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RESEARCH ARTICLE

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Molecular identification and antibiotic resistance profiling of *Vibrio* spp. in diarrhoeal patients from Dinajpur, Bangladesh

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ABSTRACT

Aim: Cholera, caused by ingestion of *Vibrio cholerae*-contaminated food or water, poses a lethal threat and remains a global public health concern. This research aimed to characterize *Vibrio* species from cholera-suspected patients in three hospitals in Dinajpur.

Methods: We collected 36 stool samples from cholera-suspected patients prior to any treatment and then isolation and identification of *Vibrio* spp. were performed using conventional cultural, morphological, biochemical tests, and polymerase chain reaction (PCR)-targeting the 16S ribosomal RNA (rRNA) gene of *Vibrio cholera.* The antibiogram study of the isolated bacteria was conducted using the disc diffusion method.

Results: The highest Prevalence was observed in children (18.18%), compared to adolescents (0%), adults (16.67%), and senior adults (16.67%). Cultural identification revealed yellow colonies on thiosulphate citrate bile salts sucrose agar, hemolytic colonies on blood agar, pale non-lactose fermenting colonies on MacConkey agar, and white, opaque colonies on nutrient agar. Gram staining showed gram-negative, curved rod-shaped bacteria. Biochemical tests indicated positive reactions for oxidase, catalase, methyle red, triple sugar iron, citrate utilization, motility indole urea, and indole, while voges-proskauer was negative. Molecular characterization by PCR and gel electrophoresis using 16S rRNA primers confirmed the presence of *Vibrio* spp. Antibiotic sensitivity testing showed that isolates were sensitive to cotrimoxazole-25 µg (21 to 30 mm), neomycin-30 µg (22 mm), gentamicin-10 µg (15 mm), streptomycin-10 µg (20 mm), and tetracycline-30 µg (17 mm), but resistant to penicillin, mecillinam, and amoxicillin.

Conclusion: The results of the current study help to identify *Vibrio cholera* and also these findings suggest streptomycin and cotrimoxazole as effective drugs for controlling cholera.

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Introduction

Cholera is one of the most significant fatal diarrheal diseases affecting humans, primarily involving the small intestine. This disease has persisted endemic across various parts of the world for centuries, resulting in millions of deaths [1,2]. In Bangladesh, diarrheal disease is estimated to be the fourth leading cause of death among children, with *Vibrio cholerae* recognized as the sole causative agent responsible for cholera outbreaks [3]. The World Health Organization [4] estimates that diarrheal diseases cause 3 to 5 million deaths annually, affecting 10% of the population in developing countries, including

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Bangladesh. They have set a roadmap to reduce the mortality rate by approximately 90% by 2030. Cholera remains a global public health threat and a key indicator of inadequate social development. The re-emergence of cholera has been noted in conjunction with the increasing size of vulnerable populations living in unsanitary conditions [5,6]. *Vibrio* species are diverse bacteria naturally inhabiting aquatic environments, particularly estuaries, marine coastal waters, sediments, and aquaculture settings in temperate and tropical climates [7]. The genus currently includes 72 species, 12 found in human clinical samples. Among these, *V. cholerae* is the etiological agent of cholera, acute dehydrating diarrhea occurring in epidemic forms worldwide, especially in developing countries [8,9]. The major sources of *V. cholerae* infections are contaminated food and water [10]. Transmission can also occur by consuming raw, undercooked, contaminated, or re-contaminated food. Humans are the only animal hosts affected. Risk factors include poor sanitation, inadequate personal and domestic hygiene practices, and poverty [11]. Traditional laboratory procedures for biochemical identification are labor-intensive and slow, whereas molecular detection via polymerase chain reaction (PCR) offers a reliable alternative. Many PCR methods have been developed targeting housekeeping genes such as 16s ribosomal RNA (rRNA), 23s rRNA, *pho*, *amiB*, *dnaJ*, *gyrB*, *rpoA*, and *rpoB* [12,13]. However, issues with sensitivity and specificity persist, leading to false-positive and false-negative results in *Vibrio* spp. PCR assays [14]. Therefore, in this study, 16s rRNA gene primers were used specifically to detect *Vibrio* spp. Antimicrobial therapy remains crucial as it can reduce the volume of diarrhea, shorten the duration of symptoms, and decrease the excretion of vibrios in the stool [15,16]. However, antibiotic-resistant strains of *V. cholerae* are increasingly reported. Resistance to various antibiotics is also common among environmental non-O1, non-O139 isolates from Malaysia, Thailand, South India, and Bangladesh [17]. In Bangladesh, patients are often treated with broad-spectrum antibiotics without species-specific detection, which may contribute to the ineffectiveness of these treatments. This study is important because species-specific detection during bacterial disease outbreaks is crucial for effective treatment and rapid patient recovery. The present study was conducted to isolate, identify, and characterize field isolates of *Vibrio* spp. using cultural, morphological, biochemical, PCR, and gel electrophoresis techniques, along with determining the identified species' antibiotic resistance and sensitivity profile.

Materials and Methods

Study area and sample collection

Fresh diarrheal stool samples were collected from patients at three hospitals in Dinajpur (Fig. 1):

Figure 1. Map of Dinajpur district with the GPS coordinates of 25° 38' 11.6664'' N and 88° 38' 10.7592'' E.

Table 1. Stool sample collection for *Vibrio* spp. from three different hospitals in Dinajpur.

Place	Age of patients (year)				
	Child (0–12)	Adolescence (13–18)	Adult (19–59)	Senior adult (60 and above)	Total number of sample collection
A	10	0	2	7	19
B	3	0	2	0	5
C	9	1	2	0	12
Total number of sample	22	1	6	7	36

Legends: A = Dinajpur 250 Bed General Hospital, B = M. Abdur Rahim Medical College, C = Hospital and Saint Vincent Hospital Dinajpur

Dinajpur 250 Bed General Hospital, M. Abdur Rahim Medical College and Hospital, and Saint Vincent Hospital Dinajpur. These patients were suspected of being infected with cholera (Table 1). A total of 36 samples were obtained before any treatment was administered. The collected stool samples were immediately transported to the Bacteriology Laboratory of the Department of Microbiology at Hajee Muhammad Danesh Science and Technology University (HSTU) in Dinajpur. Alkaline peptone water (Merck, Germany) was used for enrichment. The samples were then inoculated onto thiosulphate citrate bile salts sucrose (TCBS) agar plates (Merck, Germany) to isolate and identify *Vibrio* species. The experiment was conducted in the Bacteriology Laboratory of the Department of Microbiology at HSTU, Dinajpur.

Isolation and identification of Vibrio spp.

Collected cholera-suspected patients' stools specimens were incubated in nutrient agar medium (Hi-Media, India) for 24 hours at 37°C. After that developed in MacConkey agar (MAC) medium (Hi-Media, India), blood agar medium (Hi-Media, India), and TCBS agar plates (Merck, Germany) separately by following manufactures' guideline at 37°C for 24 hours. For more confirmation, we did biochemical tests [Methyle Red (MR test), Voges-Proskauer (VP test), indole test, catalase test, oxidase test, motility indole urea (MIU test), Simmons citrate test, and triple sugar iron (TSI Test)] according to Cheesbrough (1985), Buxton and Fraser (1977), Merchant and Packer (1967), and OIE (2004) [18–21] were then conducted to confirm the identification of *Vibrio* spp.

DNA extraction

Pure colonies from TCBS agar were grown in TCBS Broth medium overnight. We extracted DNA using the following steps: First, 1 ml of broth was taken in Eppendorf tubes and centrifuged at 5,000 rpm for 3 minutes. The supernatant was discarded, and 500 µl of PBS was added and mixed. The mixture was centrifuged again at 5,000 rpm for 3 minutes. This step was repeated: discarding the supernatant, adding 500 µl of PBS, mixing, and centrifuging at 5,000 rpm for 3 minutes. After discarding the supernatant for the final time, 200 µl of TE buffer was added, and the tubes were sealed with parafilm paper. The samples were boiled for 10 minutes and then chilled on ice for 10 minutes. After chilling, the samples were centrifuged at 10,000 rpm for 10 minutes. We collected 100 µl of the supernatant as DNA. The extracted DNA was stored in a –20°C deep freeze until used for PCR.

Primers

For PCR, we used a reference primer targeting the 16S rRNA gene of *V. cholerae*. The forward primer was 63f (5'CAGGCCTAACACATGCAAGTC3'), and the reverse primer was 763r (5'GCATCTGAGTGTCAGTATCTGTCC3'), selected according to Marchesi et al. [22] with little modification. This primer pair produced a 712 bp band in gel electrophoresis.

PCR amplification and thermal condition

In PCR amplification we used a total volume of the product was 25 µl. PCR master mix (Promega, USA) had taken 12.5 µl, 1 µl Forward primer, 1 µl reverse primer, 5.5 µl nuclease-free water, and 5 µl DNA template in each PCR tube. For thermal condition we followed initial denaturation at 95°C, 1 cycle for 5 minutes; denaturation at 94°C for 1 minute; annealing at 61°C for 30 seconds and extension at 72°C for 1 minute for cycle 30 and final extension done at 72°C for 5 minutes just for 1 cycle. Then, hold the product at 4°C and prepare for further initiatives.

Agarose gel electrophoresis

After completing the thermal cycle, PCR products were analyzed by gel electrophoresis (MGU-402T, CBS Scientific, United Kingdom) using 1×TAE buffer. Final products were run 1.5% agarose gel (Promega, USA). 5 µl of each PCR product was mixed with 1 µl of loading dye (Promega, USA) and the sample was loaded to the appropriate well of the gel, where the gel was stained in ethidium bromide (0.5 µg/ml). Electrophoresis was accomplished for 40 minutes at 100 V. After that, the DNA band was visualized with gel documentation.

Antibiotic sensitivity tests

All bacterial isolates were subjected to an antibiotic sensitivity test by the Kirby-Bauer Disc diffusion method [23] according to The European Committee on Antimicrobial Susceptibility Testing standard (EUCAST) guidelines [24]. The antimicrobial discs (Oxoid, UK) (10 antibiotic discs; tetracycline 30 µg, erythromycin 15 µg, azithromycin 15 µg, streptomycin 10 µg, gentamicin 10 µg, cotrimoxazole 25 µg, penicillin 10 µg, mecillinam 25 µg, neomycin 30 µg, and amoxicillin 30 µg) were applied to the nutrient

Table 2. Antimicrobial agents with their disc concentration and zone diameter interpretive standards (CLSI) for *Vibrio* spp.

Sl. No.	Name of antibiotics with disc concentration (µg/disc)		Interpretation of results (zone of diameter in mm)		
			S	I	R
1	Tetracycline	30	≥ 15	12-14	≤ 11
2	Erythromycin	15	≥ 23	14-22	≤ 13
3	Azithromycin	15	≥ 18	14-17	≤ 13
4	Streptomycin	10	≥ 15	12-14	≤ 11
5	Gentamicin	10	≥ 15	13-14	≤ 12
6	Cotrimoxazole	25	≥ 16	11-15	≤10
7	Penicillin	10	≥ 29	-	≤ 28
8	Mecillinam	25	≥ 17	12-15	≤ 14
9	Neomycin	30	≥ 15	13-16	≤ 12
10	Amoxicillin	30	≥ 18	14-17	≤ 13

Legends: Sl = Serial, No. = Number, µg = Microgram, mm = Millimeter, S = Susceptible, I = intermediately resistant, R = Resistant, ≥ = Greater than or equal to, ≤ = Less than or equal to.

agar and Mueller-Hinton agar plates. McFarland 0.5 standard was maintained for each culture suspension of bacterial isolates before the antibiogram study. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. After the disks were placed on the plate, the plate was incubated at 37°C for 24 hours. After incubation, antimicrobial testing results were recorded as sensitive, intermediate, and resistant according to the National Committee for Clinical Laboratory Standards [25] and Cappuccino and Sherman [26]. zone diameter interpretative standards are presented in Table 2.

Ethical approval

Ethical permission was not required because the stool samples were collected from the individual pan used by the patients; however, before collecting the samples, verbal permission was taken from each patient.

Results

Results of isolation and identification

The culture of the organism produced small colonies on nutrient agar. The characteristics of these colonies included white, discrete, opaque, circular, and translucent in appearance (Fig. 2A). The organism also produced small to moderate sized (1–3 mm) and appeared as or slightly pink, dew colorless water drop-like colonies on MAC agar (Fig. 2B). The culture of the organism produced small colonies on blood agar with a zone of beta-hemolysis surrounds

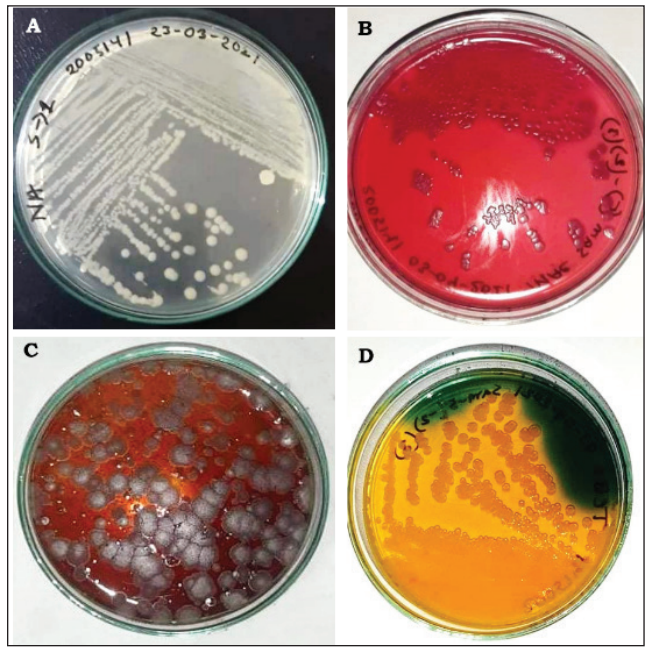

Figure 2. Cultural colonies of *Vibrio* spp. on different culture media. (A) Small colonies on nutrient agar, (B) water drop like colonies on MAC (C). hemolytic colonies (β-hemolysis) on Blood agar, and (D) yellow color colonies on TCBS ..agar

colonies (Fig. 2C). After 24 hours, at a temperature of 37°C. Hemolytic colonies had clear zones around them where red blood cells had been lysed. On TCBS agar culture organism produced large (2–4 mm) in diameter, slightly flattened yellow colonies with opaque center and translucent peripheries.

The media was green when prepared and turned yellow because of sucrose fermentation after 24 hours (Fig. 2D). The isolated organisms were

characterized by morphological characterization using Gram's staining techniques. It was revealed that all of the isolates were Gram's negative pink color, comma, or short red arranged in single or pairs. The isolated organisms were also characterized by a series of biochemical tests like; oxidase catalase, MIU, MR-VP, Indole, TSI, and Simmons citrate test utilization tests, in which all isolates were positive for *Vibrio* spp. In biochemical reactions it was observed (Table 3) that all isolated organisms were positive for oxidase (color changes), catalase (presence of bubble), MR (bright red), TSI (glucose, sucrose, and lactose fermented with acid and gas H_2S), citrate utilization (turned deep green to blue color), MIU (presence of turbidity), Indole (red ring is present), and negative for VP (no color change).

Prevalence of Vibrio spp.

Age categories screened a total of 36 diarrheal stool samples. The patients were categorized as child (0–12 years), adolescence (13–18 years), adult (19–59 years), and senior adult (60s above), in which the overall prevalence of *Vibrio* spp. was detected as 54.29% (Table 4). The prevalence varied according to age group. It was revealed that the highest prevalence observed in child (18.18%) and adult (16.67%) than adolescents (0%), and senior adult (16.67%), respectively.

Molecular identification

Genomic DNA was extracted from suspected samples (diarrhoeal stools) of *Vibrio* species. PCR identification was performed on cultures to before genomic DNA extraction using whole cells and genomic DNA extracted by primer pair 63f and 763r for 16S rRNA gene. Among 36 samples 6 samples were suspected as *Vibrio* spp. by cultural, morphological, and biochemical study but only six samples were confirmed as *Vibrio* spp. after PCR and gel-electrophoresis. The expected 712 bp fragment of a single compact band were visualized under UV light and documented by gel documented system (Macrogen) (Fig. 3).

Result of antibiogram study

Out of 36 samples (fresh diarrhoeal stool) 6 (16.67%) were confirmed as positive for Vibrio spp. Antibiotic sensitivity test of the 6 isolated Vibrio

Table 3. Result of different biochemical tests of study isolates.

Test performed	Reaction pattern/observation		Result	Remarks
MR test	Bright red		+ve	
VP test	No color change		–ve	
Indole test	Red ring is present		+ve	
Catalase test	Presence of bubble		+ve	
Oxidase test	Changes in color		+ve	
MIU test	Presence of turbidity		+ve	*Vibrio* spp.
Simmons citrate test	Turned deep green to blue color		+ve	
TSI Test	Slant	Yellow and red	+ve	
	Butt	Black and red	+ve	
	H_2S	Presence	+ve	

Legends: Methyle Red = MR test, Voges-proskauer = VP test, Indole test, Catalase test, Oxidase test, Motility Indole Urea = MIU test, Simmons citrate test, and Triple Sugar Iron = TSI Test

Table 4. Prevalence of *Vibrio* spp. in the study area.

Age categories (year)	Total number of sample tested	No. of positive isolation	Prevalence (%)	Overall prevalence (%)
Child (0–12)	22	4	18.18	
Adolescence (13–18)	1	0	0	
Adult (19–59)	6	1	16.67	16.67
Senior Adult (≥ 60s)	7	1	16.67	
Total	36	6	51.52	

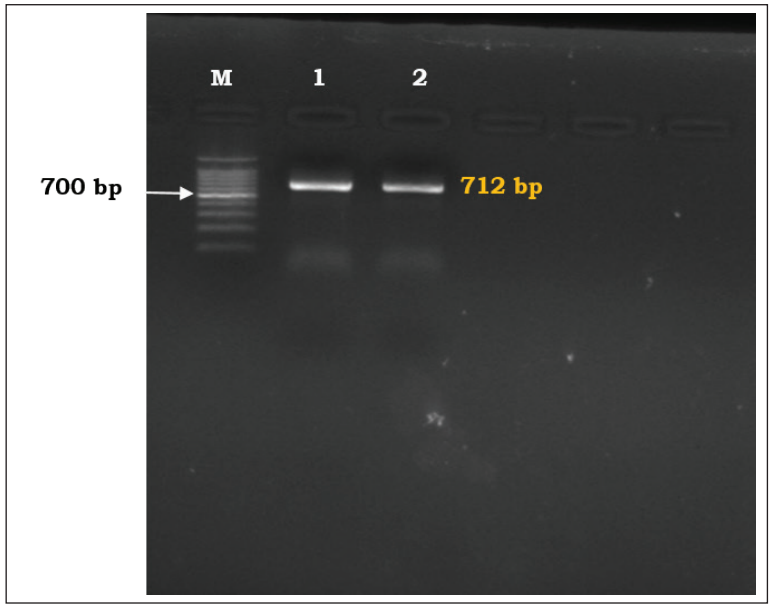

Figure 3. *Vibrio* spp. showing 712 bp in 1.5% agarose gel. M=100 bp DNA ladder (Promega Corporation, Madison, WI). Representative sample lane (1, 2) showing positive result for *Vibrio* spp.

Table 5. Name of antibiotics with disc concentration (µg) and measurement of their zone of inhibition(mm) by disc diffusion method.

Sl. No.	AZM (15 µg)		GEN (10 µg)		E (15 µg)		S (10 µg)		TE (30 µg)		COT (25 µg)		P (10 µg)		MEL (25 µg)		N (30 µg)		AMX (30 µg)	
	S	I	S	R	S	I	S	R	S	R	S	R	S	R	S	R	S	R	S	R
A		16							17		21									
B			15			17	20													
C							20							R		R				
D											30						22			R

Legends: AZM = Azithromycin, GEN = Gentamicin, E = Erythromycin, S = Streptomycin, TE = Tetracycline and COT = Cotrimoxazole, P = Penicillin, MEL= Mecillinam, N = Neomycin, AMX= Amoxicillin. S= susceptible, I = Intermediately resistant, R = resistant, Sl. No. = Serial number of agar plate.

spp. were performed against nine commonly used antibiotic disc belonging to different groups. The result of antimicrobial sensitivity testing of Vibrio spp. by disc diffusion method with 10 chosen antimicrobial agents are presented in Table 5. All the isolates were sensitive to cotrimoxazole (25 µg) 21 to 30 mm, neomycin (30 µg) 22 mm, gentamicin (10 µg) 15 mm, streptomycin (10 µg) 20 mm, and tetracycline (30 µg) 17 mm. Resistant to penicillin (10 µg), mecillinam (25 µg), and amoxicillin (30 µg). Azithromycin (15 µg) 16 mm, and erythromycin (15 µg) 17mm are intermediately resistant (Fig. 4).

Discussion

Bangladesh is extremely overpopulated, with many people living in poor hygienic conditions in both rural and urban areas. Diarrheal disease, linked to these unsanitary conditions, is a major cause of death among children under five [27]. The collected samples were subjected to morphological, cultural, biochemical, and antibiogram studies. In addition, the identified isolates underwent molecular characterization using PCR and gel electrophoresis techniques. In this study, the prevalence of *Vibrio* spp. was observed based on the age of patients. The overall prevalence of *Vibrio* spp. was 16.67% by age of the patient. The prevalence was higher in children (18.18%) compared to adolescents (0%), adults (16.67%), and senior adults (16.67%), which is supported by the earlier observations of Keske et al. [28]. Our present study isolated the bacterium from diarrheal stool on nutrient agar, MAC, blood agar, and TCBS agar. We observed white, opaque,

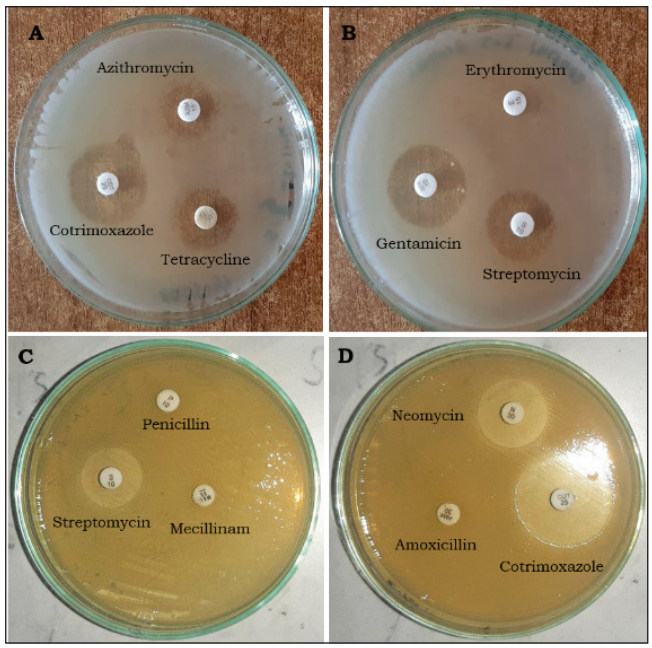

Figure 4. Result of antimicrobial sensitivity test on Nutrient agar (A) and (B) and Mueller- Hinton agar (C) and (D) plates of different antibiotic discs.

and translucent colonies on nutrient agar; pale, non-lactose fermenting, flat colonies on MAC; hemolytic colonies on blood agar; and flattened, shiny, yellow colonies on TCBS agar, similar to the findings of Uddin [29] and Choopun et al. [30]. Cholera is a significant concern for Bangladesh, especially due to the multidrug-resistant (MDR) *Vibrio* spp., which has received increasing worldwide attention. Antibiotic resistance is a major global public health concern [31,32]. To combat any bacterial disease, it is crucial to know the sensitivity and resistance profile of the bacteria to recommend effective antibiotics. Our isolated *Vibrio* spp. were subjected to an antibiogram study using ten commonly used antibiotic discs from different groups. The antibiotic sensitivity data showed the highest levels of sensitivity to cotrimoxazole (21 to 30 mm), neomycin (22 mm), gentamicin (15 mm), streptomycin (20 mm), and tetracycline (17 mm). Okoh and Igbinosa [33] revealed that *Vibrio* spp. was resistant to cotrimoxazole, streptomycin, and gentamicin. Agboola et al. (2023) [34] also observed the resistance pattern of tetracycline, erythromycin, and cotrimoxazole against *Vibrio* spp. The isolated Vibrio spp. were completely resistant to penicillin (10 µg), mecillinam (25 µg), and amoxicillin (30 µg). Azithromycin (15 µg, 16 mm) and erythromycin (15 µg, 17 mm) showed intermediate resistance. These findings are supported by Dutta et al. [16] and Gxalo et al. [35]. The conscious use of antibiotics is essential to tackle antibiotic resistance. Several factors contribute to the increase in bacterial resistance, including overuse, non-prescribed medications, and incomplete dosages [36]. The primary reason for the growing resistance is the easy availability and widespread use of antibiotics, especially in preventive treatments, which leads to resistance mainly through selective pressure [37]. *V. cholerae* remains a significant pathogen due to its increasing resistance to various antibiotics [8]. Studies have shown that the spread of antibiotic-resistant *V. cholerae*, particularly the O139 strain, is linked to mutations and the mobilization of drug-resistant genetic elements [8,38]. In our research, tetracycline was found to be effective against *Vibrio* spp., although Mandal et al. [15] observed resistance with a minimum inhibitory concentration (MIC) of less than 1 µg/ml. A major limitation of our work is that we did not determine the MIC of the tested antibiotics. Further studies are needed to detect and characterize antibiotic-resistant genes in these *Vibrio* spp.

Conclusion

This research isolated and identified *Vibrio* spp. from diarrheal stool samples collected from three hospitals in Dinajpur. Using cultural, morphological, and biochemical methods, 6 out of 36 samples (16.67%) tested positive for *Vibrio* spp., confirmed by PCR with 16S rRNA primers. Antibiotic sensitivity testing revealed that all isolates were resistant to penicillin, mecillinam, and amoxicillin, while most were sensitive to sensitive to cotrimoxazole, streptomycin, neomycin gentamicin, and tetracycline. The current study highlights the Prevalence of *V. cholerae* in northern Bangladesh. Due to its availability and effectiveness, it establishes reliable identification techniques, recommending streptomycin and cotrimoxazole as primary treatment options.

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Authors' contributions

Concept: MRA, MS; Research, Data arrangement, and analysis: M, MRA; Initial draft: MS; Critical review and writing the final version: MS. All authors approved the final version of the manuscript.

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Conflict of interest

The author(s) declare no conflict of interest.

Data Availability Statement

Not applicable.

References

1. Tauheed I, Ahmed T, Akter A, Firoj MG, Ahmmed F, Rahman SI, et al. A snap-shot of a diarrheal epidemic in Dhaka due to enterotoxigenic *Escherichia coli* and *Vibrio cholerae* O1 in 2022. Front Public Health. 2023 Apr 14; 11:1132927; doi:10.3389/fpubh.2023.1132927
2. Montero DA, Vidal RM, Velasco J, George S, Lucero Y, Gómez LA, et al. *Vibrio cholerae*, classification, pathogenesis, immune response, and trends in vaccine development. Front Med. 2023 May 5; 10:1155751; doi:10.3389/fmed.2023.1155751
3. Khan AI, Islam MT, Amin MA, Khan ZH, Qadri F. Outbreak of diarrheal diseases causes mortality in different geographical locations of Bangladesh during the 2021 COVID-19 era. Frontiers in Public Health. 2023 Jan 27; 11:1103518; doi:10.3389/fpubh.2023.1103518
4. World Health Organization. Diarrhoeal disease (WHO). 2017. Available via http://www.who.int/mediacentre/factsheets/fs330/en/
5. Billah SM, Raihana S, Ali NB, Iqbal A, Rahman MM, Khan AN, et al. Bangladesh: a success case in combating childhood diarrhoea. J Global Health. 2019 Dec; 9(2):3566; doi:10.7189/jogh.09.020803
6. Almagro-Moreno S, Pruss K, Taylor RK. Intestinal colonization dynamics of *Vibrio cholerae*. PLoS Pathogens. 2015 May 21; 11(5):e1004787. Available via https://doi.org/10.1371/journal.ppat.1004787
7. Sampaio A, Silva V, Poeta P, Aonofriesei F. Vibrio spp.: life strategies, ecology, and risks in a changing environment. Diversity. 2022 Jan 29; 14(2):97. Available via https://doi.org/10.3390/d14020097
8. Zereen F, Akter S, Sobur MA, Hossain MT, Rahman MT. Molecular detection of *Vibrio cholerae* from human stool collected from SK Hospital, Mymensingh, and their antibiogram. J Adv Vet Anim Res. 2019 Dec; 6(4):451; doi:10.5455/javar.2019.f367
9. Faruque SM, Nair GB. Molecular ecology of toxigenic *Vibrio cholerae*. Microbiol Immunol. 2002 Feb; 46(2):59–66. Available via https://doi.org/10.1111/j.1348-0421.2002.tb02659.x
10. Patrick GB, Nishibuchi M, Tunung R, Son R. Molecular characterization of clinical isolate of *Vibrio cholerae* isolated from outbreaks cases in Malaysia. Int Food Res J. 2012 Sep 1;19(3):1267.
11. Ojeda Rodriguez JA, Hashmi MF, Kahwaji CI. *Vibrio cholerae* Infection. In: StatPearls Internet. Treasure Island, FL: StatPearls Publishing; 2024 Jan. Available via https://www.ncbi.nlm.nih.gov/books/NBK526099/
12. Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, et al. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl Environ Microbiol. 2005 Sep; 71(9):5107–15. Available via https://doi.org/10.1128/AEM.71.9.5107-5115.2005
13. Nhung PH, Shah MM, Ohkusu K, Noda M, Hata H, Sun XS, et al. The dnaJ gene as a novel phylogenetic marker for identification of Vibrio species. Syst Appl Microbiol 2007 Jun 15; 30(4):309–15. Available via https://doi.org/10.1016/j.syapm.2006.11.004
14. Maurer JJ. Rapid detection and limitations of molecular techniques. Ann Rev Food Sci Technol 2011 Apr 10; 2(1):259–79. Available via https://doi.org/10.1146/annurev.food.080708.100730
15. Mandal J, Dinoop KP, Parija SC. Increasing antimicrobial resistance of *Vibrio cholerae* OI biotype EI Tor strains isolated in a tertiary-care centre in India. J Health Population Nutr. 2012 Mar; 30(1):12; doi:10.3329/jhpn.v30i1.11270
16. Dutta D, Kaushik A, Kumar D, Bag S. Foodborne pathogenic vibrios: antimicrobial resistance. Front Microbiol 2021 Jun 30;12:638331. Available via https://doi.org/10.3389/fmicb.2021.638331
17. Igere BE, Okoh AI, Nwodo UU. Non-serogroup O1/O139 agglutinable *Vibrio cholerae*: a phylogenetically and genealogically neglected yet emerging potential pathogen of clinical relevance. Arch Microbiol. 2022 Jun; 204(6):323; doi:10.1007/s00203-022-02866-1
18. Cheesbrough M. Medical laboratory manual for tropical countries. Microbiology 1985; 2:400–80.
19. Buxton A, Fraser G. Animal microbiology. Volume 1: immunology, bacteriology, mycology, diseases of fish, and laboratory methods. Blackwell Scientific Publications, Hoboken, NJ, 1977.
20. Merchant IA, Packer RA. Veterinary Bacteriology and Virology Seventh Edition. The Iowa University Press, Ames, IA, 1967, pp 286–306.
21. OIE. OIE Manual Part-2: pullorum disease and fowl typhoid in OIE manual diagnostic tests and vaccines for terrestrial animals. 4th OIE, Paris, France, 2004.
22. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, et al. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environment Microbiol. 1998 Feb 1; 64(2):795–9.
23. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966

Apr 1; 45(4_ts):493-6. Available via https://doi.org/10.1093/ajcp/45.4_ts.493
24. Giske CG, Turnidge J, Cantón R, Kahlmeter G. Update from the European committee on antimicrobial susceptibility testing (EUCAST). J Clin Microbiol. 2022 Mar 16; 60(3):e00276–21. Available via https://doi.org/10.1128/jcm.00276-21
25. CLSI. Performance standards for antimicrobial susceptibility testing, twenty-third informational supplement, CLSI Document M100-S23. Clinical and Laboratory Standards Institute, Wayne, PA, 2013.
26. Cappuccino JC, Sherman N. Microbiology. A Laboratory manual. 10th edition, USA, 2014; pp 293–9.
27. Ahmed M, Abedin J, Alam KF, Al Mamun A, Paul RC, Rahman M, et al. Incidence of acute diarrhea-associated death among children <5 years of age in Bangladesh, 2010–2012. Am J TropMed Hygi. 2018 Jan; 98(1):281. Available via https://doi.org/10.4269/ajtmh.17-0384
28. Keske Ş, Zabun B, Aksoy K, Can F, Palaoğlu E, Ergönül Ö. Rapid molecular detection of gastrointestinal pathogens and its role in antimicrobial stewardship. J Clin Microbiol. 2018 May; 56(5):10–128. Available via https://doi.org/10.1128/jcm.00148-18
29. Uddin M. A study of food microorganism which are involved in food borne illness (Doctoral dissertation, East West University, Bangladesh); 2009.
30. Choopun N, Louis V, Huq A, Colwell RR. Simple procedure for rapid identification of *Vibrio cholerae* from the aquatic environment. Appl Environ Microbiol. 2002 Feb; 68(2):995–8. Available via https://doi.org/10.1128/aem.68.2.995-998.2002
31. Das B, Verma J, Kumar P, Ghosh A, Ramamurthy T. Antibiotic resistance in *Vibrio cholerae*: understanding the ecology of resistance genes and mechanisms. Vaccine. 2020 Feb 29; 38:A83–92. Available via https://doi.org/10.1016/j.vaccine.2019.06.031
32. Liu J, Wu Q, Xu H, Pan Y, Malakar PK, Zhao Y, et al. Change of antibiotic resistance in Vibrio spp. during shrimp culture in Shanghai. Aquaculture. 2024 Feb 15; 580:740303. Available via https://doi.org/10.1016/j.aquaculture.2023.740303
33. Okoh AI, Igbinosa EO. Antibiotic susceptibility profiles of some Vibrio strains isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa. BMC Microbiol. 2010 Dec; 10:1–6. doi:10.1186/1471-2180-10-143
34. Agboola TD, Nmema EE, Odetoyin BW. Distribution and antibiogram of Vibrio species from hospital wastewater in Southwest, Nigeria. Pan Afr Med J. 2023;45(1):80; doi:10.11604/pamj.2023.45.80.35773
35. Gxalo O, Digban TO, Igere BE, Olapade OA, Okoh AI, Nwodo UU. Virulence and antibiotic resistance characteristics of Vibrio isolates from rustic environmental freshwaters. Frontiers Cell Infect Microbiol. 2021 Aug 19; 11:732001. Available via https://doi.org/10.3389/fcimb.2021.732001
36. De R, Mukhopadhyay AK, Ghosh M, Basak S, Dutta S. Emerging resistome diversity in clinical *Vibrio cholerae* strains revealing their role as potential reservoirs of antimicrobial resistance. Mole Biol Rep. 2024 Dec;51(1):409. Available via https://doi.org/10.1007/s11033-024-09313-y.
37. Chiș AA, Rus LL, Morgovan C, Arseniu AM, Frum A, Vonica-Țincu AL, et al. Microbial resistance to antibiotics and effective antibiotherapy. Biomedicines. 2022 May 12; 10(5):1121; doi:10.3390/biomedicines10051121
38. Yu L, Zhou Y, Wang R, Lou J, Zhang L, Li J, et al. Multiple antibiotic resistance of *Vibrio cholerae* serogroup O139 in China from 1993 to 2009. PloS One. 2012 Jun 11; 7(6):e38633; doi:10.1371/journal.pone.0038633