

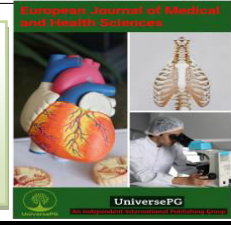


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## Antibiotic Resistance Analysis of *Vibrio spp* Isolated from Different Types of Water Sources of Bangladesh and Their Characterization

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### ABSTRACT

Cholera is an acute type of the diarrheal disease that torments human civilization over the centuries. The wickedness of human sufferings led physicians, and researchers to carry out extensive research on cholera and *Vibrio cholerae* leading to potential discoveries that opened up new areas of research or new disciplines in biomedical sciences. An attempt is made here to summarize some of these breakthroughs and outline their significance in broader perspectives. In the present study, a total of 12 samples were collected from four types of water sources for separation of *Vibrio spp*. Water samples are enrichment into alkaline peptone water then inoculate into culture media such as Nutrient agar, MacConkey, and Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar medium. After incubation of TCBS plates for 24h at 37°C yellow and green colonies are screened out for biochemical identification. No-sucrose fermenting *Vibrio parahaemolyticus*, *Vibrio mimicus*, and *Vibrio vulnificus* show green colonies and sucrose fermenting *Vibrio cholerae* and *Vibrio alginolyticus* shows yellow colonies on TCBS. To identify the *Vibrio spp* extracts biochemical test was carried out and typical *Vibrio spp* give a positive result. The majority of *Vibrio spp* are avirulent, but certain strains may be sporadically human pathogenic. The antibiotic resistance studies represented that among the 9 extracts were resistant against Erythromycin, Penicillin, Cephalexin, Vancomycin and 4 extracts resistant against Tetracycline. To expand present knowledge of the occurrence, ecological niche and persistence of significant human pathogenic *Vibrio spp* in water environments, occurrence, and laboratory studies were tested.

**Keywords:** Isolation, *Vibrio cholera*, Water sources, Pathogenic, Diarrhea, and Identification.

### 1 INTRODUCTION

Water is the necessity for life and an adequate, safe and accessible supply is of paramount importance. Life is possible on earth due to the presence of water. The impact of water on health is associated with unwholesome sources contaminated with faces

containing pathogenic microorganisms. The quality of drinking water is closely associated with human health and providing safe drinking water is a major public health priority. It is the dispersion medium for all biochemical reactions of the living process and takes part in many of these reactions. Freshwater bodies

serve as the main water resources in rural areas used for drinking, cooking, and irrigation for agriculture in most communities that have little or no access to potable, safe water. *V. cholerae* is known to be normal inhabitants of surface water. Illnesses in humans occur after ingestion or direct wound contact with contaminated food or water (Cho *et al.*, 2008; Turner *et al.*, 2013). At least twelve *Vibrio* species are of clinical significance for the human, being *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* the three main species (Gomez-Gil *et al.*, 2014).

Over the years, several researchers have focused on the sternness of diseases caused by *V. cholerae* leaving out relatively minor *Vibrio* species of medical interest, some of which are described as emerging pathogens able to cause mild to severe human diseases. *Vibrio spp* has emerged as a serious threat to human health worldwide. The growing number and severity of food poisoning incidents around the world have greatly increased public awareness about food safety (Forsythe, 2000). Many *Vibrio* species are pathogens to human and have been implicated in foodborne disease. Five types of diseases such as tail necrosis, shell disease, red disease, loose shell syndrome (LSS) and white gut disease (WGD) is caused by *Vibrio spp* in *Penaeus monodon* (Jayasree *et al.*, 2006). *Vibrio spp* has also been implicated in mortality events in the wild (Li *et al.*, 2006). Pathogenic *Vibrio* causes acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, and fever (Yang, *et al.*, 2008). *V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus* is among the well-known fish pathogen worldwide (Haenen *et al.*, 2014; Azila *et al.*, 2017; Ina-Salwany *et al.*, 2018).

*Vibrio spp* is rod-shaped Gram-negative bacteria that are widespread in coastal and estuarine environments around the world. *V. cholerae* is a usual inhabitant of the environment and causes severe diarrheal disease contaminating thousands of people each year in developing countries. *V. parahaemolyticus* was first identified as a foodborne pathogen in the 1950s, and, in subsequent decades, became globally recognized as an etiological agent of diarrheal disease associated with seafood consumption (Parveen *et al.*, 2008).

*Vibrio* is a Gram-negative halophilic bacterium found abundantly in marine and estuarine environments (Thompson *et al.*, 2004; Raissy *et al.*, 2014). Among the different species, *V. parahaemolyticus* has emerged as a significant pathogen for *M. rosenbergii* (Khuntia *et al.*, 2008). The genus *Vibrio* includes some species that behave as opportunistic pathogens and can be involved both in cultured gilthead sea bream outbreaks and in cases of human disease (Austin and Zhang, 2006). Typical symptoms of the gastroenteritis may include abdominal pain, diarrhea, nausea, and fever (Tan *et al.*, 2017).

Bacteria can have an impact on the quality of water by controlling the amount of oxygen and other elements and by causing diseases in aquatic organisms and humans. Antibiotic-resistant bacteria may represent a potential threat to human health due to direct transmission through the food chain (Duran and Marshall, 2005; Rahman *et al.*, 2019) or by transferring the acquired antimicrobial resistance to human pathogens by mobile genetic elements (Guglielmetti *et al.*, 2009). *Vibrio spp* usually, are said to be highly susceptible to most clinically used antibiotics (Mala *et al.*, 2014; Shaw *et al.*, 2014). The number of *Vibrio spp*. classified as pathogenic strains are at least 11, including *V. cholerae* as the main cause of diarrhea, *V. parahaemolyticus* as the cause of foodborne gastroenteritis (Ozer, *et al.*, 2008). The aim of this study was to identify, characterize, and determine antibiotic resistance of *Vibrio* species of clinical importance in water samples and determine their pathogenicity, which will help to control *Vibrio*-associated gastroenteritis from Bangladesh.

## 2 MATERIALS AND METHODS

**2.1 Sample Collection and Cultural** - The present work was divided by separation and Identification of *Vibrio spp* from different types of water sources. The sample was collected from different region like as beside the Niribili area, Savar and Megna River from Daudkandi, Comilla, Bangladesh. For the separation of bacteria the enrichment, streak and spread plate approaches were followed by alkaline peptone water, Nutrient agar MacConkey's agar and TCBS agar were used for the isolation purpose.

**Table 1:** Water was collected from different region.

Sample No.	Sampling source	Sample collection area	Sampling Time	Total sample No.
1	Tap water	Hostel, Niribili area, Daudkandi	3	12
2	Pond water			
3	River water			
4	Sewage water			

**2.2 Enrichment in Alkaline Peptone Water (APW) -** *Vibrio spp* grows very rapidly in APW and at 6 to 8 hours will be present in greater numbers than non-*Vibrio* organisms. Enrichment in APW enhances the separation of *V. cholerae* when few organisms are present, as in specimens from convalescent patients and asymptomatic carriers. An enrichment medium containing a selective agent may not offer any advantage over APW if it is used with short incubation time (6 to 8 hours).

**2.3 Nutrient and Mac Monkeys Agar-** Nutrient agar was used for cultivating of non-fastidious microorganisms. A differential medium was also used for the separation of Coliforms and intestinal pathogens in water and biological specimens.

**2.4 TCBS Agar -** TCBS is the medium of choice for the separation of *V. cholerae* and is widely used worldwide, it has a relatively short shelf life once prepared (3 to 5 days) unless plates are carefully protected against drying. TCBS agar is green when prepared. Overnight growth (18 to 24 hours) of *V. cholerae* will produce large (2 to 4 mm in diameter), slightly flattened, yellow colonies with opaque centers.

**2.5 Morphological Characterization by Gram's Staining -** The most widely used staining procedure in microbiology is the gram stain. The procedure is based on the ability of microorganisms to retain the color of the stains used during the gram stain reaction.

Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolonization step and a

counter stain is used to impart a pink color to the decolorized gram-negative organisms.

**2.6 Biochemical Test for the Identification of the Organism:** The biochemical identification of the organism was done by performing the biochemical tests. The biochemical tests were done as stated on Berge's Manual of Determinative Bacteriology.

Several types of biochemical tests were performed in this study such as Sugar Fermentation Test, Oxidase Test, Catalase Test, Indole Test, Methyl Red-Catalase Test (MR-VP), Triple Sugar Iron Agar (TSI), Simmons Citrate Agar (SCA), Urea Test, Kligler Iron agar (KIA) Test and Gelatin Hydrolysis Test, Starch Hydrolysis Test, etc

**2.7 Preparation of a Hanging Drop Slide -** A hanging drop slide allows viewing live bacteria under a microscope. This approach allows for maintaining their natural shape and makes it possible to observe their behavior. If an organism is motile, its activity is clearly apparent, and directional, place locomotion will be observed. This test was performed according to the approach described by Cowan to differentiate motile bacteria from non-motile one.

**2.8 Antibiotic Sensitivity Test -** The list of commercially available antimicrobial disc used in this study with their concentration is given bellow After placing of the disc on the plate, the plates were inverted and incubated at 37°C for 16 to 18 hours after incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the undersurface of the plate without opening the lid.

**Table 2:** Different antimicrobial agents with their disc concentration for Antibiotic sensitivity test.

Antimicrobial agents	Symbol	Disc concentration (ug/disc)
Erythromycin	E	15
Tetracycline	TE	30
Chloramphenicol	C	30
Gentamycin	GEN	10
Ciprofloxacin	CIP	5
Vancomycin	VA	30
Nitrofurantoin	F	300
Penicillin	P	10
Sulphamethoxazole	SXT	25
Cephalexin	CN	30

### 3 RESULTS AND DISCUSSION

**3.1 Separation and study of cultural characters:** In the present study, 12 water samples were collected from different sources such as tap water, pond water, river water and sewage water for isolation and identification of *Vibrio spp* gram negative bacteria. All samples should be tested before enrichment and after enrichment. Before enrichment samples were inoculated into culture media such as Nutrient agar, MacConkey's and TCBS agar used for separation of *Vibrio spp*. After enrichment into alkaline peptone water can be used to enhance the recovery of *Vibrio spp* where there are few organisms present. In the present study, specific biochemical tests were used for the detection of Gram-negative bacteria.

### 3.2 Nutrient, Mac Conkey, and TCBS Agar:

Nutrient agar represented circular, smooth and translucent colonies. MacConkey agar represented colorless colonies after prolonged incubation pink color colonies were seen. This is a highly selective medium which is used to extract and to differentiate the sucrose fermenting from non-sucrose fermenting species of *Vibrio*. TCBS agar represented that large 2 to 3 mm, smooth, yellow, shiny, convex and slightly flattened with opaque centers. Among the 12 samples, 2 samples did not show any growth of an organism in the media. Ten-fold dilutions of water samples were made down to 10<sup>-8</sup>, before 0.1 ml of the aliquot of each serial dilution was plated onto TCBS agar in triplicate (Letchumanan et al. 2015).



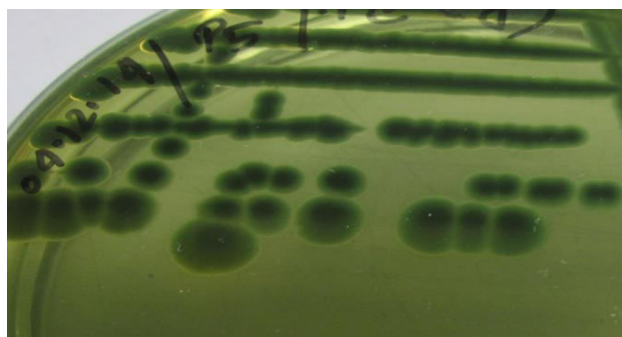
**Fig 1:** Culture of organism on Nutrient 's Agar (Left), Culture of organism on MacConkey Agar (Middle), and Culture of organism on TCBS agar (right).

**3.3 Cultural Characteristic on TCBS Agar -** Among the 15 extracts, 9 isolates represented green colony and 6 extracts represented yellow colony. Initial bacterial separations were made from the hemolymph of infected moribund animals using TCBS agar plates (Zhou et al., 2012). 12 samples were collected from different region for separation and identification of

*Vibrio spp* in this present investigation. We extracted 2 types of *Vibrio spp* colony such as yellow and green colony. Yellow colonies of Sucrose fermenting *Vibrio* like *V. cholera* and *V. alginolyticus*. Green colonies of non-sucrose fermenting *Vibrio* like *V. parahaemolyticus*, and *V. mimicus*.

**Table 3:** Different extracts of *Vibrio spp* with their various color of colony in the growth media.

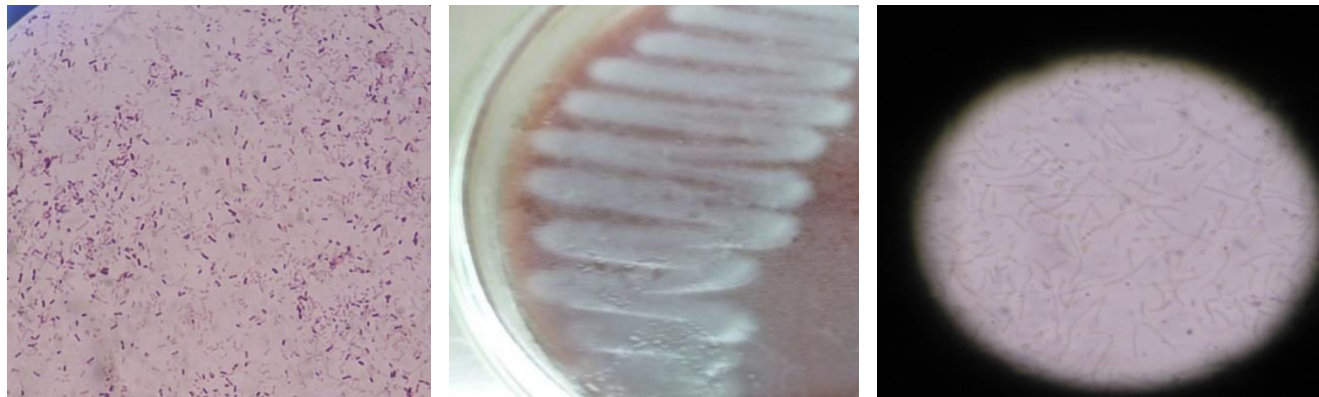
Sample no	Color of colony
1	Yellow, Green
2	Green
3	Green
4	Large yellow, Green
5	Green, yellow
6	Yellow
7	Green
8	Yellow, Green
9	Yellow, Green
10	Green



**Fig 2:** Blue green colonies of non sucrose fermenting *Vibrio* on TCBS agar.

**3.4 Microscopic Examination:** Microscopic observation was performed to observe the shape and the gram reaction of the extracts. All the extracts were found to be gram-negative, curved, comma and rod shape. In Gram’s staining, the morphology of extracted *V. species* is gram-negative, curved rod-shaped

bacteria with the single polar flagellum. We worked with 15 extracts of 12 samples of water after 3 times of sampling we get 15 extracts from 4 sources of water such as tap water, pond water, river water, and sewage water. Among 15 extracts, 8 extracts of 4 samples represented a zone of inhibition on blood agar media. The motile and non-motile organisms were identified by observing motility.



**Fig 3:** Morphology of *Vibrio spp* under light microscopy (left); Growth on Blood agar (right) and Hanging drop slide examined fewer than 40x power microscope.

**Table 4:** Haemolytic activity of *Vibrio spp* in the Blood agar.

Isolate no	Blood Agar	Isolate no	Blood Agar
1	$\beta$ -haemolytic	6	$\alpha$ -haemolytic
2	$\beta$ -haemolytic	7	$\alpha$ -haemolytic
3	$\beta$ -haemolytic	8	$\alpha$ -haemolytic
4	No haemolytic	9	$\alpha$ -haemolytic
5	No haemolytic	10	No haemolytic

**3.5 Biochemical Characteristics of the Extracts** - All the isolates were tested for selective biochemical tests which are presented in **Table 5**. Among 12 samples of 15 extracts, 15 extracts represented oxidase-positive. Among 15 extracts, 15 extracts represented catalase-positive. Among 15 extracts, 15 extracts represented oxidase and catalase positive.

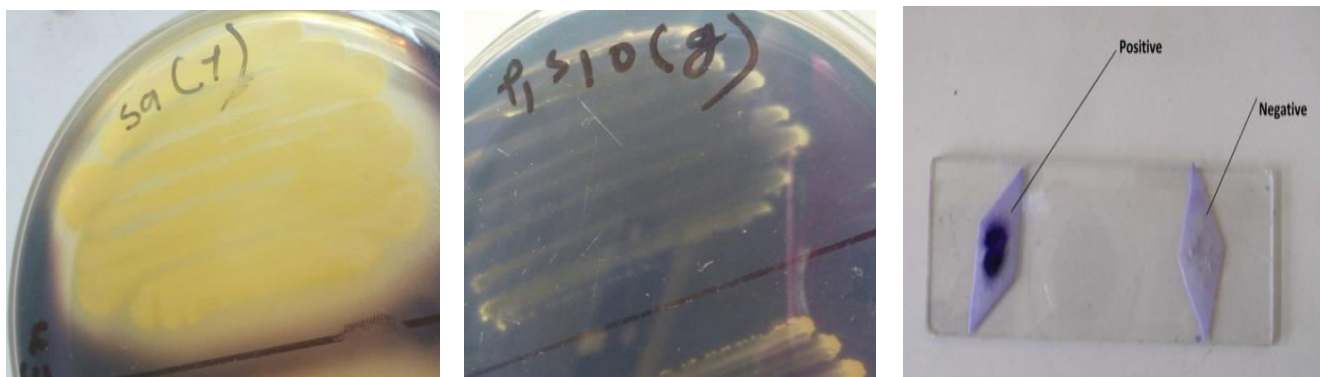
Among the 15 extracts of 12 samples MIO represented a positive result, 15 extracts represented urease negative, a yellow colony of *Vibrio spp* represented starch positive and green colonies represented starch

hydrolysis negative, Among 7 extracts 2 extracts represented citrate negative, 1 extract represented indole positive, 3 extracts represented indole negative. Among the 4 extracts, 2 extracts MR positive, 2 negative. 15 extracts of 2 sample VP positive result. Sugar fermentation test of *Vibrio spp* fermented dextrose, sucrose and Mannitol produce acid and gas but did not ferment lactose. 7 extracts, 6 extracts of 12 samples represented positive KIA test. Among 15 extracts of 12 samples represented small, curved, rods with darting motility of wet mount test.

**Table 5:** Enrichment results of the representative test extracts.

Isolate name	KIA				MIO	Urease test	Citrate utilization	TSI
	Slant	Butt	Gas production	H <sub>2</sub> S				
1	Red	Yellow	+	-	+	-	+	-
2	Red	Yellow	+	-	+	-	+	+
3	Red	Yellow	+	-	+	-	+	-
4	Red	Yellow	+	+	+	-	+	-
5	Red	Yellow	+	+	+	-	+	-
6	Yellow	Yellow	+	+	+	-	-	-
7	Red	Red	-	-	+	-	-	-
8	Red	Yellow	+	+	+	-	-	-
9	Red	Yellow	+	+	+	-	+	-
10	Red	Yellow	+	+	+	-	+	-

Symbols: +: positive and -: negative g: green colony y: yellow colony



**Fig 4:** Starch hydrolysis test shows positive (left) and shows negative result (middle) and Oxidase test represented positive result (right).

**Table 6:** Gelatin hydrolysis, starch hydrolysis by representative test extracts:

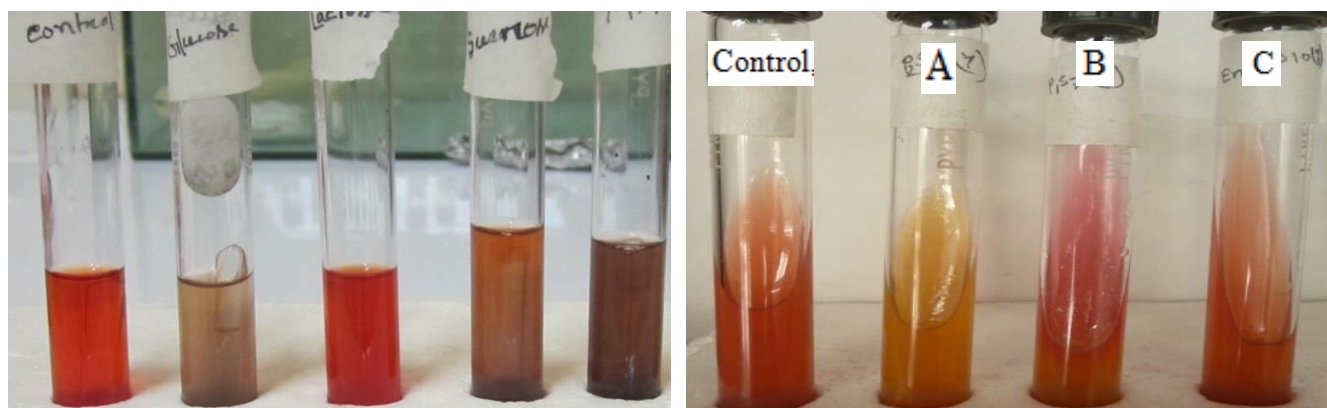
Isolate name	Gelatin hydrolysis	Starch hydrolysis
1	-	-
2	+	-
3	+	+
4	-	-
5	+	-
6	+	+
7	-	-
8	+	+
9	+	+
10	-	-

Symbol: + = positive, - = negative

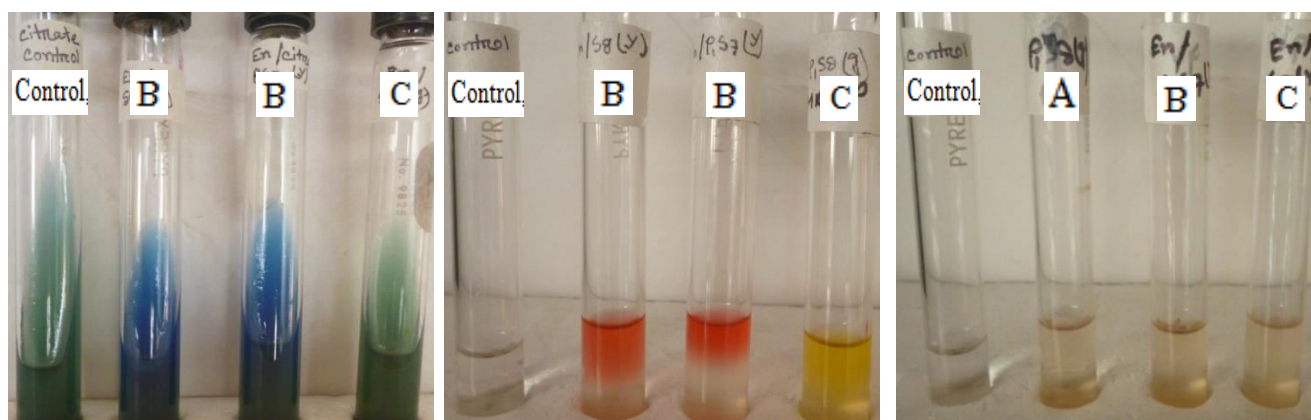
**Table 7:** Sugar fermentation reaction profile of the extracts with interpretation.

Sample No	Sugar fermentation profile				Interpretation
	DX	L	S	MN	
1	+	-	-	+	<i>Vibrio spp</i>
2	+	-	-	+	
3	+	-	+	+	
4	+	-	+	+	
5	+	-	-	+	
6	+	-	+	+	
7	+	-	+	+	
8	+	-	+	+	
9	+	-	-	+	
10	+	-	-	+	

Symbols: DX=Dextrose, L=Lactose, S=Sucrose, MN=Mannitol,



**Fig 5:** Result of sugar fermentation tests *Vibrio spp* fermented dextrose, sucrose and mannitol produce acid and gas but did not ferment lactose (left); KIA test (right, Control, A, B showing positive result and C showing negative result).



**Fig 6:** Citrate test (Left, Control; B. showing positive and C. showing negative result); MR test (Middle, C. shows MR negative and B. shows positive result); and Result of Voges- Proskauer test represented no color change (right).

**Table 8:** Result of starch, citrate, and indole, KIA, MR, VP without Enrichment.

Isolate name	Starch hydrolysis	Citrate utilization test	Indole	KIA	MR	VP
5	-	-	-	+	+	-
6	+	-	-	+	+	-
7	-	-	-	+	+	-
8	+	+	-	+	+	-
9	+	+	+	+	+	-
10	-	-	+	-	+	-

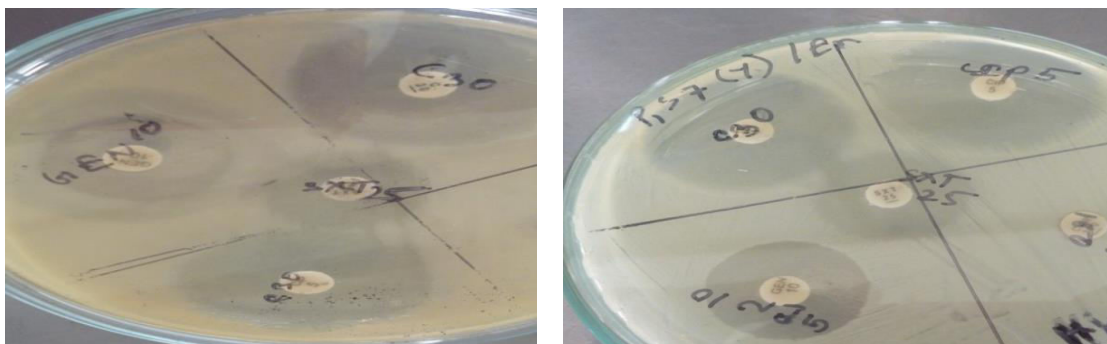
Symbols: + : positive, - : Negative

**Table 9:** Antibioqram profile against test extract of *Vibrio spp.*

Sample name	Antibiotic name									
	E	TE	C	GEN	P	CN	VA	F	SXT	CIP
1	R	S	M	R	R	R	A R	S	S	R
2	R	S	R	R	R	R	R	M	S	R
3	R	M	S	R	R	R	M	S	M	S
4	R	S	S	S	R	R	R	S	S	S
5	R	R	S	S	R	R	R	M	S	S
6	R	R	R	S	R	R	R	M	S	M
7	R	R	S	S	R	R	R	S	S	S
8	M	M	S	S	R	R	R	S	S	S
9	R	M	S	S	R	R	M	S	S	M
10	R	M	S	S	R	R	R	M	S	S

Key to the abbreviation of the antibiotics: E-Erythromycin, TE-Tetracycline, C-Chloramphenicol, GEN-Gentamycin, P-Penicillin, CN-Cephalexin, VA-Vancomycin, F-Nitrofurantoin, CIP-Ciprofloxacin, SXT-Sulfamethoxazole, R-Resistant, and S-Sensitive.



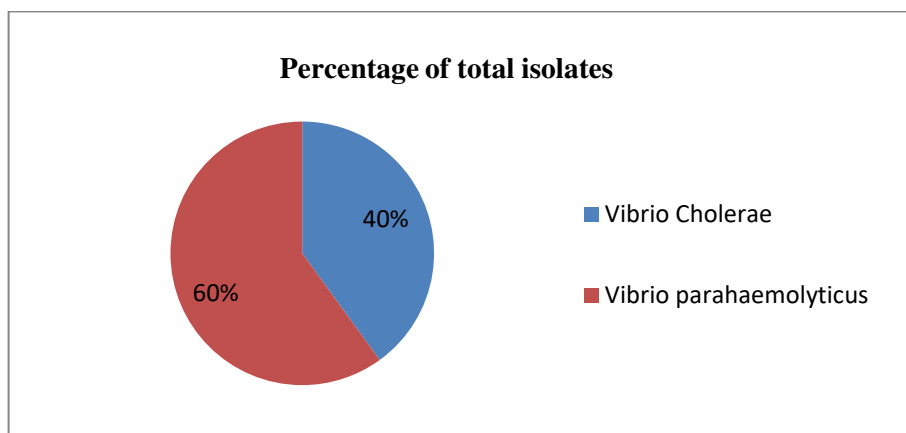


**Fig 7:** Result of antibiotic sensitivity test.

We investigate the antibiotic susceptibility; *Vibrio* species were extracted and identified from the sample of water. Among the 15 extracts, 9 extracts represented green and 6 extracts represented yellow colony. Susceptibility of these *Vibrio* species was assayed by the disc diffusion approach against 10 antibiotics. Among the 9 extracts, 6 extracts represented sensitive against Chloramphenicol, 9 extracts represented highly sensitive against Sulphamethoxazole, Gentamicin,

Nitrofurantoin, and 5 extracts sensitive against ciprofloxacin.

Among the 9 extracts were resistant against Erythromycin, Penicillin, Cephalexin, Vancomycin and 4 extracts resistant against Tetracycline. *Vibrio* species extracted in this study represented good susceptibility to most of the antimicrobial agents that are commercially available for the treatment of causing disease.



**Fig 8:** Graphical representation of Total extracts.

#### 4 CONCLUSIONS

The present study focuses on the incidence of *Vibrio spp* in water bodies which are used by people in surroundings for their daily needs. For all living organisms, water is the most vital and significant matter for survival. Despite modern techniques for disinfection, sanitation, and water purification, waterborne diseases still threaten human health. Studies represented that ingestion of water

contaminated with *Vibrio spp* like *V. cholerae* can create serious symptoms including diarrhea, enteritis, and even death, leading to high economic losses. These bacteria are found almost everywhere. Humans, animals, and sewage can be sources of these bacteria. Contaminated water plays a significant role in the transmission of bacteria to human body. The primary goal of this work the separation and identification of *Vibrio spp* from a different region of water such as tap water, pond water, river, and sewage water and was

conducted to determine the separation and identification of gram-negative *Vibrio spp* organisms by cultural study, staining procedure and different type of biochemical test. The purpose of the study was to extract and identify the presence of *Vibrio spp* in the water bodies in different areas of Bangladesh as well as determine the pattern of antibiotic resistance. Finally, more work should be done on the molecular level to confirm extracts as they are not being extracted using the cultural approach.

## 5 ACKNOWLEDGEMENTS

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## 6 CONFLICTS OF INTEREST

The authors declared no potentially conflicts of the interest with respect to the research, authorship and publication of this article.

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