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DISSEMINATION OF DIARRHEAGENIC BACTERIA IN COMMUNITY-BASED NATURAL WATER SOURCES AND WASTEWATER ENVIRONMENTS: A COMPREHENSIVE ANALYSIS IN UNIVERSITY OF NIGERIA, NSUKKA AND ITS METROPOLIS

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ABSTRACT

The presence of pathogenic strains abounds in our environment, arising from many sources. The study was aimed at identifying *Vibrio* species, *E. coli*, and other coliforms in various water sources within a university setting and its metropolis. Approximately 20 samples from roof-collected rainwater, municipal piped tap water, borehole water and 60 samples of sewage effluent from the central effluent water of University of Nigeria, Nsukka were systematically collected at intervals and subjected to bacteriological analysis. The samples underwent dilutions, and aliquots were inoculated on chromocult and thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Simultaneously, 100 ml of each natural water sample underwent membrane filtration, followed by the inoculation of TCBS agar plates in duplicates. Both sets of culture plates were incubated at 37 °C for 24 h. Further biochemical characterization was performed after microscopy. Susceptibility profile was done using disk diffusion assay. Descriptive statistics were used in the aggregation of the results where necessary. Results revealed that each sample, when plated on chromocult agar, yielded a substantial number of colonies of *E. coli* (bluish colour) and other coliforms (pink-red colonies). Notably, wastewater gave a maximum yield of *Vibrio cholera*. Conversely, all significant pathogenic *Vibrio* species were recovered on TCBS agar inoculated with natural water samples, wastewater and sewage effluent, with isolation rates appearing independent of both time (in weeks) and collection points. Antimicrobial susceptibility studies disclosed that some of the isolates exhibited antibiotic resistance. The study underscores the need to install appropriate measures to mitigate the contamination of the environment.

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INTRODUCTION

The integration of wastewater into the hydrological cycle poses a critical concern, given its potential to introduce pathogenic bacteria into natural water reservoirs. Wastewater refers to raw sewage derived from anthropogenic activities [1], whereas sewage effluent is described as treated or untreated wastewater generated from a treatment plant [2]. In contemporary urban environments, the issue of sewage contamination of natural water reservoirs gains particular significance due to the interconnection between wastewater and community water sources. Within the confines of university settings, where diverse water outlets coexist, understanding the extent and nature of this contamination becomes paramount for safeguarding public health. The establishment of the sewage treatment facility is one of the large-scale epidemiological action plans that minimizes the risk of water-borne disease outbreaks in a human population [3].

Coliform bacteria occupy a distinct niche in the transmission of diseases through wastewater contamination [4]. They are the class of facultative anaerobic, Gram-negative rods, that are non-spore-forming and are very rapid lactose fermenters. This group of bacteria are members of the *Enterobacteriaceae* genus: *Enterobacter cloacae*, *Enterobacter aerogenes*, *E. coli*, and *Klebsiella pneumoniae*. Some coliform bacteria are associated with the intestines (colon) of warm-blooded animals (called faecal coliforms), while others are related to plant material. Consequently, these faecal coliforms are used as indicator organisms for faecal contamination of water, food, and other related products. In particular, *E. coli*, given its ubiquity in the intestinal microbiota, was adopted as the general indicator organism [5, 6]. Although some strains of *E. coli*, are considered non-pathogenic, as resident flora, their pathogenic switch is very high due to the dynamic nature of the intestinal microbiome.

Microbial acquisition of resistance/virulence can be attributed to drug abuse among human and animal populations and poses a high risk of circulation of resistance strains in sewage water contaminated by the faecal coliforms, following their shedding in the stools [7, 8].

The sewage effluent arguably, can serve as a point and nonpoint source of pollution, propagation and dissemination of human-associated bacteria, thereby leading to public health threats [9]. The effluents, known to contain various microorganisms, pose a potential health risk to the community [10].

In Nigeria, most of the cities lack municipal sewage disposal and treatment plants, whereby this trend has resulted in a chronic scourge of infection outbreaks with high mortality rates [11]. However, the University of Nigeria, Nsukka, is one of the few population communities with a sewage treatment plant. Limited epidemiological data exists on waterborne disease epidemics, their causes, and exposure routes. The contribution of these diseases to the overall illness burden is uncertain. The research conducted by various authors in Nigeria, particularly in Enugu state and the University of Nigeria, reveals a concerning pattern of water contamination and microbial presence. Ohanu et al. [12] isolated Coliform, *Clostridium* sp, and *Penicillium* sp from tap water in Enugu State, with an average recovery rate of 66 % *Clostridium* sp from tap water across three different locations. Ughamba et al. [13] sampled boreholes and pipe-borne water in the Nsukka metropolis, identifying various bacteria, including *Escherichia coli*, *Citrobacter* spp., *Bacillus cereus*, *Micrococcus varians, Proteus mirabilis*, and *Salmonella* spp. Okoro et al. [14] conducted a comparative analysis of three borehole water sources in the Nsukka urban area, detecting *E. coli* in one of the borehole water sources. Emurotu et al. [15 detected *Enterococcus faecalis* in wastewater facilities at the University of Nigeria, while Chigor et al. [16] reported the presence of enterotoxigenic and multidrug-resistant *Escherichia coli* in treated wastewater used for fresh produce irrigation in Nsukka, Southeast Nigeria.

Despite the extensive research conducted by these authors, none isolated *Vibrio* species from the effluent wastewater at the University of Nigeria. Also, there is no report on the isolation of *Vibrio* species from tap, rain, and borehole water. Thus, this study aims to examine the distribution of *Vibrio* species, *E. coli*, and coliforms within the central sewage effluent of the University of Nigeria, Nsukka as well as in tap, rain, and borehole water sources.

MATERIALS AND METHODS

Materials

Equipment/laboratory tools used include Petri dish, sterile propylene 100ml bottles spatula, measuring cylinder, beaker, marker, conical flask, bijou bottles, cotton wool, sterile swap stick, aluminium foil, sterile hand gloves, autoclave, masking tapes, wire loop, gas cylinder, Bunsen burner, test tube rack, incubator, glass rod, spreader, microliter pipette, microscope. The reagents include acetone-ethanol, distilled water, oxidase reagent, Gentian violet, Lugol's Iodine, Safranin solution were the reagents used. The culture media include Thiosulfate-citrate-bile salts-sucrose (TCBS) agar, chromocult agar, Nutrient Agar and Mueller-Hinton agar were used.

Study Area

This study was undertaken at the University of Nigeria, Nsukka Community, latitude 6°51'52.038" and longitude 7°24'29.8368", in Enugu state Nigeria, when the school was in session and during the rainy season. The community is facilitated with a sewage treatment plant, which serves the entire student hostels and staff quarters. The influx of solid and effluent wastes into the plant increases when the school is in session, due to larger anthropogenic activities, compared to vacation months.

Sample Collection

Raw sewage (S) and effluent (E) samples were collected from the central sewage treatment plant of the University of Nigeria, Nsukka (UNN), in a clean sterile 100 ml bottle, each labelled with their distinctive codes. The samples were immediately transferred to the laboratory for analysis.

For the natural water, samples were collected from roof-caught rainwater, municipal piped tap water, and borehole water (**Figure 1**). All the samples were collected in sterile bottles, mid-stream from the collection points and at weekly intervals and transported to the laboratory for bacteriological analysis.

Sample Size and Sampling Technique

During the entire period, a total of 80 samples were obtained, including 60 wastewater samples collected from the central sewage plant and 20 samples collected from roof-caught rainwater, municipal piped tap water and borehole water. The samples were sealed, labelled, and transported in an icebox maintained (at 4 ^{o}C) to the Pharmaceutical Microbiology and Biotechnology Laboratory, University of Nigeria, Nsukka. The sampling protocol was scrupulously done following the standards of the American Public Health Association (APHA).

Isolation of *Vibrio* spp., *Escherichia coli* and other Faecal Coliforms

The sewage samples were serially diluted up to 10^{-4}, in normal saline solution (0.85 % NaCl g/ml). About 100 µl of the dilutions were inoculated on chromocult agar (28 g/L; Sigma Aldrich, Merck, Germany), and thiosulphate-citrate-bile salts-sucrose [TCBS (88 g/L] agar media plates. Membrane filtration of the different water sources was done by passing 100 ml, using a suction pump. The membrane filters were aseptically placed on TCBS agar (88 g/L) plates in duplicates. After inoculation, the samples were incubated at 37 ^{o}C for 24 h.

Identification of the Isolates

The resulting colonies were differentiated based on their characteristic colours on the chromocult and TCBS agar media. The isolates were sub-cultured on the same media to obtain pure and discrete colonies. Microscopy and other definitive biochemical tests including oxidase test on the presumptive isolates were done according to established technique [17].

Figure 1: Water collection points and processing. **A**: collection of pipe-borne water, **B**: collection of rainwater, **C**: water sample processing

RESULTS AND DISCUSSION

Sewage water is a known reservoir of microorganisms and other particles of varied origins. It is a matrix of organic and inorganic matter [18]. In sewage water, coliforms are the major group of commonly isolated bacteria. Furthermore, *E. coli* is the predominant bacterial species in the wastewater microenvironment. Other species of coliform bacteria and non-

coliform bacteria occur in significant proportion in the same environment, whereby *Shigella*, *Klebsiella*, and *Proteus* are usually found in close association with *E. coli* [19].

Isolation and Identification of Coliforms and *Vibrio* spp. from Wastewater and other Natural Water Sources

From the current study, the target organisms are prevalent. Comparatively, there is a higher prevalence of other coliform bacteria than *E. coli*. Our study utilized the efficiency of chromocult agar, to identify *E. coli* and other coliform species, while differentiating the non-coliform group. *Escherichia coli* and other coliforms were isolated and identified by their blue and pink colours respectively on the chromocult media as shown in **Figure 2**. The characteristic appearance of *Vibrio* spp. isolates, on the TCBS agar plates were as shown **in Figure 3**. Each of the isolates was characterized as Gram-negative, vibrio-shaped rods. From the result, the accuracy and precision of the chromogenic media were optimal, as there was no noticeable overlap among the colony appearances on the plate. This was corroborated by other studies using similar isolation media [20, 21]. As indicated by the microscopy, and oxidase test, the chromocult media is specific for selection and differentiation of coliforms (Gram-negative rod bacteria) from environmental samples [22].

Distribution of the *E. coli* and other coliforms, from different times/points in the sewage treatment plant, and the period of collection, are presented in **Figure 4**. The point distribution of *Vibrio* species is also presented in **Table 1**, with the total occurrence of *Vibrio* spp. (**Figure 5**) belonging to *Vibrio alginolyticus* (11 %), *Vibrio cholerae* (10 %), *Vibrio parahaemolyticus* (37 %), *Vibrio vulnificus* (10 %), and *Vibrio fluvialis* (32 %).. No difference in the burden or profile of the coliforms was observed between the time/points of collection. This suggests that both the raw sewage and the effluents have similar profiles of microbial quality. Although some studies have reported variation in the quality and quantity of microbial loads between the sewage influent and effluent, other contrasting observations have been made, consistent with the present observation [22]. Given the homogeneity of the population releasing the wastewater, especially in the regimented school activities and eating habits, discharge of similar pathogens in the wastewater could be anticipated.

Similarly, the weeks of sample collection coincided with the week of resumption for a new academic session (Mid-August – third week of September 2023). Consequently, the sewage treatment plant experienced a simultaneous surge in the volume of the raw sewage in-flow, and effluent flow. This could further account for the minimal variation in the coliform profile between the two collection points.

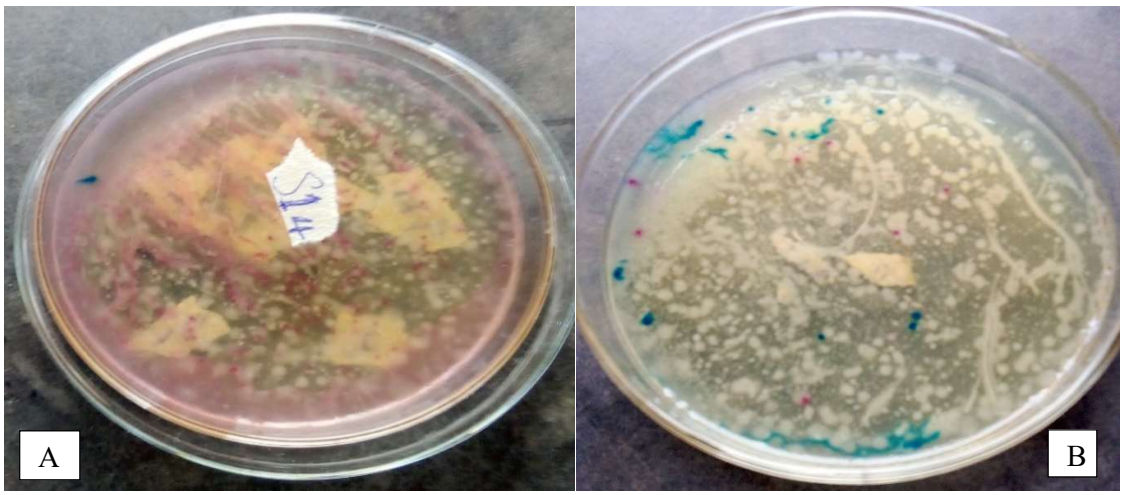

Figure 2: The characteristic appearance and colour of the coliforms on chromocult agar.
Plate A *shows the pink colour of non-E. coli coliforms, while* ***Plate B*** *shows the blue colour of E. coli and the colourless colonies of non-coliforms.*

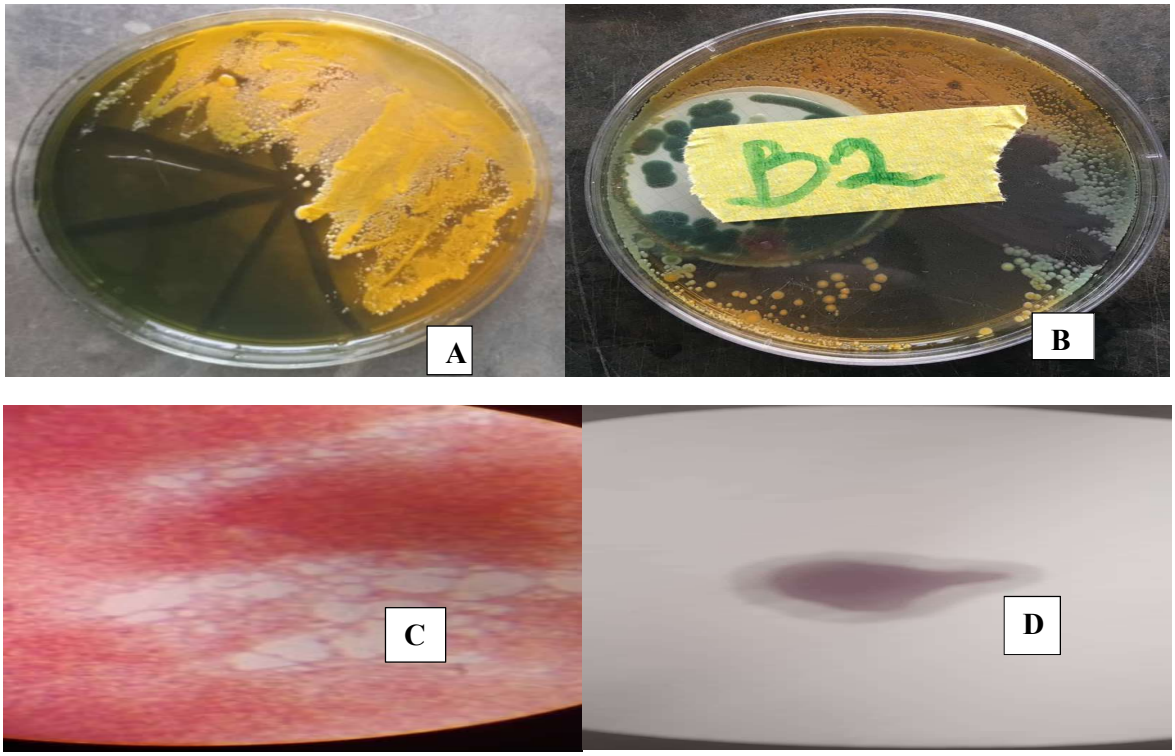

Figure 3. The characteristic appearance of *Vibrio* spp. isolates, on the TCBS agar plates
A: *Flat yellow colonies of Vibrio cholerae on TCBS agar media,* ***B****: Translucent colonies of Vibrio fluvialis, and colourless colonies with green centers, for V. parahaemolyticus,* ***C****: Gram-negative curved rods under x100 of the microscope,* ***D****: Oxidase positive test for the Vibrio spp*

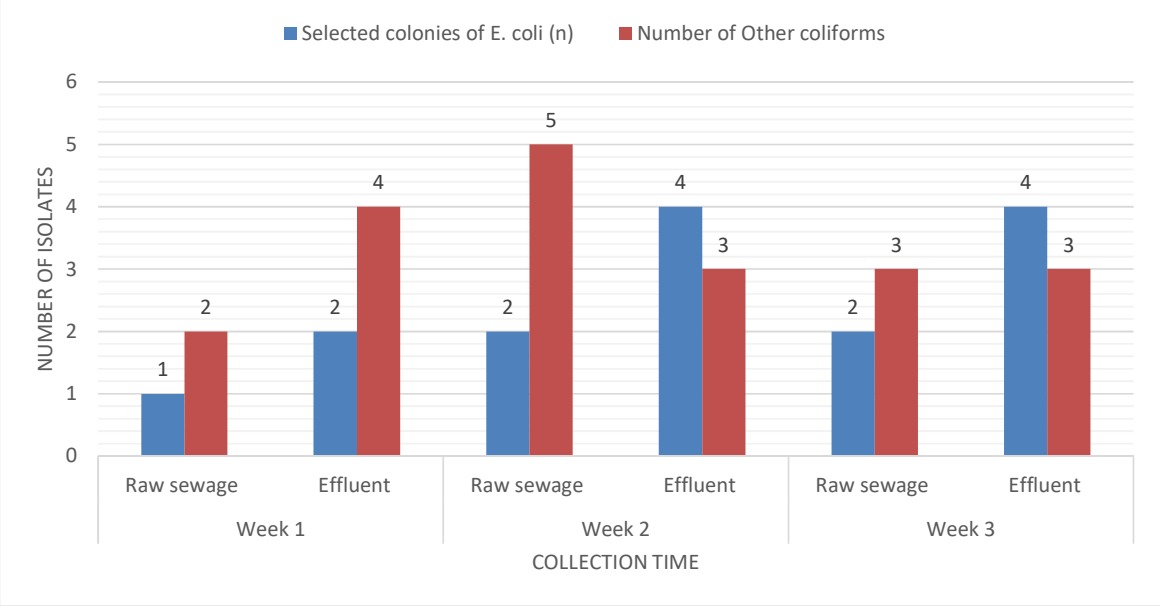

Figure 4: Distribution of the coliform isolates according to collection time

Table 1: Water source distribution of *Vibrio* species

Water Source	*Vibrio* species						
	V. cholerae	*V. vulnificus*	*V. alginolyticus*	*V. parahaemolyticus*	*V. fluvialis*	Non-*Vibrio* species	Total
B1		+			+		2
B2	+		+	+	+		4
B3		+	+		+		3
T1				+	+	+	3
T2				+			1
T3				+			1
R1				+		+	2
R2	+			+	+		3
R3				+	+	+	3
Total	2	2	2	7	6	3	22

Key: *(+) = presence of Vibrio; B1, B2, B3 = Borehole water, T1, T2, T3 = Tap water, R1, R2, R3 = Rain water*

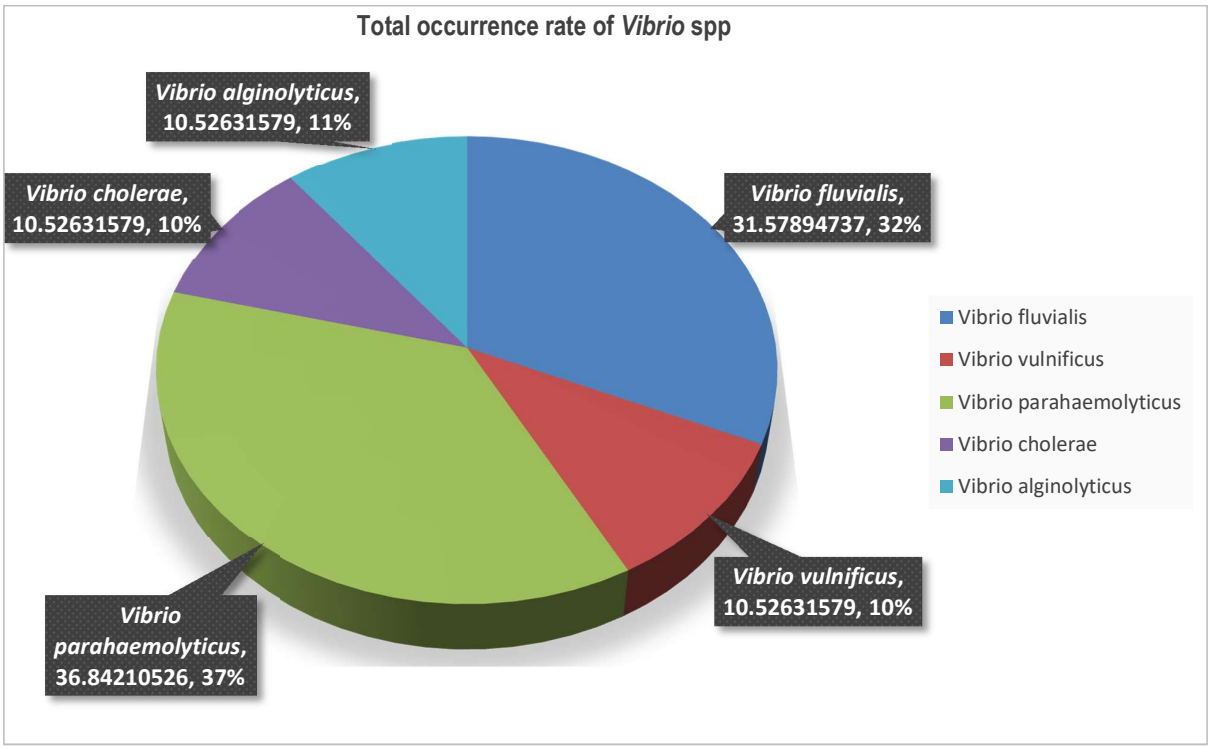

Figure 5: Total occurrence rate of *Vibrio* spp.

Table 2: Antibiotic susceptibility profile of coliform bacteria

Antibiotics	Coliform susceptibility profile (%)		
	S	I	R
LEV	100	0	0
CEF	50	0	50
SP	100	0	0
CPX	100	0	0
AM	12.5	12.5	75
AU	0	12.5	87.5
CN	75	12.5	12.5
PEF	0	12.5	87.5
OFX	100	0	0
AZ	50	25	25

***Key**: S = sensitive, I = intermediate, R = resistant*

Table 3: Antibiotics susceptibility of *Vibrio* spp.

Vibrio spp.	SP	AM
R1	S	I
R2	I	I
R3	S	S
B1	S	R
B2	S	R
B3	R	R
T1	S	R
T2	S	S
T3	S	S

***Key**: SP = sulfonamides; AM = ampicillin, B1, B2, B3 = Borehole water, T1, T2, T3 = Tap water, R1, R2, R3 = Rain water*

Table 4: Sensitivity test results of sewage effluent isolated *Vibrio cholera*

Isolate	LEV	CF	SP	CPX	AM	AU	CN	PEF	OFX	AZ
	Inhibition zone (mm)									
V1	**23**	**R**	**22**	**23**	**R**	**R**	**22**	**R**	**15**	**20**
V2	**18**	**15**	**20**	**20**	**20**	**15**	**23**	**R**	**5**	**5**

Key: LEV: levofloxacin CF: cefotaxime, SP: sparfloxacin, CPX: ciprofloxacin, AM: amoxicillin, AU: augmentin, CN: gentamycin, PEF: pefloxacin, OFX: Ofloxacin, AZ: azithromycin

Table 5. Antibiotic susceptibility of coliform bacteria from the sewage samples

Isolate	LEV	CEF	SP	CPX	AM	AU	CN	PEF	OFX	AZ
EFE	S	R	S	S	R	R	S	R	S	S
EFE	S	R	S	S	R	R	S	R	S	S
NEF	S	S	S	S	R	R	S	I	S	S
NEF	S	S	S	S	S	S	R	R	S	S
ES	S	R	S	S	R	R	S	R	S	I
ES	S	R	S	S	I	R	I	R	S	I
NES	S	S	S	S	R	R	S	R	S	R
NES	S	S	S	S	R	R	S	R	S	R

***Keys:** (S) = Sensitive, (R) = Resistant; EFE = E. coli from effluent, NEF = non-E. coli coliform from effluent; ES = E. coli from sewage, NES = non- E. coli from sewage*

The antibiotic susceptibility profile of the coliforms (**Table 2**) showed that they were most resistant to augmentin and pefloxacin with each having 87.5 %, followed by amoxicillin (75 %) and cefotaxime (50 %) and were 100 % sensitive to levofloxacin, sparfloxacin, ciprofloxacin, and ofloxacin. For the *Vibrio* spp (**Table 3**) against sulfonamide and amoxicillin, the most resistance was recorded against amoxicillin, while only one was resistant to sulfonamides. The sensitivity test of the two

Vibrio cholera (V1 and V2) isolated from the sewage effluent (**Table 4**) showed total resistance to pefloxacin. V1 was however resistant to cefotaxime, amoxicillin, augmentin, and pefloxacin. The coliforms isolated from the sewage samples (**Table 5**) were most resistant to pefloxacin, followed by amoxicillin and cefotaxime. They, however, were all sensitive to sparfloxacin and ciprofloxacin.

CONCLUSION

The result indicated high circulation of the organisms in the wastewater, and which increase was attributed to increased human activity due to the large population in the university community. The isolation rate of the coliforms was independent of the collection time. The study also validates the efficiency of the chromocult agar media for this, and other related studies. As ongoing research, molecular resistance profiling of the coliform isolates will be performed to ascertain their roles in the spread of resistance genes, through sewage water. The presence of *Vibrio* species, *Escherichia coli*, and coliforms in diverse water sources emphasizes the necessity for proactive measures to safeguard public health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS

MIN designed the experiment, collected the data, wrote the first draft of the manuscript, and supervised and partook in the experiment. Ngwu MI, PEC, GIN, EEN, CAN, VIF, SCE, PDB, ENE, and RCO. reviewed and contributed to the manuscript. All authors read and approved the manuscript.

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