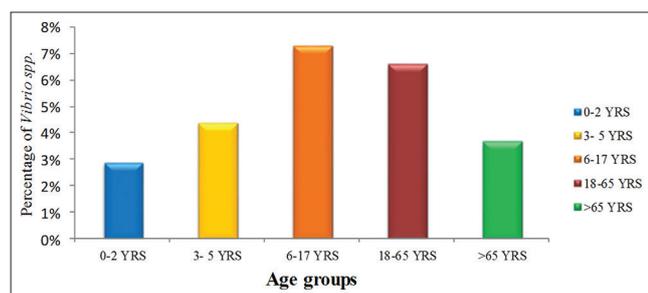


Fig. 2: Incidences of *Vibrio* spp. among different age groups of moderate-to-severe diarrhea-infected patients. 0–2 years: Infant, 3–5 years: Children, 6–17 years: Adolescent, 18–65 years: Adult, >65 years: Elderly population

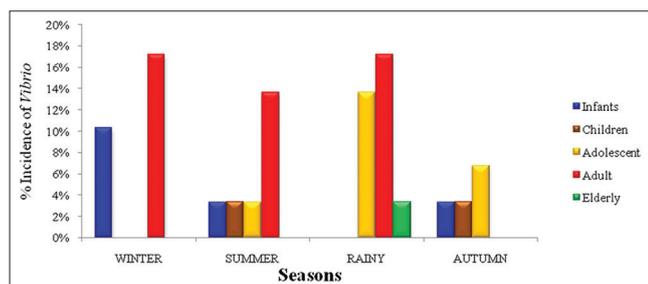


Fig. 3: Seasonal trends of *Vibrio* species incidences among different age categories of diarrheal patients

antibiotic classes, tetracycline, β -lactam, and quinolones are prescribed for the treatment of severe diarrhea cases. Treatment regimen involving norfloxacin, nalidixic acid, cotrimoxazole tetracycline, and ampicillin are specific for *Vibrio*-mediated diarrhea [24]. Other antibiotics were also screened in the study to investigate the common resistance profiles of *Vibrio* spp. Accordingly, Kirby-Bauer's disk diffusion test was performed against fifteen different antibiotics (Fig. 4a-d).

In β -lactam class, alarming resistance levels were analyzed for ceftazidime (65.5%), cefipime (62%), and ceftriaxone (55.1%) antibiotics (Fig. 4a). The proportion of intermediate isolates was found from 6.8% to 20.6% for all β -lactam antibiotics. However, lowest resistance was observed for imipenem (13.8%).

Among aminoglycosides, highest frequency of resistance was observed against streptomycin (34.4%) and kanamycin (31%) (Fig. 4b). Percentage of intermediate isolates for aminoglycosides group ranged from 6.8% to 31%. *Vibrio* spp. exhibited minimum resistance levels for netilmicin (6.8%), followed by tobramycin (13.7%) and amikacin (20.6%).

For quinolones, alarming resistance rates were observed for nalidixic acid (72.4%), followed by ofloxacin (37.9%), ciprofloxacin (37.9%), and norfloxacin (27.4%) (Fig. 4c). *Vibrio* spp. exhibited least resistance against levofloxacin (30.1%). Proportions of intermediate isolates for quinolones were observed to range between 3.4% and 20.6%.

Overall, *Vibrio* isolates were most sensitive for imipenem, netilmicin, tobramycin, levofloxacin, and norfloxacin, and hence, these antibiotics could be priority antibiotics of choice for treatment of *Vibrio* infections. Moreover, continuous monitoring of antibiotic resistance is required to access β -lactam resistance trends as third-generation cephalosporin-resistant *Vibrio* incidences are rare and anxiously alarming [11,14].

DISCUSSION

Diarrhea is a gastrointestinal illness of multi-etiology and multifactorial nature with high morbidity and mortality incidences [1]. In the past few years, increased incidences of *Vibrio* have been reported from various parts of the globe [25] with coastal regions more prone to this infectious disease [18,25]. Historically, *Vibrio* has caused many pandemic and epidemics along with several explosive outbreaks which continue to thrive in regions with contaminated water and suboptimal hygiene practices [26-28]. Diarrhea-associated morbidity and mortality rates are quite higher in Indian sub-continent than the rest parts of world [1,26], and many parts of country are endemic for cholera disease. In Southeast Asian region, India and Bangladesh have reported highest *Vibrio* infections, and in the past few years, threefold increase in infection rates has been reported [25]. Himachal Pradesh is a Northern hilly state of India with varied geographical conditions, and comprehensive studies elucidating *Vibrio* species incidences and antimicrobial surveillance are limited.

In the previous studies, we investigated the role and incidences of viral pathogens as etiological agents of diarrhea in the region [20]. The present study was focused to explore infection rates of *Vibrio* spp. in the state. Antimicrobial susceptibility patterns of *Vibrio* isolates were also determined according to standards of CLSI against commonly utilized antibiotics. The population under the study comprised of moderate-to-severe diarrhea patients admitted to regional and tertiary care hospital settings of Himachal Pradesh.

Standard biochemical assays and molecular methods were utilized to investigate incidences of *Vibrio* in diarrhea cases. Initially, *Vibrio* isolates were biochemically confirmed by IMViC and triple sugar iron agar tests followed by molecular characterization. By amplification of *Vibrio* genus-specific 16S rRNA gene, infection rate of *Vibrio* was found to be 5% (29/572) among diarrheal population (Fig. 1). In a previous study, Bora *et al.* reported 16.5% of *Vibrio* infections in an outbreak from remote areas of Himachal Pradesh [29]. In addition, such higher

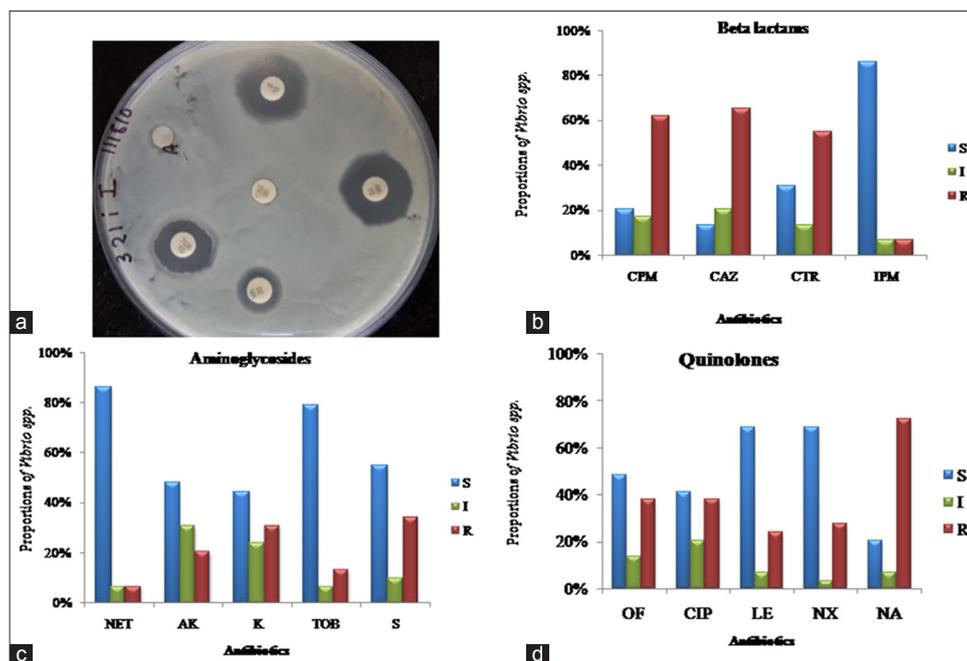


Fig. 4: Antibiotic susceptibility patterns of *Vibrio* spp. (a) antibiotic susceptibility performed by Kirby-Bauer's disk diffusion method. Proportions of sensitive (S), intermediate (I), and resistant (R) phenotypes for β lactams (b), aminoglycosides (c), and quinolones (d). CPM - cefepime, CAZ - ceftazidime, CTR - ceftriaxone, IPM - imipenem, NET - netilmicin, AK - amikacin, K - kanamycin, TOB - tobramycin, S - streptomycin, OF - ofloxacin, CIP - ciprofloxacin, LE - levofloxacin, NX - norfloxacin, and NA - nalidixic acid

Vibrio spp. incidences have also been reported from surrounding areas of state [16,17]. Another study deciphered presence of higher proportion of *Vibrio* spp. in water samples collected from different river systems [30]. Similarly, *Vibrio* incidence rates of 2–4% have also been reported from surroundings of Himachal Pradesh [31-33]. In comparison to our observation, higher *Vibrio* infection rates ranging from 10% to 15% are reported from different parts of India [34,35]. Furthermore, surveillance studies from Bangladesh reported up to 18% of *Vibrio* infections among diarrheal populations [3,37]. Overall, our study depicts low levels of *Vibrio* spp. incidences, and similar trends are observed for active surveillance studies in the country.

Incidences of *Vibrio* were found to be higher among adolescent and adult population, and similar observations were found in other report [38]. However, children <5 years age group (0–2 and 3–5 years) also suffered with similar infection rates as that of adult. CDC weekly morbidity and mortality reports also found children <5 years as most vulnerable group to *Vibrio* infection [3].

Seasonal variations for *Vibrio* incidences are driven by various factors such as recurrent floods stagnant water and suboptimal hygienic conditions [39,40]. Annually, Himachal Pradesh is fed by snow and rainfall; however, owing to its mountainous slopes, water is drained into two main basins: Indus and Gangetic river systems. Uniform seasonal distribution of *Vibrio* spp. was seen throughout the years; however, slight peaks were observed during rainy, winter, and summer seasons. Similar seasonal trends have been observed from coastal area, but frequency is much higher as compared to current study [27,37-41]. This may be due to lack of stagnant water reservoirs in the region and direct drainage of rain water into major river systems.

Vibrio is known to cause life-threatening complications, if left untreated. Therapeutic approaches involving rehydration therapy combined with antibiotics reduces duration of infection and fatality rates. So far, antibiotic resistance pattern have been utilized as key epidemiological and phenotypic markers to trace the evolution of outbreak strains. Hence, it is imperative to monitor resistance levels in *Vibrio* pathogen to regulate proper treatment regimen. Although screening for all

antibiotics utilized in the current study is not recommended by ICMR guidelines [24], the current study also elucidated common antibiotic patterns exhibited by *Vibrio* as reported in other studies [42].

Vibrio strains showed greatest sensitivity levels against imipenem (86.2%), followed by netillin, tobramycin, levofloxacin, ofloxacin, amikacin, and kanamycin (Fig. 4a-d). Similar findings have also been reported from other parts of country [27,31-33]. Moreover, our study showed current treatment regimen effective against *Vibrio* isolates.

Vibrio strains showed rising levels of resistance against beta-lactam group (cefepime, ceftazidime, and ceftriaxone) and nalidixic acid. *Vibrio* strains having resistance against cephalosporins have been reported scarcely [15], while the current study elicited alarming resistance rates against cephalosporin class in the region. The previous studies reported very higher levels of resistance against nalidixic acid, streptomycin, and ciprofloxacin antibiotics [31,32,43,44]; however, our study implicated relatively low levels of resistance against these antibiotics.

Overall, our study elucidated lower incidences of *Vibrio* species as compared to other parts of country. Our findings also provide new insight regarding incidences and prevalence of *Vibrio* spp. and its seasonal distribution and resistance management. For developing nations like India, strategic management of infectious diarrhea should be accelerated at a great pace through continuous and active surveillance of etiological agents to reduce disease-associated morbidity and mortality rates.

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