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Characterization of *Vibrio* spp Isolated from Environmental Water Sources in Benin City, Edo State

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Abstract: The study was carried out to isolate and identify *Vibrio* species obtained from water samples in different sources within Benin City. Physicochemical assessment of the water samples collected aseptically was carried out. *Vibrio* spp isolation and identification, antimicrobial susceptibility tests, phenotypic virulence determinants and molecular identification were carried out using standard microbiological technique. The results revealed that the pH ranged from 4.15±0.02 – 6.53±0.16, temperature (29.10±0.10 – 29.30±0.10^{o}C), electrical conductivity (15.67±0.58 – 142.00±1.00 µS/cm), turbidity (0.02±0.00 – 0.21±0.01NTU), Alkalinity (0.12±0.00 – 0.67±0.01), Phosphate (0.10±0.00 – 1.53±0.07mg/L), Nitrate (0.54±0.03 – 1.12±0.09mg/L), and Sulphate (0.02±0.01 – 0.53±0.10mg/L) were within acceptable range delineated by World Health Organization for drinking water. The bacteriological analysis of water samples showed that river water had the highest total heterotrophic bacteria count of 175±111.24 cfu/100ml and stagnant water had the highest total *Vibrio* spp (20±1.73 cfu/100ml). The identified *Vibrio* spp by molecular characterization were *Vibrio cholera, Vibrio fluvialis, Vibrio parahaemolyticus and Vibrio anguillarum.* The antimicrobial susceptibility testing revealed that all the isolates were resistant to Ceftriaxone, Cefoperazone, Co-Trimoxazole, Cefuroxime and Meropenem. The phenotypic virulence properties of the bacterial isolates showed that they had virulence determinants activity for Gelatinase and Lipase. However, the microbiological quality did not meet the SON standard for portable water, hence the need for quality assessment of drinking water sources and ensuring compliance with relevant standard to avoid risk to human health.

1. Introduction

Vibrio infections are a major public health concern worldwide, It is estimated that 2.4 million cases and 120,000 fatalities are recorded each year (WHO, 2016). Infection with *Vibrio* spp can take many different forms, from the notorious cholera that causes crippling diarrhea and dehydration to the serious and sometimes fatal septicemia brought on by the *Vibrio vulnificus*. Beyond just direct infection, the effects are far-reaching, devastating economies and impeding growth in areas with scarce resources (Colwell, 1996).

Vibrio species are mesophilic and chemoorganotrophic, Gram-negative, typically motile rods with facultatively fermentative metabolism (Farmer *et al*., 1992). These bacteria coexist with eukaryotes and are widely distributed in aquatic habitats (Ruby, 1996). While some vibrio species are harmless (*Vibrio fischeri*), a large number of them pose a significant threat to human health (*Vibrio cholera*), leading to a range of waterborne diseases with life-threatening consequences (Nair *et*

al., 2021). Pathogenic Vibrio species possess an advanced set of virulence characteristics that enable them to efficiently infiltrate and inflict harm on human hosts. Toxins such as cholera toxins and hemolysin cause disruptions in intestinal function, resulting in severe diarrhea and electrolyte imbalance (Barua, 2010). Lipopolysaccharides and outer membrane proteins help organisms elude immune responses while flagella and adhesion pili aid in adhering to host cells (Hussain *et al*., 2018). *Vibrio* species can severely cause damage to human health because of the complex interaction of virulence factors (Mayhungu *et al*., 2023).

The environmental conditions have a complex relationship with the distribution and abundance of *Vibrio* species. Due to their halophilic nature, they do well in salty settings such as estuaries and coastal water in which temperature is an important factor. As a result of climate change, their range is growing, forcing them into freshwater environments that were previously inappropriate and raising the possibility of human exposure (Baker-Thompson *et al*., 2016). Furthermore, human actions such as pollution and fertilizer runoff might accelerate the proliferation and virulence of *Vibrio* species (Colwell, 1996). Seeing the distorting effects of these *Vibrio* species on both humans and the economy, it becomes even more complicated by the emergence of antibiotic resistance in *Vibrio* species. These resistant strains are aided by the inappropriate or indiscriminate use of antibiotics in human medicine and aquaculture, thereby compromising the effectiveness of treatment and posing a critical challenge in the area of public health (Colliere *et al*., 2006).

Looking at the significance of the threat *Vibrio* species poses to public health, taking proactive measures are critical. Reducing the risk requires improved water quality monitoring, good sanitation techniques, and cautious antibiotic use (Jaianie *et al*., 2013). Creating successful preventative and control plans requires a thorough understanding of the ecology, pathogenicity, and resistance patterns of Vibrio species (Lee *et al*., 2019). The aim of this study was to isolate and characterized *V*ibrio species in different water sources in Benin City, Edo State, Nigeria.

2. Methodology

2.1 Samples collection

Samples were collected from four (River, well reservoir and stagnant) water sources within Benin City, Edo State. A total of 46 water samples were randomly collected from the four different sources. Collection of samples was done using sterile 1L sample bottles and water samples were collected in triplicate. The samples collected were placed in an ice cooled box and transported to the Department of Microbiology laboratory, University of Benin, Benin City for analysis as described by the American Public Health Association (1998).

2.2 Physico-chemical tests (Water Quality Test)

The evaluation of water quality involves the assessment of various physico-chemical parameters that can provide information about its suitability for different purposes such as drinking, recreational activities or industrial use. Several equipment and processes are used to measure and analyze these parameters. Different physicochemical parameters amenable to water quality assessment, namely, pH, temperature, salinity, dissolved salts measured as electrical conductivity, total suspended solid, essential elements and their corresponding compounds (nitrates, phosphates, sulphate), dissolved oxygen, biological oxygen demand and carbon-oxygen demand (NSDWQ, 2007; WHO/UNICEF, 2021).

2.3 Samples processing and enumeration of Vibrio spp

The water samples were first enriched in alkaline peptone water which allows the *Vibrio* cells to multiply and increase their number for easy detection. After enrichment with alkaline peptone water, the different samples were incubated. 100mL of the pre-enriched broth was then filtered through a sterile membrane filter which pore size is 0.45um, which helps traps Vibrio cells. The membrane filter was transferred to Thiosulfate-citrate-bile salts sucrose agar (TCBS) and incubated again at 37ºC for 24 hours. After incubation, smooth, convex, green and yellow distinct colonies with flattened texture and opaque centre was observed which is an indication of Vibriospp was observed. Also, biochemical tests were carried out, such as done are; Catalase, Indole, Citrate, Oxidase, Motility, Urease, Glucose, Sucrose, Lactose, Mannitol, Gas formation, H_2S formation, TSI (Slant/Butt) reaction and Esculin Hydrolysis were carried out.

2.4 Antibiogram:

Antimicrobial susceptibility studies were carried out by the modifiedKirby-Bauer disk diffusion method, according to the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2020). The isolates were adjusted to 0.5 McFarland turbidity standards and applied onto Mueller-Hinton (MH) agar plates usingsterile swab sticks. Single antibiotics disks (Antibiotics disks used were: CIP - Ciprofloxacin (5mcg), TET - Tetracycline (30mcg), GEN - Gentamycin (10mcg), CRX - Cefuroxime (30mcg), VAN-Vancomycin (30mcg), CTX-Ceftriaxone (30mcg), COT- Co-Trimoxazole (10mcg), AMK-Amlkacin (30mcg), CHL- Chloramphenicol (30mcg), CRX-Cefuroxime (30mcg), MEM-Meropenem (10mcg), CPZ-Cefoperazone (30mcg). They were aseptically placed on the swabbed Mueller-Hinton agarplates at a distance of 20mm apart using sterile forceps. All susceptibility test plates were incubated at 37°C for 18–24h. The zone of inhibition was measured, recorded, and interpreted as susceptible (S) and resistant (R) using standard antibiotic breakpoints as stated by the CLSI (2020). Also, multiple antibiotics resistance index (MARI) was determined.

2.5 Pathogenicity Testing

Testing for pathogenicity was used to determine a microorganism's capacity to infect or cause disease in a host organism. It assists with comprehending the virulence mechanisms and possible dangers connected to particular diseases. Lipase and Gelatinase test were carried out as described by Bergey *et al*. (2009) and Tille and Forbes (2014).

Gelatinase production/protease activity: Gelatinase activity was demonstrated using gelatinagar. The gelatin agar plates were inoculated with the individual isolates and wereincubated at 37°C for 24 hours.After incubation, the plates were flooded with mercuric chloride solution. Development of the zone of opacity surrounding the colonies was considered positive for gelatinase production. Spirit blue agar (SBA) was measured, prepared and autoclaved. The medium was poured into sterile Petri dishes after cooling. Isolates were inoculated into labeled plates by streaking with sterile wire loop and plates were incubated at 37°C for 24hrs. Positive result is observed when the bacterium breaks down the lipids, causing the medium to turn opaque or develop a chalky-white appearance (clear zone) around the organism. Negative result shows no clear zone around the organism (Nurhafizah *et al*., 2021).

2.6 Molecular identification of isolates

Colonies isolated and biochemically identified were subject to molecular identification. Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets

were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed as described by Nurhafizah *et al*. (2021). PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds; and a final termination at 72°C for 10 mins. And chill at 4oC. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific. The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

3. **Results and Discussion**

3.1 The physico-chemical parameters of the different water samples

The result of the physico-chemical parameters of the different water samples from the four location is presented on Table 1. The temperature of the study sites ranged from 29.10 – 29.57^{0}C, which is within the WHO acceptable limits. Stagnant water has the highest electrical conductivity of 86.33µS/cm but within WHO acceptable limit. Also pH, salinity, alkalinity, BOD and COD are within acceptable range delineated by WHO for drinking water. *Vibrio* spp are of global economic, health significance and a universal public health burden, producing significant morbidity and mortality in the populations. Some *Vibrio* species aremedically important, while some of which are emerging pathogens that can cause mild to severe human diseases (Osunla and Okoh, 2017)**.**

Table 1. The physico-chemical parameters of the different water samples

	River	Well	Reservoir	Stagnant	WHO limits
Temp(°c)	29.10±0.10	29.43±0.06	29.57±0.06	29.30±0.10	25 – 30
PH	4.15±0.02	4.74±0.01	5.12±0.02	6.53±0.16	6.5 – 8.5
EC(µS/cm)	142.00±1.00	15.67±0.58	37.00±1.00	86.33±1.53	500
Salinity (ppm)	70.33±0.58	6.33±0.58	17.00±1.73	42.67±0.58	250ppm of Cl & 200ppm of Na
Turb(NTU)	0.03±0.00	0.03±0.00	0.02±0.00	0.21±0.01	5
Alkalinity	0.12±0.00	0.20±0.00	0.12±0.01	0.67±0.01	200
Phosphate (mg/L)	0.10±0.00	0.14±0.00	1.53±0.07	0.75±0.05	200
Nitrate (mg/L)	0.54±0.03	0.50±0.01	1.12±0.09	0.63±0.15	50
Sulphate (mg/L)	0.13±0.02	0.15±0.05	0.53±0.10	0.02±0.01	250
BOD	0.21±0.12	0.84±0.06	1.45±0.7	0.12±0.01	4.0
COD	0.34±0.04	0.48±0.06	0.02±0.00	0.41±0.05	80
Copper(mgCU/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.00	2
Zinc(mg/L)	0.02±0.00	0.01±0.00	0.15±0.00	0.03±0.01	3

Vibrio species infestation is mostly through oral-fecal contamination. Contaminated excrement, which occasionally is excreted straight into waterways that flow into the seas and rivers could act as the primary causes of pollution both directly from the water sources and/or indirectly (Angala *et al*., 2024). Research has shown that not all *Vibrio* species are regarded as pathogenic, but the prevalence of these *Vibrio* species in environmental water bodies is frequently linked to poor waste management practices in many populations, which contaminate surface runoff, streams, rivers, wells, ponds, and seas with human waste (Teklehaimanot *et al*., 2015). This is in agreement with this study, where *Vibrio* spp were also isolated from water bodies such as river, wells, reservoirs and stagnant water. There was no significant increase in the physicochemical parameters as compared with WHO standard. Ojesanmi and Ibe (2012) reported that the occurrence of *Vibrio* spp is not dependent on increase or decrease of any physicochemical parameters. It is also observed that the *Vibrio* spp count in this study is higher than 3.84±1.76 cfu/mL 4.42±1.61 and5.66±1.12cfu/ml from sea water (Eyisi *et al*., 2013). The variation of *Vibrio* spp could be attributed to difference in sampling location, seasonal variation and evaluation methods.

3.2 Identification of bacterial isolates, antibiogram and phenotypic virulence

Table 2 represents the total bacterial count in the water samples from the different sources. The highest total heterotrophic count of 204 ± 58.94CFU/100ml and total suspected *Vibrio* spp count of 20 ± 1.73CFU/100ml was recorded from stagnant water, followed by river water and the least count was from well water. The suspected *Vibrio* spp identified by biochemical test were *Vibrio cholera, Vibrio parahemolyticus and others but after the molecular test, Vibrio cholera, Vibrio fluvialis, Vibrio parahaemolyticus and Vibrio anguillarum* were identified. The antibiogram of the *Vibrio* spp isolated was carried out as presented on Table 3. The *Vibrio* spp were observed to be resistant to Ceftriaxone (30mcg), Cefoperazone (30mcg), Co-Trimoxazole (16mcg), Cefuroxime (30mcg) and Meropenem (10mcg). The isolates were susceptible to Ciprofloxacin (5mcg), Tetracycline (30mcg), Amikacin (30mcg) Vancomycin (30mcg) and Chloramphenicol (30mcg). It was also evident that the isolates were found to have an MAR index greater than 0.2 which means that the isolates were all pathogens of public health importance.

Table 2. Total bacterial count of water samples found in different water sources in Benin City

Sample location	Number of samples	Total heterotrophic count (Mean ±SD)	Total*Vibrio*spp count (Mean ±SD)
River	13	175 ±111.24	17 ± 2.39
Well water	9	11 ± 29.22	9 ± 1.69
Reservoir water	11	11 ± 115.33	9 ± 2.10
Stagnant water	13	204 ± 58.94	20 ± 1.73

*Vibrio*s pp are largely considered to be very susceptible to most antimicrobials recommended for medical treatment (Letchumanan *et al*., 2015), but resistance among environmental isolates *are* increasingly recorded. In this study, multiple antibiotics resistance was observed in the recovered *Vibrio* spp against some endorsed and frequently used antibiotics which correlated with previous reports (Adesiyan *et al*., 2022). Resistance against the β-lactams drugs (Meropenenem and amikacin) recorded in this study is similar to that documented in environmental *Vibrio* isolates (Sony *et al*., 2021).

The observed resistance of *Vibrio* spp to tested β-lactams in this study maybe related with the location, as bacteria such as *Vibrio*, recovered from water environment have been documented to show high resistance towards β-lactams class of antibiotics (Elmahd *et al.*,2016). The recorded resistance to carbapenem in this present study raises public health concerns as this drug is one of the major drugs for medical treatment of multiple drug-resistance *Vibrio* infections. Moreso, carbapenemases responsible for resistance to carbapenems has been associated with mobile genetic elements which can perhaps be transferred to humans through food interface (Meletis, 2016). The level of *Vibrio* isolate resistance to many of the antibiotics examined in this study suggests a probable misuse or overuse of antimicrobials around the study area for a variety of purposes beyond what the environment can absorb, thereby resulting in the maintenance of unabsorbable residue in the water bodies. Such practices have been suggested to impact the likelihood of bacteria to develop resistance to commonly used antibiotics (Titilawo *et al.*, 2015). The phenotypic virulence properties of the *Vibrio* spp isolates showed that they had virulence determinants evaluated in the study (Table 4).

Table 3. The Antibiotic sensitivity of *Vibrio* spp isolates from different water sources in Benin City.

Isolates	CTX	CIP	CPZ	TET	AMK	COT	GEN	VAN	CRX	CHL	MEM	MARI
Vibrio cholera	R	S	R	S	R	R	R	S	R	S	R	0.55
Vibrio parahemolyticus	R	S	R	S	R	R	S	S	R	S	R	0.45
Vibrio species	R	S	R	S	R	R	S	S	R	S	R	0.45

Keys: R=Resistant, S=Susceptible, MARI=Multiple Antibiotic Resistance Index, GEN- Gentamycin (10mcg), CIP-Ciprofloxacin (5mcg) TET-Tetracycline (30mcg), VAN-Vancomycin(30mcg) , CTX-Ceftriaxone (30mcg) , COT- Co-Trimoxazole , AMK-Amikacin (30mcg) , CHL- Chloramphenicol (10mcg), CRX-Cefuroxime (30mcg), MEM-Meropenem (10mcg), CPZ-Cefoperazone (30mcg).

Table 4. The phenotypical virulence properties of *Vibrio* spp from different water sources in Benin City

Isolates	Lipase	Gelatinase
Vibrio cholera	+	+
Vibrio parahemolyticus	+	+
Vibrio species	-	+

Key: + = Positive; - = Negative

In this study, we were able to look at virulence levels of the isolates (Gelatinase and lipase), which are indicators for bacterial virulence and pathogenesis and it was observed that all the *Vibrio* spp exhibit virulence markers. Pathogenic bacteria excrete extracellular products (ECPs) for successful host colonisation and absorption of nutrients from the host. These virulence factors affect the host tissues by causing total damage that ultimately leads to malfunction of the tissues. The positive findings of virulence factors in this study are in agreement with other work who reported excretion of ECPs in *Vibrio* spp. (Costa *et al.*, 2013; Kumaran and Citarasu, 2016; Teng *et al.*, 2017; Nurhafizah *et al.*, 2021).

3.3 Molecular identification of *Vibrio* spp from the different water samples

Figure 1 showed the 16S DNA sequence analysis on Gel electrophoresis. The isolates identified were *Vibrio cholera, Vibrio fluvialis, Vibrio parahaemolyticus* and *Vibrio anguillarum* at molecular weight of 1500bp. In this study *Vibrio* spp were identified by TCBS culturing biochemical test and molecular method; using of *Vibrio* housekeeping *16S rDNA* gene primers. It is reported that molecular detection approaches had enhance the incidence of finding harmful microbes while conventional culture-based detection methods might not be highly efficient in detection of *Vibrio* spp (Azwai *et al.*, 2016; Hasan *et al.*, 2017; Al-Saady *et al.*, 2020; Church *et al.*, 2020). *Vibrio cholera*, *Vibrio fluvialis*, *Vibrio parahaemolyticus* and *Vibrio anguillarum* were identified in this study, which is in line with the work of Eyisi *et al.* (2013) and El-Zamkan *et al.* (2023).

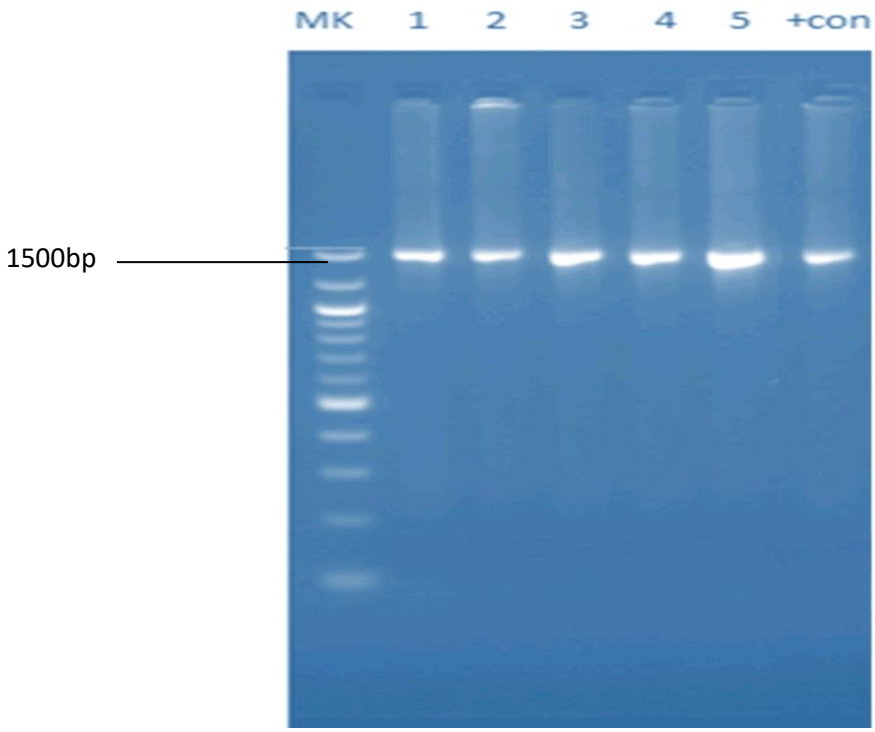

Figure 1. the 16S rDNA sequence analysis on Gel electrophoresis. The samples were identified as; 1 and 2-*Vibrio cholerae*, 3- *Vibrio fluvialis*, 4- *Vibrio parahaemolyticus*, 5- *Vibrio anguillarum*, +con- positive control (*Vibrio cholerae* typed strain) and Mk- molecular ladder.

Conclusion

The present study shows the prevalence of *Vibrio* species from different water bodies in Benin City. Health campaigns should be carried out to enlighten the populace about the risks involved in the prevalence of this infection in order to reduce its burden and the need for concerted environmental surveillance.

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