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Antibiogram Profiles and Virulence Factors Associated with Water-borne Gram-negative Bacteria from Ogbese river, Edo State, Nigeria

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Abstract

Surface waters such as flowing rivers, fresh water lakes and hand dug wells are the major sources of water for most rural dwellers in Nigeria. These are often consumed without testing their potability. This study was conducted to assess the bacteriological quality of surface water sampled from five (5) sampling locations on Ogbese river in Ogbese and Olumoye towns, Ovia North East Local Government Area, Edo State. Water samples were collected in duplicates from the sampling locations during the months of June 2018 to February 2019. Bacteriological analysis of the water samples was conducted using the membrane filtration technique and isolates identified by standard cultural and genomic procedures. Disc diffusion technique was utilized to determine the antibiotic sensitivity profiles of the identified bacteria. Other microbiological analysis which comprised of screening for enterotoxin production, invasiveness, hemolysin production and serum resistance, which are all virulence factors were performed using relevant methods. The total heterotrophic bacterial count varied from $1.0 \pm 32.0 \times 10^4 cfu/ml$ *and* $1.0 \pm 0.12 \times 10^4 cfu/ml$ *to* $9.7 \pm 2.23 \times 10^4 cfu/ml$. *The total coliform count ranged from* $0.1 \pm 0.02 \times 10^2 cfu/ml$ *to* $5.5 \pm 0.05 \times 10^2 cfu/ml$. *The observed differences between the mean heterotrophic bacterial and coliform counts recorded for Ogbese and Olumoye sampling points was significant (P< 0.05). The identified bacterial isolates included; Escherichia coli, Enterobacter cloacae, Shigella flexneri, Salmonella enterica, Vibrio cholerae, Klebsiella pneumoniae and Pseudomonas aeruginosa Both E. coli (60.0%) and E. cloacae (60.0%) were the most frequently isolated water borne isolates cultured from Ogbese sampling point. All E. coli and S. enterica isolates exhibited sensitivity towards ciprofloxacin and streptomycin. All S. flexneri isolates were invasive and serum resistant. There is an urgent need by relevant Governmental and Non –Governmental agencies to conduct advocacy programs targeted at these communities on the necessity of treating and disinfecting*

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abstracted water sourced directly from the water body prior to drinking.

Key Words: Antibiotic Susceptibility, Gram-Negative Bacteria, Ogbese River, Virulence

INTRODUCTION

Fresh water from surface water bodies, such as rivers and fresh water lakes, is utilized for a plethora of activities which include agricultural and domestic purposes. Water from these fresh water sources are often consumed without any form of treatment and this could pose risks of acquiring water-borne infections. Anthropogenic activities around the catchment area of water bodies usually introduce microorganisms into the water bodies, some of which are virulent and could cause water-borne infectious diseases. In developing countries, four-fifths of all illnesses are caused by water-borne diseases, with diarrhea causing dehydration as the major cause of childhood death (WHO, 2018).

About 70 million Nigerians are unable to access potable water and 335,000 die annually from water-borne illnesses (UNICEF, 2014). Fecal coliforms, opportunistic pathogens and heterotrophic bacteria have all been isolated from raw surface waters. Bacteria isolated include *Klebsiella oxytoca, Escherichia coli, Enterobacter asburiae, Salmonella enterica, Vibrio cholerae* and *Shigella spp.* (Mulamattathil *et al*., 2014; Seiyaboh *et al*., 2017). Some of these have been implicated in gastrointestinal diseases and epidemic outbreaks (Sharma, 2003). Illness is contracted by faecal-oral route through direct drinking water or foods such as vegetables contaminated by water used for irrigation and washing (Dahiru and Enabulele, 2015). Several types of antibiotic resistant bacteria are continuously isolated from different environmental niches which range from terrestrial to aquatic niches (Ayandiran *et al*., 2014). It has been speculated that there is an elevated possibility of antibiotic resistance being spread by environmental borne drug resistant bacteria to related human pathogenic bacteria through several transmission routes thereby suppressing the effectiveness of commonly utilized antibiotics (Ayandiran *et al*., 2014).

A lot of anthropogenic activities occur along the banks of the Ogbese river as it flows through several communities which include; Ayede, Ogbese and Olumoye in both Ondo and Edo States respectively. Consequently, agricultural, human and animal wastes pollute its waters and may render it unfit for human consumption (Orjiekwe, and Chinedu, 2013). Data on bacteriological quality of this water body is scant and where available, it is not comprehensive. Orjiekwe and Chinedu (2013) identified coliforms but not individual bacterial species as contaminants of the river. There is also a report on its water quality index (Akinbile and Omoniyi, 2018).

This study attempts to provide data to augment these available reports, as a comprehensive assessment of the bacteriological quality of water including studies on potentially pathogenic species that can be transmitted by water. Such reports are necessary for safeguarding public health and planning strategies for control and prevention of water-borne infections.

MATERIALS AND METHODS

Study Area

River Ogbese in Edo State is a part of Osse river which has its origin in Apata Hills in Ekiti State Nigeria. Osse river flows through Ondo State to several towns and villages including Ogbese and Olumoye in Ovia North East Local Government Area of Edo State, emptying

through several intricate creeks and lagoons, into the Atlantic (Idowu *et al.*, 2017). In Ogbese and Olumoye, where this study was done, it is known as Ogbese river and in some other parts of Edo State, Nigeria, it is also known as Ovia river. Sampling site selection was done based on the extent of accessibility to the water body in both Ogbese and Olumoye towns and the water samples were collected from June 2018 to February 2019.A sampling map indicating the respective sampling points was drawn using geo-referenced GPS coordinates of the sampling locations collected with the aid of a GPS meter

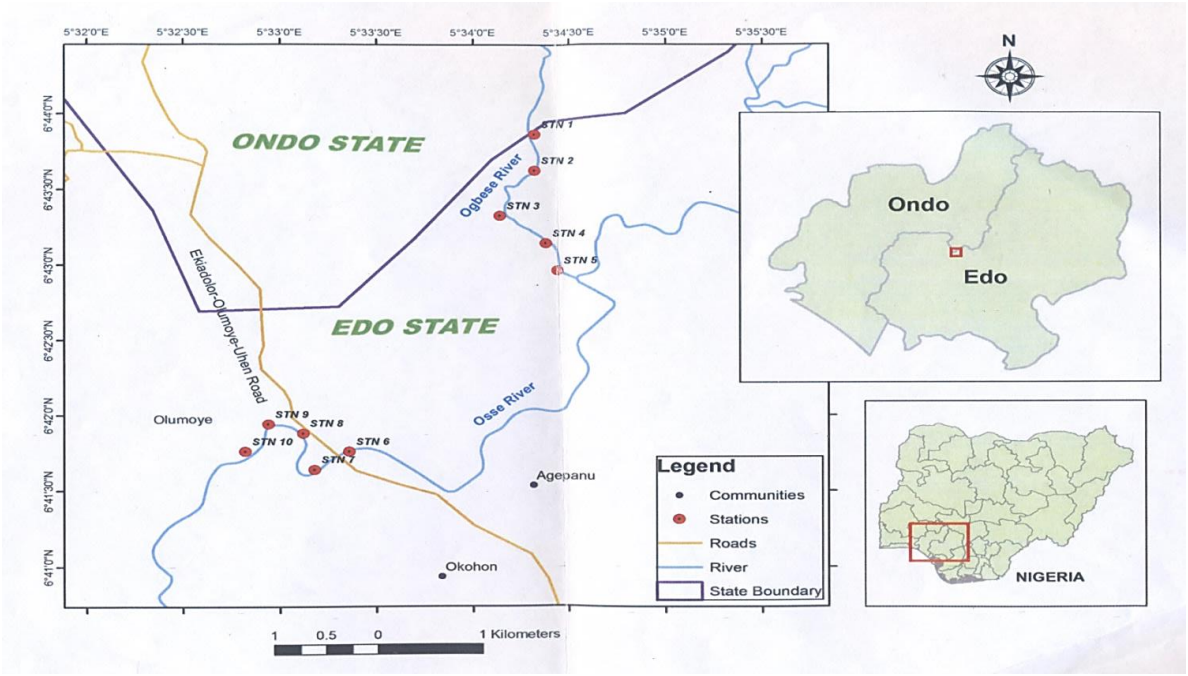

Fig. 1: Map of Ogbese River revealing the sampling sites

Sample Collection

Ten water samples each were collected from sampling points in both Ogbese and Olumoye towns along Ogbese river in Ovia North East Local Government Area of Edo State. The water samples were collected in clean sterilized glass bottles from five randomly selected points of 50 metres apart at each station. Samples were collected by dipping the 500ml bottles below the water surface, opening the lids for water to gush in and closing immediately when out of the river.

Bacteriological Analysis of Water Samples

One hundred millimeter each of the respective surface water samples was filtered through 0.45mm pore sized nitrocellulose membrane filters (Cheesebrough, 2006), which were then cultured in plates of nutrient agar (NA), MacConkey agar (MCA), *Salmonella- Shigella* agar (SSA) and Thiosulfate Citrate Bile salts agar (TCBS). Nutrient agar and MacConkey were utilized for the isolation of heterotrophic bacterial flora and coliforms present in the water samples (Bridson, 2006). The SSA and TCBS were used for the isolation of *Salmonella*, *Shigella* and *Vibrio* species present in the water samples (Bridson, 2006). The cultured agar plates were incubated at 37^{O}C for 24 – 48hr after which distinct representative colonies were enumerated, sub-cultured and stored in NA slants for further characterization.

Conventional bacteriological procedures such as; Catalase, Gram staining, oxidase and indole production tests, citrate utilization and various sugar fermentation tests as described by Cappuccino and Welsh (2020) were used for the tentative identification of the purified bacterial isolates. This was followed by definitive genomic identification which procedurally

comprised of bacterial DNA extraction and Polymerase Chain Reaction (PCR) wherein universal primers 27 forward $^{5'}$AGAGTTTCCTGG$^{3'}$ CTCAG and reverse $^{5'}$ACGGCTACCTTGTTACGATT$^{3'}$ which corresponded to similar positions on the 16S rRNA gene of *E. coli* was utilized (Marchesi *et al.,* 1998). The PCR cocktail mix was made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270); 1µL each of 10 µM forward and reverse primer; 2µL of DNA template using 8.5 µl nuclease free water. The PCR conditions were; initial denaturation (94°C for 5 minutes), succeeded by thirty six (36) cycles of denaturation (94°C for 30 seconds), annealing (55°C for 30 seconds) and elongation (72°C for 45 seconds), followed by a final elongation step at 72˚C for 7 min and holding temperature at 10 ˚C. The amplified DNA fragments were then visualized on a safe view-stained 1.5% agarose electrophoresis. The PCR thermal cycler utilized was GeneAmp PCR system 9700. The PCR products were sequenced and the sequences were compared in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) for definitive identification of the bacterial isolate utilizing BLAST algorithm analysis.

Antibiotic Susceptibility Testing

The bacterial isolates, diluted to the equivalent of 0.5 McFarland standard (1.5×10^8cfu/ml) were screened for antibiotic susceptibility using the modified Kirby-Bauer disc diffusion method as described by Vandepitte *et al.* (2003) and the emergent inhibitory growth zones were interpreted with the aid of the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2020). The antibiotics used were pefloxacin (10µg), gentamicin (30µg), ampiclox (30µg), ceftriaxone (30µg), ciprofloxacin (10µg), streptomycin (30µg), septrin (30µg), erythromycin (10µg) and carbapenem (30µg).

Screening for Virulence Factors in Bacterial Isolates.

Hemolysin production was detected by the agar plate method (Drews *et al.*, 2005). Serum resistance of isolates was determined by incubating standardized bacterial suspension with pooled normal human serum and performing bacterial counts at 3hr intervals (Kumar and Mathur, 1997).

Tests for tissue invasiveness were performed by inoculating the right eyes of albino mice with a standardized suspension of bacterial isolates (0.5 MacFarland's standard) and screening for evidence of inflammation as compared with the left eye (control) which was inoculated with normal saline (Murayama *et al.*, 1986). Isolates were also screened for ability to produce enterotoxin. The rabbit – ileal loop method as described by (Everest *et al.*, 1993) was utilized.

Statistical analysis

Unpaired student T test was used to ascertain if the differences in the heterotrophic bacterial and coliform counts was significant (α=0.05). This was conducted with the aid of Microsoft excel software.

RESULTS

The total heterotrophic and coliform counts of surface water samples collected from the respective sampling stations are presented in Table 1. The maximal heterotrophic bacterial count was recorded for a water sample collected during the dry season from a sampling point in Olumoye ($9.7 \pm 2.23 \times 10^4$cfu/ml) and minimal counts of $1.0 \pm 32.0 \times 10^4$cfu/ml and $1.0 \pm 0.12 \times 10^4$cfu/ml were recorded for water samples obtained during the wet season from sampling points in both Ogbese and Olumoye respectively. The highest coliform count was recorded for a sample collected from a sampling point in Olumoye during the wet season ($5.5 \pm 0.05 \times 10^2$cfu/ml) The lowest count, obtained during the wet months was 0.1 ± 0.02

$\times 10^2$cfu/ml for sample collected from a sampling point in Ogbese.

The observed differences between the mean heterotrophic bacterial and coliform counts recorded for Ogbese and Olumoye sampling points was significant ($P< 0.05$). The differences between the mean heterotrophic bacterial and coliform counts recorded for the water samples collected in the wet and dry seasons for both Ogbese and Olumoye stations was insignificant ($P> 0.05$).

The frequency of occurrence of the Gram-negative bacteria (Table 3) showed that *E. coli* and *E. cloacae* were the most frequently isolated organisms from the river at Ogbese sampling sites as they were recovered from 60% and 20% of samples during the wet and dry seasons respectively. *S. enterica* and *K. pneumoniae* were isolated from 40% of the samples; *S. flexneri* from 20% while *V. chlorerae* was not detected during the dry season. *V. cholera* and *S. flexneri* were not detected during the wet season, but all others were present in 20% of the samples respectively. These same organisms were also isolated from sampling points in Olumoye town. *E. coli*, *E cloacae*., *S. enterica*, *V. cholerae*, *P. aeruginosa* and *S. flexneri* were all isolated from 40% of the samples during the wet season. *K. pneumoniae* (20%) was least isolated. *E. cloacae* and *V. cholerae* were not detected during the dry season but all others but *P. aeruginosa* (40%) were detected in 20% of the samples.

The isolates were screened for potential virulence attributes such as invasiveness, hemolytic activities and serum resistance. Varying proportions of the isolates possessed these features (Table 4). All isolates of *S. flexneri* and *K. pneumoniae* were invasive with *S. enterica* having the lowest percentage (55.5%) of invasive isolates. *S. flexneri* had the highest number of hemolytic strains (66.7%) followed by *P. aeruginosa* (60.0%) while *K. pneumoniae* had the least (25.0%). Fluid accumulation in rabbit illeal loops, an indication of enterotoxin production, was detected in 83.3% of *E. coli* isolates followed by *S. enterica* and *V cholerae* both of which had 66.7% gentamicin (100.0%), streptomycin (96.7%), pefloxacin (93.3%) were the most effective antibacterial agents against the isolates. meropenem, (a relatively new antibiotic) was effective against 83.3% of the isolates. All but one isolate; *V. cholerae* were resistant to cefuroxime (Table 5).

Table 1: Total Heterotrophic bacterial counts for surface water samples from Ogbese River, Edo State (Mean ± SD $\times 10^4$cfu/ml)

	Stations			
	Ogbese		**Olumoye**	
SP	Wet season	Dry season	Wet season	Dry season
1	8.5 ± 11.50	5.9 ± 1.17	1.0 ± 0.12	9.7 ± 2.23
2	7.5 ± 9.80	4.2 ± 0.25	8.9 ± 2.14	7.7 ± 0.75
3	8.0 ± 0.00	6.5 ± 1.26	5.6 ± 0.20	4.5 ± 1.21
4	8.5 ± 21.20	6.6 ± 0.89	7.4 ± 4.80	5.6 ± 0.19
5	1.0 ± 32.00	6.8 ± 0.98	9.4 ± 2.00	7.6 ± 0.12

KEY: SP; Sampling point

Table 2: Coliform counts for surface water samples from Ogbese River, Edo State (Mean ± SD × 10^2cfu/ml)

	Stations			
	Ogbese		Olumoye	
SP	Wet season	Dry season	Wet season	Dry season
1	0.6 ± 0.02	0.2 ± 0.15	5.5 ± 0.05	3.7 ± 0.42
2	0.1 ± 0.02	0.9 ± 0.22	3.5 ± 0.05	2.5 ± 0.52
3	2.2 ± 0.45	1.5 ± 0.15	2.9 ± 0.00	2.1 ± 0.12
4	3.1 ± 0.58	2.6 ± 0.65	0.3 ± 0.00	2.6 ± 0.17
5	4.3 ± 0.28	3.2 ± 0.52	4.2 ± 0.15	4.0 ± 0.00

KEY: SP; Sampling point

Table 3: Frequency of Occurrence of Gram-negative enteric bacteria from Ogbese river, Edo State (%)

Bacterial isolates	**Ogbese (n=5)**		**Olumoye (n=5)**	
	Wet	Dry	Wet	Dry
Escherichia coli	3(60.0)	1(20.0)	2(40.0)	1(20.0)
Enterobacter cloacae	3(60.0)	1(20.0)	2(40.0)	0(0.00)
Salmonella enterica	2(40.0)	1(20.0)	2(40.0)	1(20.0)
Vibrio cholerae	2(40.0)	0(0.0)	2(40.0)	0(0.00)
Klebsiella pneumoniae	2(40.0)	1(20.0)	1(20.0)	1(20.0)
Pseudomonas aeruginosa	2(40.0)	1(20.0)	2(40.0)	2(40.0)
Shigella flexneri	1(20.0)	0(0.00)	2(40.0)	1(20.0)

KEY: n: Number of water samples tested

Table 4: Virulence factors of surface water-borne Gram negative bacterial isolates

Isolate	Number of tested isolates	% No of invasive strains	% No of Serum-resistant strains	% No of hemolytic strains	% Entero toxin producing strains
Escherichia coli	6	66.7	50.0	33.3	83.3
Salmonella enterica	9	55.0	55.0	33.3	66.7
Vibrio cholerae	6	66.7	83.3	50.0	66.7
Klebsiella pneumoniae	4	100.0	50.0	25.0	NT
Pseudomonas aeruginosa	5	80.0	40.0	60.0	NT
Shigella flexneri	6	100.0	100.0	66.7	35.3

NT = Not Tested

Table 5: Antimicrobial Sensitivity Profiles of surface water borne Gram-negative Bacterial Isolates

Bacterial species	No of isolates (n)	CIP (%)	STR (%)	SXR (%)	ERY (%)	PEF (%)	CN (%)	APX (%)
Escherichia coli	4	100.0	100.0	100.0	50.0	75.0	100.0	25.0
Salmonella enterica	6	100.0	100.0	50.0	100.0	100.0	100.0	50.0
Vibrio cholerae	6	100.0	100.0	100.0	50.0	100.0	100.0	66.7
Klebsiella pneumoniae	4	50.0	100.0	75.0	50.0	75.0	100.0	25.0
Pseudomonas aeruginosa	5	80.0	80.0	60.0	40.0	100.0	100.0	0.0
Shigella flexneri	5	100.0	100.0	100.0	50.0	100.0	100.0	0.00)

Key: CIP – Ciprofloxacin, CN – Gentamicin, STR – Streptomycin, APX - Ampiclox SXT – Trimethroprim-sulfamethoxaxole, CEF - Cefuroxime ERY – Erythromycin, CET – Ceftriaxone, PEF – Pefloxacin, MEM – Meropenem

DISCUSSION

Access to safe-drinking water is of utmost 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. Available research evidence however showed that in the absence of fecal contamination, there is no direct relationship between HPC values in ingested water and human health effects in the population (Allen *et al.*, 2003). However, HPC greater than 500 cfu/ml would indicate a decrease in water quality that should trigger further investigation (Verhille, 2013). The *E. coli* or thermotolerant coliform count should be less than 1 per 100ml of sample (CDC, 2003). Both counts fall short of the acceptable limits by these regulatory bodies at all stations screened along Ogbese river. Heterotrophic plate counts can be used in assessing the cleanliness of water in distribution system. It could pose health risks if virulent bacteria are present (Verhille, 2013). The range of mean heterotrophic plate counts obtained in this report are lower than values recorded for surface water samples collected from Ikpoba River, Edo State (Ekhaise and Anyasi, 2005) and River Nun, Bayelsa State (Seiyaboh *et al.*, 2017) The range of mean coliform counts observed in this study were a lower than the range of values earlier reported by Orjiekwe and Chinedu (2013) with respect to surface water samples abstracted from the same Ogbese river but the values were comparatively higher than the acceptable standard of 10 cfu/100ml prescribed by (SON, 2007) with respect to drinking water. The comparative differences between the mean microbial counts recorded for this study and previous cited researches could be due to the differences in both natural geographical structures and anthropogenic activities obtained in and around these water bodies. The bacterial species obtained in this study included *E. coli, S. enterica, S. flexneri, K. pneumoniae* and *V. cholerae.* The detection of these isolates is in agreement with some earlier reports on surface water in various parts of Nigeria (Aliyu *et al.,* 2016; Seiyaboh *et al.*, 2017).

Some of the isolates possessed pathogenic potentials as indicated by their ability to produce virulence factors such as hemolysin, fluid accumulation in illeal loops, invasiveness and serum resistance. The presence of *E. coli* and coliforms indicated feacal contamination and the consequent possibility of contamination by enteric pathogens. The detection of typical enteric pathogens such as *S. enterica, S. flexneri* and *V. cholerae,* some of 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 ampiclox but also, a few were resistant to the relatively new β - Lactam, meropenem. An identical trend with respect to antibiotic resistant bacterial isolates cultured from surface water samples collected from Oluwa River, Ondo State has also been reported (Ayandiran *et al.*, 2014).

Usage of antibiotics and other chemotherapeutic agents in veterinary medicine and animal husbandry could result in contamination of natural surface water bodies through run-offs and waste discharges. This study highlights the risk of transmission of antibiotic resistant pathogenic bacteria by this river. Health risks to users should be minimized by advocating point-of-use disinfection, such as boiling, for water intended for drinking purposes.

CONCLUSION

All the examined surface water samples collected from the respective sampling locations on Ogbese river harbored a variety of Gram negative bacteria which would indicate that the Ogbese river is exposed to microbial contamination from both natural and anthropogenic sources. Based on the complete reliance of the human communities residing within the catchment area of the river on the water body as source of water for both drinking and domestic purposes, there is an urgent need by relevant Governmental and Non – Governmental agencies to conduct awareness and advocacy programs specifically targeted at

these communities on the necessity of treating and disinfecting abstracted water sourced directly from the water body prior to drinking.

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