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# Plasmid Curing and Antibiotic Susceptibility Test of Bacteria Isolated From RiverOsin, Ila Local Government Area, Ila-Orangun, Osun State, Nigeria

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***Abstract***
*Water is an essential natural resource and a basic need of lives. Its qualities and sustenance is threatened by pollution, resulting into serious public health concern. Polluted or contaminated water could harbor pathogenic microorganisms. Therefore, this study aimed at subjecting isolated bacteria from River Osin, Ila Local Government, Ila-Orangun, Osun State, Nigeria, to antibiotic susceptibility test and plasmid curing following standard microbiological procedures. The mean value of total viable bacteria1 count and total coliform count obtained ranged between {2.41x $10^3$ - 1.81x$10^4$}cfu/ml and {120-150}cfu/100ml for February and July, 2019 respectively and were both found to exceed WHO standard of 1.0x$10^2$cfu/ml and (0) zero cfu/100ml respectively. A total of (9) nine bacteria were isolated and identified to include; Staphylococcus aureus, Escherichia coli, Klebsiellasp, Salmonella sp, Bacillus sp, Streptococcus sp, Proteus sp, Alcaligensp, and Pseudomonas aeruginosa. All isolates tested for susceptibility were resistant to Cotrimaxazoleand Amoxicillin, and showed varied susceptibility to Levufuroxime, Tetracycline, Levofloxacin and Amikacim. Many were susceptible to Ciprofloxacin, Gentamicin and Ofloxacin. The plasmid curing done shows that the resistance genes were both plasmid and chromosomallyborne. It was concluded from this work that all bacteria isolated from this River resist Cotrimazole and Amoxicillin, and their resistant genes were both plasmid and chromosomally borne.*
***Keywords:****Chromosome, Bacteria, Plasmid Curing and Antibiotic Susceptibility.*

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## I. Introduction

Water is an excellent polar solvent that dissolves many substances, and basic needs of human being an all forms of life (Singh, *et al*., 2011; Daghara, *et al.,* 2019). Virtually, the physiological activities of humans and microorganisms cannot hold without water (Shittu, *et al.,* 2008).

Rivers are surface water sources and its quality are influenced by the environment, economic growth, humans anthropogenic and developmental activities. The tremendous increase in human population, indiscriminate waste disposal, urbanization and explosive industrialization along the rivers have put serious pressure on water sources and other qualities (Veakatesharaju, *et al.,* 2010). Pollution of water is already a great problem in many developing countries. River serves as dump site of majority of waste generated by humans such as waste water, industrial waste and indiscriminate sewerage disposal (Kolawole, *et al.,* 2013). Potable water scarcity amongst rural dwellers has made people depend on surface water sources like river, well, stream and pond water for domestic uses. The uses of river water can serve as source of contamination to the river bodies leading to its deterioration that threatened its sustenance, resulting into a serious public concern (Wang *et al.,* 2015). Keeping the river or aquatic environment healthy depends on the physiochemical and biological variability of that particular river water body. Preventing the water from becoming deteriorating depends on timely monitoring of the water body, especially microbiologically so as to prevent infectious diseases outbreaks (Venkatesharaju, *et al.,* 2010).

The incidence of water related illness in Africa is based on lack of providing water quality for its citizens. WHO (1984) reported that eighty percent (80%) of ill health in underdeveloped countries was as a result of lack of safe water and inadequate sanitation. Obviously, a lot of ill health is due to diseases caused by contamination of water.

Bacteriological assessment of river water is a way of checking the presence of pathogenic bacteria that can pose health risk to human. Bacteria normally used as indicators of water quality is coliform bacteria specifically faecalcoliform (WHO, 1996; Nnane, *et al,* 2011). The presence of coliform bacteria in any water is an indicative of faecal pollution in water which is of public concern.

This study will give information on the bacteriological quality of River Osin in Ila-Orangun, Osun State, Nigeria. The objectives are to determine the total variable bacteria, total coliform, characterize and identify the bacterial isolates, determine the antibiotic susceptibility pattern of the bacterial isolates, and determine the effect of cured plasmid on the bacterial isolates.

## II. Methodology

### 2.1 Collection of Water Sample

Water sample from the working River were collected from three sampling points in the month of February and July, 2019 representing dry and raining season sample collections. The samples were collected into sterile glass vessels with a twist cap (Neiwolak, 1998). The three sampling points were 500meters apart. All samples after collection between 7 - 9am in the morning were aseptically transported to the laboratory on an ice pack for microbiological analysis (WHO 1998).

### 2.2 Enumeration of Total Viable Bacterial Count from Collected River Water Samples

The river water samples were diluted serially to $10^{-4}$ dilutions using sterile pipettes and poured onto already set solidified sterile nutrients agar plates in duplicates. All the inoculated plates were incubated at $37^0$C for 24 - 48 hours. After incubation period, the plates were examined for colony formation and the numbers of discrete colonies was counted and expressed in cfu/ml (Adebowale*et al*, 2010).

### 2.3 Enumeration of Total Coliform Count

Mutitude fermentation tube (MT) method was used to enumerate total coliform present in water samples. In this technique, series of tubes containing MacConkey broth was used. Three boiling tubes containing 10ml double strengthMacConkey broth with inverted Durham tubes were used and were all inoculated with 10ml water samples each. Another two sets of 3 test tubes containing 10mls single strengthMacConkey broth with inverted Durham tubes were also inoculated with 1ml and 0.1ml each of test water samples respectively (forming 3-3-3 regimen). All inoculated tubes were incubated for 24 - 48 hours for acid and gas production for presumptive positive test (APHA, 2002); (Fawole and Oso, 2004). Positive tube indicates possible presence of coliform. Bacterial concentration in the samples was estimated using MacCrady statistical table. Positive tubes from presumptive test were confirmed by inoculation on Eosine Methylene Blue Agar (EMB) to observe for greenish metallic sheen for the presence of *E.coli* (Fawole and Oso, 2004). To complete the test, positive colonies on EMB agar were inoculated on a tube of lactose broth with inverted Durham tubes and incubated at $37^0$C for 24 - 48 hours. Gas production after incubation further confirms the presence of coliform.

### 2.4 Purification and Preservation of Bacterial Isolates

Discrete colonies from nutrient agar were sub-cultured severally on sterile nutrient agar (NA) plates to obtain pure isolates. The pure isolates were then inoculated onto sterile NA slants, incubated at $37^0$C for 24 - 48 hours to observe visible growth, and then stored at $4^0$C in a refrigerator.

### 2.5 Characterization and Identification of Bacterial Isolates

The characterization of the bacterial isolates was done by the determination of their colonies and cellular morphology and biochemical characteristics. The identification of the isolates was obtained (Buchanan and Gibbons, 2004; Garrity*et al*, 2004; and Cheesbrough, 2000).

### 2.6 Antibiotic Susceptibility Test

Preparation of 0.5 McFarland standard was done (Movahedi*et al*, 2019). Then 18 hours old culture of the isolates was inoculated into sterile normal saline with turbidity matched with 0.5 McFarland standard. The standardized culture was spread plates on sterile set plate of Mueller Hinton agar using sterile swab stick. Antibiotic discs were then placed on the agar and pressed firmly on the surface for efficient activity. The multiple antibiotics employed were manufactured by rapid labs of which set CM-12NR100 was used for the gram-negative organism and CM-12-8PR100 was used for gram-positive bacteria.

The multiple antibiotic discs used and their concentrations are; Cefuroxime (CFX) 30µg, Gentamycin (GEN) 10µg, Ciprofloxacin (CPR) 5µg, Ofloxacin (OFL) 5µg, Amoxycillin/Clavulinate (AUG) 30µg, Nitrofurantoin (NIT) $30^0$µg, and Ampicillin (AMP) 10µg. after placing the discs firmly on the surface of the agar, they were subsequently incubated for 18-24 hours before the diameter of zone of inhibition was taken in millimeter using a ruler.

### 2.7 Plasmid Curing of the Bacterial Isolates

Bacterial isolates were subjected to plasmid curing using acridine orange (Ezeokoli*et al*, 2016) with slight modification. An amount of 5ml aliquot of overnight suspension cultures of bacterial isolates were sub-

cultured into tubes containing 5ml of double strength nutrient broth supplemented with 0.1mg/ml acridine orange and incubated at $37^0$C for 24 - 28 hours (it was assumed based on scientific literature that such acridine concentration and exposure time was sufficient to cure plasmids). Subsequently, bacterial cultures were then plated out on Muller-Hinton agar and test against the set of antibiotic disc used for sensitivity test, followed by disc diffusion method and again, the zone of inhibition was measured. The changes in resistance pattern was noted. The bacteria that displayed clear changes in resistance pattern after curing were regarded as having their resistance gene in the plasmid (Movahedi*et al*,. 2019).

**2.8 Statistical Analysis**

1BM-SPSS version 20.0 was used to carry out the statistical analysis. Duncan's multiple range test at = 0.05 was used to separate the means.

## III. Results

**3.1 Bacteriological Results**

The River water was collected from 3 points at 500meters apart during each sampling period. Water samples were collected in February and July 2019. The total viable bacterial count and total coliform count for February 2019 sampling period ranged between ($2.41 \times 10^3 - 1.87 \times 10^4$) cfu/ml and (120-150) cfu/100ml respectively. Also, the total coliform counts for July 2019 sampling period was between ($4\text{-}1 \times 10^5 - 2\text{-} \times 10^6$) cfu/ml and (1100 – 2400) cfu/ml respectively (Table 1)

**3.2 Identification of Bacterial Isolates**

A total of nine (9) bacterial were isolated and identified to include; *Staphylococcus aureus, Escherichia coli, Klebsiellasp, Salmorellasp, Bacillus sp, Streptococcus sp, Proteus sp, Alcaligenssp,* and *Pseudomonas aeruginosa.*

**Table 1: Total Viable Bacterial Counts (cfu/ml) and Total Coliform Counts (cfu/100ml) from Sampled River Water**

| Sampling points | TVB (cfu/ml) February | TVB (Cfu/ml) July | TCL (cfu/100ml) February | TCL (cfu/100ml) July |
|---|---|---|---|---|
| Point A | $1.15\text{x } 10^4$ | $4.1\text{x } 10^6$ | 150 | 2400 |
| Point B | $2.4\text{x } 10^3$ | $4.1\text{x } 10^5$ | 128 | 1100 |
| Point C | $1.87\text{x } 10^4$ | $1.8\text{x } 10^4$ | 120 | 1100 |

Point A, B, C are sampling points along the River flow and are 500 meters apart;
**TVB**= Total viable bacterial count
**TCL**= Total coliform count
Figures are mean of duplicate values

**Table 2: Antibiotics susceptibility patterns of bacterial isolates**

| Organism/Antibiotics disc(m/m) | CRX | GEN | COT | TET | AMX | CIP | LEV | AMK | OFL |
|---|---|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | R | R | R | R | R | R | R | R | R |
| *Escherichia coli* | R | 13 | R | R | R | R | R | R | R |
| *Klebsiellaspp* | R | 13 | R | R | R | R | R | R | R |
| *Salmonella spp* | 15 | R | R | R | R | 27 | R | R | 23 |
| *Bacillus spp* | R | 22 | R | 16 | R | 29 | 20 | 25 | R |
| *Streptococcus spp* | R | R | R | R | R | R | R | R | R |
| *Proteus spp* | R | 10 | R | R | R | 14 | R | R | 22 |
| *Alcaligensspp* | 15 | R | R | R | R | R | R | R | 24 |
| *Pseudomonas aeruginosa* | R | 10 | R | R | R | R | R | R | R |
| *% Resistivity* | 78 | 44 | 100 | 89 | 100 | 67 | 89 | 89 | 67 |

**Key: R=** Resistant, COT= Cotrimaxazole, CIP= Ciprofloxacin, OFL= Ofloxacin, CRX= Cefuroximne, TET= Tetracyclin, LEV= Levofloxacin, GEN= Gentamicin, AMX= Amoxicillin, AMK= Amikacin.

**Table 3: Antibiotic susceptibility patterns of plasmid cured bacterial isolates**

| Bacterial isolates/Antibiotic disc (mm) | CRX | GEN | COT | TET | AMX | CIP | LEV | AMK | OFL |
|---|---|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | 12 | 14 | 12 | R | 12 | R | 14 | R | 14 |
| *Escherichia coli* | 18 | R | R | R | R | R | R | 14 | R |
| *Klebsiellasp* | R | 14 | R | R | R | R | R | R | R |
| *Salmonella sp* | 21 | R | R | R | R | R | R | R | R |
| *Bacillus sp* | R | R | 22 | R | R | 21 | 25 | 23 | 21 |
| *Streptococcus sp* | 14 | 12 | R | R | R | R | R | R | 18 |
| *Proteus sp* | 18 | 19 | R | R | R | 7 | R | R | 24 |
| *Alcaligenssp* | 23 | 20 | R | R | R | R | R | R | 22 |
| *Pseudomonas aeruginosa* | R | R | R | R | R | 15 | R | R | R |
| % resistance | 33 | 44 | 88 | 100 | 89 | 67 | 77 | 89 | 33 |

**Key: R=** Resistant, COT= Cotrimaxazole, CIP= Ciprofloxacin, OFL= Ofloxacin, CRX= Cefuroximine, TET= Tetracyclin, LEV= Levofloxacin, GEN= Gentamicin, AMX= Amoxicillin, AMK= Amikacin.

**Antibiotic susceptibility test of bacterial isolates**

All bacterial isolates were resistant to cotrimaxazole and Amoxicillin. *Salmonella sp* and *Alcaligenssp* are susceptible to Cefuroximine; *Bacillus sp* only susceptible to tetracyclin, levofloxacin and Amikacin (Table 2). All other isolates had varied susceptibility patterns to all other used antibiotic disc (Table 2).

**Plasmid cured bacterial isolates.**

All isolates resisted cotrimaxazole and Amoxicillin before curing of plasmid, after plasmid cured, 22% and 11% of isolates were susceptible which are; *Staphylococcus aureus*to both antibiotic and *Bacillus sp*to Amoxicillin alone. For these two isolates their resistant factor is plasmid borne. *Bacillus sp*, aftertreated with acridine orange resist Tetracyclin and hence it's resistant factor was borne chromosomally. All other isolates are inhibited by all the antibiotics to varying extent.

## IV. Discussion

Almost all the bacteria isolated from this working water samples were known to be pathogenic. Their presence in water can pose serious harm to the people when consumed the water raw and to the environment when the water is used to irrigate plant that will adversely affect the human when the irrigated plant is consumed (EPA, 1994).

The bacteriological examination of River Osin was studied and the total viable bacterial count (TVBC), Total coliform count (TCC), Antibiotic susceptibility pattern and plasmid curing on an isolated bacterial were determined.

The total viable bacterial count obtained from the six samples collected at three different spatial points in February and July 2019 ranged from ($2.41 \times 10^3$ - $1.87 \times 10^4$) cfu/ml and ($1.8 \times 10^4$ - $4.1 \times 10^6$) cfu/ml respectively. The highest viable bacterial count of $4.1 \times 10^6$ cfu/ml obtained in the study was higher than $2.7 \times 10^3$cfu/ml obtained by Olanrewaju, (*et al*., 2017) from domestic water used in Ila-Orangun, Osun State Nigeria. Also, the higher values obtained for total viable counts in this study was higher than recommended value by WHO,(1996) for domestic water and was an indication that the river water could be dangerous when used.

The total coliform counts obtained for February and July (2019) from water samples ranged between (120 -150) cfu/100 ml and (1100- 2400) cfu/100ml respectively. These values have greatly exceeded the recommended limit of 1 cfu/ 100ml by WHO,(1996), This indicated the high level of coliform presence in the studied river water which potentially poses a high health risk for human use (Kolawole*et al*., 2011).

The bacterial isolated in this study were identified to include; *Staphylococcus aureus, Escherichia coli, Klebsiellasp, Salmonella sp, Bacillus sp, Streptococcus sp, Alcaligensp and Pseudomonas aeruginosa.* Most of these identified bacteria in these studywas from soil and sewage origin (Wilson and Miles, 1915). *Escherichia sp and Klebsiellasp*isolated belongs to coliform group of bacteria and is an indication that the river water is

seriously polluted with faecal matter. *Bacillus sp and Pseudomonas sp* are of soil origin and their members are able to metabolize pollutant in water environment (Nani, *et al*., 2003). Other isolated bacteria; *Staphylococcus aureus, Salmonella sp, Streptococcus sp*and *Alcaligensp* are pathogens associated with the presence of coliform (WHO,2019) that can pose health ill effect if the water is used without been treated.

Before plasmid was cured, all bacteria isolated were resistant to cotrimaxazole and Amoxicillin while 89% of the isolate were resistant to tetracycline, levofloxacin and Amikacin. Also (78%, 67% and 44%) of isolated bacteria were resistant to cefuroximine, ofloxacin, ciprofloxacin and gentamycin respectively.

The antibiotic sensitivity of some isolated bacteria in the study were lost after the cured of plasmid showing that their resistant factors is in plasmid. However, *Streptococcus*sp did not show serious change in antibiotic resistance profile after plasmid cured and this indicates that its resistance gene was chromosomally borne. It was reported that plasmid treated bacteria could have lost 75% resistant of the initially tested antibiotic (Akpe, *et al.*, 2018).

In this work, acridine orange was used to cured plasmid, this chemical agent is one of the intercalating agents that remove plasmid from bacteria. The wide spread of bacterial resistance to commonly used antibiotics in human was confirmed in the study.

The public health importance of this study is that the polluted water results into water borne illness which might not be able to combat when commonly antibiotics is use because of the problem of antibiotics drug resistant of most bacterial species.

## V. Conclusion

It is concluded from this study that river water as a source of water for human uses should be discouraged for use because of high presence of coliform that indicates presence of pathogenic organisms of which most demonstrated multiple antibiotics resistance where the resistant genes can be both plasmid and chromosomally borned.

## VI. Recommendation

(1) The river water should be discouraged not to use raw without undergo any sort of treatment.
(2) The government at local, state and national levels should provide safe water for every community.
(3) The indiscriminate waste disposal should be discouraged by initiating binding laws on waste disposal.
(4) Waste water or sewage should be adequately treated before disposing.

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