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Enumeration and Phenotypic traits of Microorganisms from Environmental Water Sources in Benin City, Edo State.

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Abstract
Recognizing the pivotal role of water in sustaining life and health, it becomes imperative to investigate and monitor the microbial content of various water sources. Water quality is a critical determinant of public health, and the presence of heterotrophic bacteria in water sources poses potential threats to human well-being. The study was carried out to enumerate bacteria from environmental water sources in Benin City, Edo State, Nigeria. Physicochemical assessment of the water samples collected aseptically was carried out. Also, bacteria isolation and identification, antimicrobial susceptibility tests, phenotypic virulence determinants were carried out using standard microbiological technique. The results revealed that the pH ranged from 4.15 -6.53, while temperature (29.10 – 29.57 ^{o}C), electrical conductivity (15.67 – 142 µS/cm), turbidity (0.02 – 0.21 NTU), Alkalinity (0.12 – 0.67), Phosphate (0.10 – 1.53 mg/L), Nitrate (0.50 – 1.12 mg/L), and Sulphate (0.02 – 0.53 mg/L) were all within acceptable range delineated by WHO for drinking water. Bacteria species isolated from the different water sources include; Bacillus spp, Vibrio spp, Klebsiella spp, Salmonella spp and E. coli. Bacillus spp had the highest bacterial count of 70% CFU/100ml from stagnant water sample. While Vibrio spp and Salmonella spp had the lowest count of 38% CFU/100ml from well and reservoir respectively. The antibacterial susceptibility testing revealed that all the isolates were resistant to Ceftriaxone (30mcg), Cefuroxime (30mcg) and Meropenem (10mcg). The isolates were found to have an MAR index greater than 0.2. The phenotypic virulence properties of all the isolates showed that they had virulence determinants. The presence of pathogenic bacteria in the water samples calls for the need for quality assessment of drinking water sources and ensuring compliance with relevant standard to avoid risk to human health.

Keywords; *Vibrio* spp, Physicochemical, Virulence Properties

INTRODUCTION
Portable water remains an essential ingredient for the maintenance of good health. The human body is made up of about 67% of water. The human body requires up to seven litres of water per day to avoid dehydration and ensure its physiological functions. This makes water indispensable to man. Water is never free of contamination; rather, it inevitably contains traces of other substances, ranging from organic compounds, microorganisms, particles, gasses, to minerals and ions which affect its bacteriological, chemical and even physical characteristics

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(Jidauna *et al*., 2014). The quality of water depends on its physical, chemical and biological characteristics, which determine its utility for different purposes (Giri and Qiu, 2016).

The quality of water is continuously changing due to majorly human activities. Water is said to be fit for human consumption is referred to as potable or drinking water. It is required that the water does not pose any significant health risk over lifetime consumption (Water and WHO, 2002; Yusuf *et al*., 2015).

However, as populations increase, the problem becomes more serious and as such, water can endanger the health and life of human beings because when polluted by faecal materials it becomes a potential carrier of pathogenic organisms (Micheal *et al*., 2021). Also, it has been increasingly noted that poor microbial quality water can be harmful to aquatic organisms and ecosystem function (Craun *et al*., 2006; Betiku *et al*., 2021). Water quality is a critical determinant of public health, and the presence of heterotrophic bacteria in water sources poses potential threats to human well-being. Benin City, like many urban areas, faces challenges in maintaining water purity, leading to concerns about the prevalence of waterborne diseases. Recognizing the pivotal role of water in sustaining life and health, it becomes imperative to investigate and monitor the microbial content of various water sources. (Adeleye *et al*., 2001).

Benin City, like many urban areas, has been the subject of significant attention in previous studies addressing waterborne diseases and their impact on public health (Okafor *et al*., 2019). Access to clean and safe water is a fundamental requirement for sustaining life and ensuring public health. Waterborne diseases, often associated with microbial contamination, continue to pose significant challenges globally, particularly in urban areas like Benin City (Onanuga and Okafor, 2022). Heterotrophic bacteria, a diverse group of microorganisms reliant on organic compounds for growth, serve as valuable indicators of water quality and play a crucial role in understanding the microbial landscape of water sources.

The presence of heterotrophic bacteria in these sources raises concerns about potential microbial contamination, necessitating comprehensive investigations into their diversity and abundance. Heterotrophic bacteria, while not inherently pathogenic, can create conducive environments for the proliferation of other potentially harmful microorganisms (Onanuga and Okafor, 2022). Therefore, identifying potential sources of contamination of water bodies is essential in assessing the overall microbial quality. The purpose of this study was to identify, evaluate the antibiogram and phenotypic traits of bacteria isolated from environmental water sources in Benin City, Edo State. Nigeria.

MATERIALS AND METHODS

Samples collection

Samples were collected from four water sources (River, well, reservoir and stagnant water) 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).

Physico-chemical tests (Water Quality Test)

The evaluation of water quality involves the assessment of various physic-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).

Samples processing and enumeration of microorganisms

Water samples were analyzed immediately after collection, for the presence of bacteria and total heterotrophic count was carried out using membrane filtration method (USEPA, 2009). Aliquots of100ml from each sample were filtered using 0.45 µm paper filters, for *Vibrio* spp, the water samples were first enriched in alkaline peptone water. The filters were placed on Nutrient agar, MacConkey agar, Shigella Salmonella agar Thiosulfate-citrate-bile salts sucrose agar (TCBS) and Eosin methylene blue agar plates and were incubated aerobically at 37 °C for 24 hrs. after incubation, the isolates were subjected to both physical observation, preliminary Gram staining and confirmatory biochemical identification tests such as; indole, oxidase, citrate utilization, Triple Sugar Iron tests and Esculin Hydrolysis were carried out (Prescott, 2001).

Antibiogram:

Antimicrobial susceptibility studies were carried out by the modified Kirby-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 using sterile swab sticks. Single antibiotics disks (Antibiotics disks used were: 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). They were aseptically placed on the swabbed Mueller-Hinton agar plates 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 calculated.

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 gelatin agar. The gelatin agar plates were inoculated with the individual isolates and were incubated 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 bacterium. No any clear zone around the bacteria is taken as negative result.

RESULTS

The results of physiochemical properties of the different water samples from different sampling sources in Benin City was evaluated as presented on Table 1. The pH of river water (4.15±0.02), well (4.74±0.01) and reservoir (5.12±0.02) were below the WHO limits. While temperature (29.10 – 29.57 ^{o}C), electrical conductivity (15.67 – 142 µS/cm), turbidity (0.02 – 0.21 NTU), Alkalinity (0.12 – 0.67), Phosphate (0.10 – 1.53 mg/L), Nitrate (0.50 – 1.12 mg/L), and Sulphate (0.02 – 0.53 mg/L) were within acceptable range delineated by WHO for drinking water. Figure 1 shows the Percentage distribution of the bacterial isolates from the different water samples. Bacteria isolated from the different water sources includes; *Bacillus* spp, *Vibrio* spp, *Klebsiella* spp, *Salmonella* spp and *E. coli*. *Bacillus* spp had the highest bacterial count of 70% CFU/100ml from stagnant water sample. While *Vibrio* spp and *Salmonella* spp had the lowest count of 38% CFU/100ml from well and reservoir respectively.

Table 2 represent the antibiotic sensitivity of bacterial isolates from different water sources in Benin City. It was observed that all the isolates were resistant to Ceftriaxone (30mcg), Cefuroxime (30mcg) and Meropenem (10mcg). 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. The phenotypic virulence properties of the isolates (*Vibrio* spp, *Klebsiella* spp, *Salmonella* spp and *E. coli*) showed that they had lipase and Gelatinase activity (Table 3).

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 and 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	0.10±0.00	0.14±0.00	1.53±0.07	0.75±0.05	200
Nitrate	0.54±0.03	0.50±0.01	1.12±0.09	0.63±0.15	50
Sulphate	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

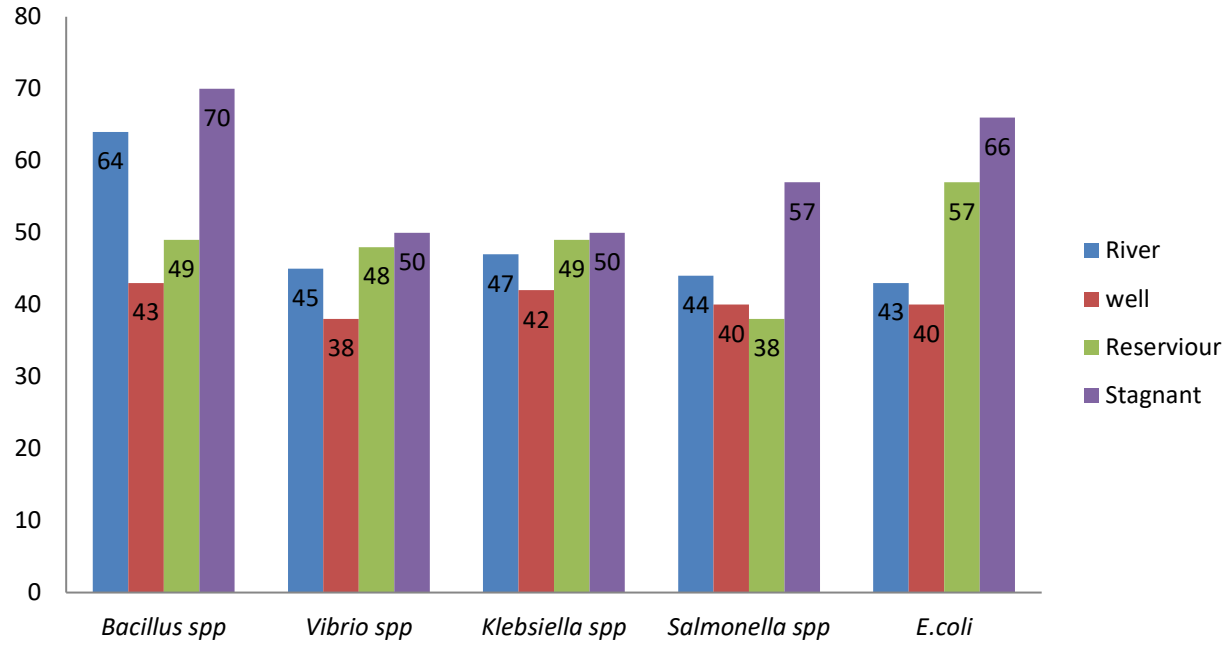

Figure 1: Percentage distribution of the bacterial isolates from the different water samples

Table 2: The Antibiotic sensitivity of bacterial isolates from different water sources in Benin City.

Isolates	CTX	CIP	CPZ	TET	AMK	COT	GEN	VAN	CRX	CHL	MEM	MARI
Bacillus spp	R	S	S	R	S	R	S	S	R	S	R	0.34
Vibrio spp	R	S	R	S	S	R	S	S	R	S	R	0.45
Klebsiella spp.	R	S	R	S	R	S	S	R	R	R	R	0.64
Salmonella spp.	R	S	S	R	S	R	S	R	R	S	R	0.55
E. coli	R	S	R	S	R	S	S	R	R	R	R	0.64

Key: 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 3: Phenotypic virulence properties of bacterial isolates from different water sources

Isolates	Lipase	Gelatinase
Bacillus subtilis	-	+
Vibrio spp	+	+
Klebsiella spp.	+	+
Salmonella spp.	+	+
Serratia spp	+	+

Key: + = Positive, - = Negative

DISCUSSION

The WHO Guidelines for Drinking-water Quality (GDWQ) is based on the absence of detectable levels of certain pathogens in drinking water. For example, the GDWQ typically recommend that there should be no detectable presence of *Escherichia coli (E. coli)* or thermo-tolerant coliform bacteria in drinking water samples. According to WHO, water should have a PH of 6.5-8.5, temperature of 25 °C -30 °C, TDS of 500, turbidity of 5, alkalinity of 200, phosphate of 200, nitrate of 50, phosphate of 200, BOD of 4, COD of 80 . The pH of water is an indicator parameter used to ascertain the state of wholesomeness of any water. The concentration values of pH obtained from the study ranged from pH 4.15±0.02 to 6.53±0.16 which were below WHO and USEPA permissible limits. This finding is not in agreement with the work of Asionye *et al*. (2023) who reported pH of 5.62 to 7.81 from portable water in Delta State. Also, not in agreement with the report of Igwe *et al*. (2021) whose investigation obtained a range of values between 6.8 to 6.9 while the control samples had a range from 6.0 to 7.1. As reported by Agbalagba *et al*. (2011)when the concentration values of pH of potable water is low then it could be categorized as being acidic which suggest that it may lower metabolic activities or impair fecundity rate of living things. It may also induce a number of biochemical reactions including corrosion of pipes, clogging of pipes, and poor taste of water. Osayande *et al*. (2015) observed the acidity of potable water and its potential to increase the risk of gastroenteritis especially *Helicobacter pylori*. Although USEPA (2019) recommends that the pH of status of potable water may not have any direct detrimental effect on humans.

Access to safe-drinking water is of greatest importance in safe guarding the health of any community. Total Heterotrophic Plate Counts (HPC) has been used for a long time to access the quality of water supplies. The microbial quality of river, well water and reservoir water sources in Benin City during the course of this research revealed that the water sources were not within the permissible limits of WHO guidelines (<1cfu/100ml). The bacterial isolates identified include *Bacillus* spp, *Vibrio* spp, *Klebsiella* spp, *Salmonella* spp and *E. coli*. This is in line with the work of Adesakin *et al.* 2020, who reported the isolation of Salmonella, Shigella and E. coli from domestic water sources from Zaria City. Also, in agreement with Miah *et al.* (2022) who isolated *E. coli* from different water sources including well water. A study by Eboh *et al*. (2017) reported the following organisms *Escherichia* sp., *Enterobacter* sp., *Alcaligenes* sp, *Klebsiella* sp., *Staphylococcus* sp, *Bacillus* sp., *Proteus* sp., *Micrococcus* sp., *Serratia* sp., *Acinetobacter* sp., *Alcaligenes* sp. and *Pseudomonas* sp. from ground water at Ukwuani LGA in Delta State. *Escherichia coli* is an indicator of fecal pollution. *Shigella* and *Salmonella* are enteric organisms responsible for Shigellosis and Salmonellosis which may cause life threatening diseases. *Vibrio* species could lead to severe gastrointestinal conditions.

Due to the possible health dangers, certain pathogenic bacteria including *Salmonella*, *Serratia*, and *Klebsiella* are generally not allowed in drinking water. If consumed, these pathogens can result in a number of illnesses and infections. Due to the well's location, which exposed it to ongoing pollution from human and animal waste, the heterotrophic bacterial counts seen in the water are not implausible. A well needs to be built correctly and placed a fair distance away from bathrooms, dumping sites, and pit latrines in order to be safe from pollution. Nevertheless, it was found that the well under investigation had been exposed to a variety of environmental contaminants. The well's improper construction, shallow depth, and lack of a well cover allowed for the pollution. The findings of this study support those of a few other studies that have been conducted both in Nigeria and elsewhere. Stanley, (2015) reported the presence of *Escherichia coli*, which is also a pathogenic heterotrophic bacteria from well water in Afikpo. He used the membrane filtration method for the enumeration and isolation of *Escherichia coli*. Also Enabulele *et al*. (2022) isolated *Salmonella* spp., *Klebsiella* spp., *Serratia*

marcescens, and *Bacillus subtilis* from the Ikpoba river. The observed discrepancies in mean microbial counts within this study may be attributed to variations in the natural geographical structures and human activities found in and around these water bodies.

Ezienyi *et al*. (2022) have also detected bacterial resistance to antibiotics from the New Calabar river. They used the standard spread plate for bacterial isolation and they isolated various pathogenic heterotrophic bacteria from the New Calabar river. all the identified isolates were 100% susceptible to ofloxacin and levofloxacin. *Salmonella* isolates in Ezienyi *et al*. (2022) research was resistant to augmentin and ampiclox. In contrast to this research, the *Salmonella* isolate was resistant to cefotaxime,co-trimoxazole, tetracycline, ceftriaxone, meropenem, vancomycin, and cefuroxime.

The antibiogram carried out using the disc diffusion technique in Awe and Ohikere (2014) research showed that *Bacillus* sp. was most sensitive to Streptomycin and Rocephin and least to Ampiclox. *Salmonella* sp. and *Klebsiella* sp. were highly sensitive to all antibiotics. In this research, *Bacillus subtilis* was sensitive to tetracycline, co-trimoxazole, and vancomycin and was resistant to the remaining antibiotics use. In this research, *Salmonella* and *Klebsiella* was sensitive to some antiobiotics. *Salmonella* spp. was sensitive to these antibiotics: ciprofloxacin, cefoperazone, amikacin, gentamicin, and chloramphenicol. *Klebsiella* spp. was sensitive to these antibiotics: ciprofloxacin, tetracycline, co-trimoxazole, and gentamicin. They also exhibited resistance to the following antibiotics; for *Salmonella* spp. (cefotaxime, tetracycline, co-trimoxazole, cefuroxime, meropenem, and ceftriaxone) while for *Klebsiella* spp. (cefotaxime, cefoperazone, amikacin, vancomycin, cefuroxime, chloramphenicol, meropenem, and ceftriaxone). In the study by Awe and Ohikere (2014), it can seen that *Salmonella* and *Klebsiella* showed no resistant or multiple drug resistance to any of the antiobiotics used but in this research all the isolates had multidrug resistant to some of the antibiotics used.

The detection of typical enteric pathogens such as *Vibrio* spp, *Klebsiella* spp, *Salmonella* spp *E. coli* and *Bacillus* spp which possessed virulence attributes is of utmost concern. This might pose a major health risk to consumers. Most of the isolates were resistant to not just the commonly used antibiotics such as Chloramphenicol but were also resistant to the relatively new β – Lactam (meropenem).

In this research, most of the bacterial isolates possessed pathogenic potentials as indicated by their ability to produce virulence factors such as lipase and Gelatinase. Through waste discharges and runoffs, the use of antibiotics and other chemotherapeutic medicines in veterinary medicine and animal husbandry may contaminate natural surface water sources. This study emphasizes the danger of antibiotic-resistant pathogenic heterotrophs spreading. Promoting point-of-use disinfection, like boiling, for water meant for drinking will reduce the health hazards to users.

CONCLUSION

A variety of pathogenic bacteria such as *Vibrio* spp, *Klebsiella* spp, *Salmonella* spp *E. coli* and *Bacillus* spp were found in all of the water samples that were examined. This suggest that these various water sources are exposed to microbial contamination from both natural and anthropogenic sources. These identified bacterial isolates were also found to exhibit multiple drug resistance and this may be a threat to the people living within these communities. Given that the human communities living in the water sources' catchment area completely rely on

them as a source of water for domestic and drinking needs, relevant governmental and non-governmental organisations must act quickly to raise awareness among these communities about the need of treating and disinfecting water that has been extracted straight from the water body before drinking.

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