

# Original Research Article

## Plasmid Profiles of Antibiotic Resistant Bacteria Associated with Biofilms from Ground Water Sources in Ado-Ekiti, Ekiti State, Nigeria

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### ABSTRACT

**Introduction:** The presence of microbial biofilms in our drinking water sources poses a significant health risks to human because biofilm serves as an environmental reservoir of pathogenic microorganisms; the bacteria in biofilms are usually antibiotic resistant and therefore their multiple resistance genes may be harbored on the resistant plasmid.

**Aim:** The study investigated the plasmid profiles of antibiotic resistant bacteria associated with biofilms from ground water sources in Ado-Ekiti, Ekiti State Nigeria.

**Methodology:** One hundred samples of water were collected randomly from wells and boreholes, isolation and identification of bacteria from the biofilm of the water samples was carried out by using standard microbiological procedures, antibiotic sensitivity of the isolates was carried out and multiple antibiotic resistant indexes of the bacterial isolates were calculated. The plasmid profiles of the bacterial isolates was also determined.

**Results:** Results showed that a total of 209 bacteria were isolated from the biofilms of the two ground water sources; these include *Streptococcus faecalis*, *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella typhi* and *Shigella dysenteriae*. *S. faecalis* from the borehole and well water samples had the highest occurrence of (37.5%) and (49.5%) respectively. *Shigella dysenteriae* had the lowest occurrence of (1.8%) from borehole water while *Staphylococcus aureus* from well occurred least (2.1%). Both Gram positive and the Gram negative bacterial isolates showed considerable resistance to the antibiotics. The percentage occurrence of the multiple antibiotic resistant (MAR) bacterial isolates was 106 (52.5%) with the highest percentage (63.4%) from the biofilms of borehole water samples. The MAR indexes of the majority of the bacterial isolates were above 0.2, this revealed a high prevalence of MAR indexes which indicates high risk source of contamination in the areas. Of the 10 MAR isolates selected and examined for plasmid analysis, it was discovered that only six isolates harbored plasmids with molecular size range of 300-950 bp. Plasmid curing and antibiotic sensitivity test after curing showed that curing of plasmids was effective in four isolates (*P. vulgaris* B11, *E. coli* W21, *Enterobacter aerogenes* W25 and *Strept. faecalis* B25) and partially effective in two isolates (*Salmonella typhi* B4 and *E. coli* B37).

**Conclusion:** Well and borehole water must be treated at the point of use and water stored tanks or storage vessels) must be washed regularly, there should be public enlightenment on indiscriminate use of antibiotics in order to eradicate the incidence of antibiotic resistance

*Keywords: Antibiotic resistance, Bacteria, Biofilms, Ground water sources, Plasmid profile and Plasmid curing*

### 1. INTRODUCTION

A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material known as extracellular

polymeric substance (EPS) [1]. The presence of microbial biofilms in our drinking water sources poses a significant health risks to human because biofilm serves as an environmental reservoir of pathogenic organisms [2]. Therefore, it is essential to ensure that drinking water is safe by preventing the formation of biofilms since water is vital to life.

The presence of biofilm in our drinking water can lead to occasional outbreak of waterborne and water-related diseases. According to WHO [3], the two main categories of relevant microorganisms that are usually involved in biofilms are: microorganisms with pathogenic properties which have been shown to be associated with water-related illness and outbreaks, and bacteria which are primarily used as indicator organisms in water analysis, indicating the presence of pathogenic organisms of faecal origin. Water-related and waterborne diseases are caused by the presence of microorganisms most especially bacteria such as *Streptococcus faecalis*, *Escherichia coli*, *Salmonella* spp. and *Shigella dysenteriae* in the water [4].

Others are opportunistic pathogens which cause disease in sensitive human subgroups such as the elderly, children, immuno-compromised individuals, patients with preexisting disease or other predisposing conditions which facilitate infection by these organisms. These organisms may attach to surfaces as primary colonizers and actively establish biofilms alone or in combination with other microorganisms. Biofilms in drinking water such as well and bore hole can be responsible for a wide range of water quality problems such as increased bacterial levels, taste and odour changes [5]. Biofilms may develop within the different drinking water sources as a result of contamination, regrowth of microorganisms or from microorganisms that survived disinfection and this may lead to occurrence of waterborne diseases.

Previous studies have demonstrated the presence of biofilms in water pipe network distribution systems as well as in sachet water in Abakaliki area of Ebonyi State, Nigeria [6], but there is no information about the plasmid analysis of bacteria that are associated with biofilms in ground water sources in Ekiti State, Nigeria. Since plasmids have always been associated with antibiotic resistance, it becomes imperative to assess the plasmid profiles of bacteria that are associated with biofilms from ground water sources in Ado-Ekiti, Ekiti State, Nigeria.

## **2. MATERIAL AND METHODS**

### **2.1 Collection of water samples**

One hundred samples of water were collected randomly from well and borehole within Ado-Ekiti metropolis in sterilized bottles and brought into the laboratory and kept in a safe place.

### **2.2 Isolation of bacteria from the biofilms of drinking water**

The water samples were allowed to stand and stored for a period of three weeks, isolation of bacteria from the biofilm samples obtained from the ground water sources was carried out using pour plate method as described by Sam [7]. The bacteria from the biofilms of water samples were isolated at the third week of storage when the total bacteria counts became significant (above 40); this was done to ensure that biofilms had actually formed in the drinking water samples. Biofilm samples were collected by swabbing the inner side of the surface in contact with the drinking water supplies at interval of seven days (weekly) until the total bacteria counts were significant.

### **2.3 Identification of bacterial isolates**

The identification of the bacterial isolates was carried out by cultural, morphological examinations and different biochemical tests using standard microbiological techniques as described by Fawole and Oso [8].

#### **2.4 Antibiotic sensitivity test**

The antibiotic sensitivity testing was carried out using disc diffusion techniques as described by CLSI [9]. The test was carried out by using gram positive and gram negative disks containing different antibiotics which were placed aseptically on the inoculated Muller Hinton agar plates. The plates were incubated at 37 °C for 24 hours and observed for the presence of growth and zones of inhibition.

#### **2.5 Multiple antibiotics resistant index of bacterial isolates**

The multiple antibiotic resistance (MAR) index of the bacteria isolates was determined according to the method used by Oluyeye *et al.* [10]. It was calculated by dividing the number of antibiotics to which each isolate was resistant to by the total number of antibiotics used.

#### **2.6 Determination of plasmid profiles of bacterial isolates**

Plasmid extraction was carried out based on the methods of Molina-Aja *et al.* [11] with little modification. Ten (10) representative multiple antibiotics resistant isolates (MAR) which included four isolates from biofilms of well water (*Streptococcus faecalis* W27, *Enterobacter aerogenes* W23, *E. coli* W21 and *E. aerogenes* W25) and six isolates from biofilms of borehole water (*Pseudomonas aeruginosa* B7, *Salmonella typhi* B4, *Proteus vulgaris* B11, *Staph. aureus* B3, *E. coli* B37 and *Strept. faecalis* B25) were selected for plasmid analysis. Five milliliter of overnight broth culture of each isolate was centrifuged in an Eppendorf tube at 10,000 rpm for 2 minutes, the plasmid DNA of the bacterial isolates was isolated and electrophoresed. The electrophoresis was run at 60- 100 V until loading dye has migrated about three- quarter of gel, the gel was removed from the tank and visualized in UV- transilluminator (FOTO UVI- 1430).

#### **2.7 Plasmid curing of the resistant isolates**

The curing of the resistant isolates was carried out according to the method used by Ebele *et al.* [12] by exposing the overnight grown culture of each representative resistant isolate to an elevated temperature of 37 °C and 10% sodium deodecyl sulphate in order to remove their antibiotics resistance ability.

#### **2.8 Antibiotics sensitivity of the cured isolates**

The antibiotics sensitivity of the cured isolates was carried out by inoculating broth cultures of each cured isolate on sterilized Muller Hinton agar plates by using spread plate technique. Both gram positive and gram negative antibiotics disks were placed separately on the inoculated plates and incubated at 37 °C for 24 hours. The plates were then observed for the presence of zones of inhibition.

#### **2.9 Statistical analysis of data**

Data obtained from this study were analyzed by descriptive statistical method and two-way analysis of variance (ANOVA) using SPSS version 22 and tukey HSD (honest significance difference) test at 95% confidence level.

### 3. RESULTS AND DISCUSSION

A total of 209 bacteria were isolated from the biofilms of the two ground water sources; these include *Streptococcus faecalis*, *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella typhi* and *Shigella dysenteriae* (Fig. 1 and 2). Their presence may be as a result of inadequate water treatment practices by the individual households, lack of good personal hygiene practiced by individuals and contamination of the drinking water with faecal materials possibly by digging of wells or boreholes very close to septic tanks which may permit the growth of bacteria in them or seepage of faecal materials from the septic tank into it. The presence of these bacterial isolates in the biofilms of the drinking water indicate that the water is unsuitable for drinking and implies the likelihood of waterborne diseases until the water is treated. This result corroborates the findings of Etido [13], where the author observed that the water samples from borehole, well and pipe-borne available to students in Nasarawa State University Keffi were found to harbor bacteria such as *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, and *Citrobacter* sp. This result is also in accordance with PanelSahar *et al.* [14] where the authors observed the presence of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species from the biofilm resistome in drinking water distribution systems.

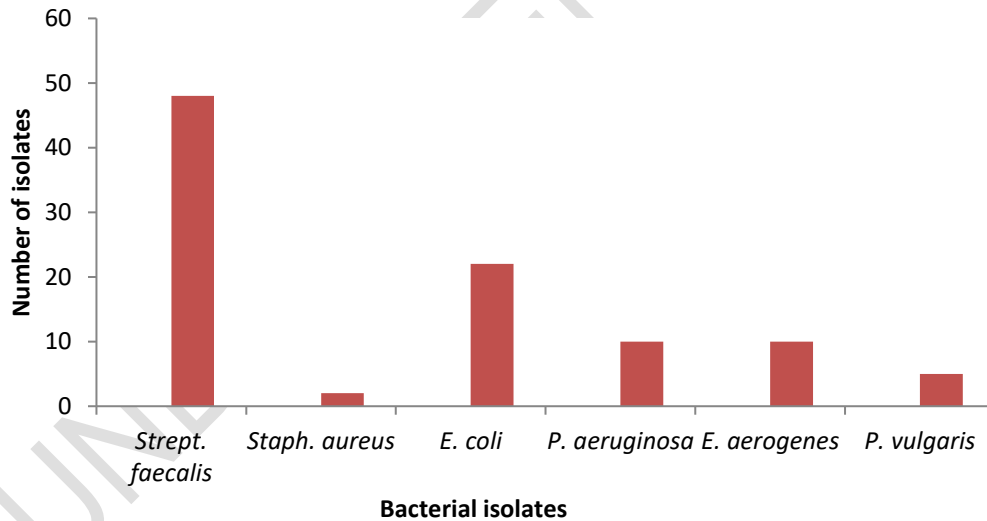
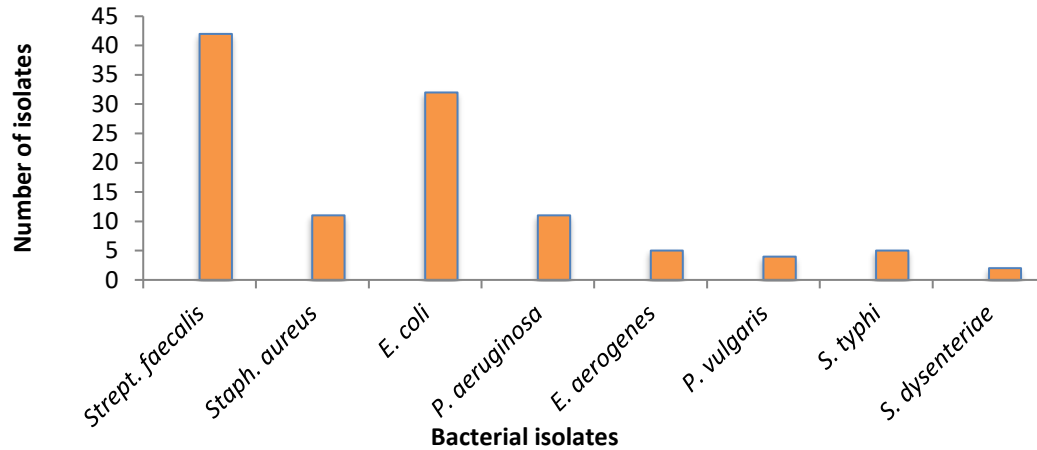
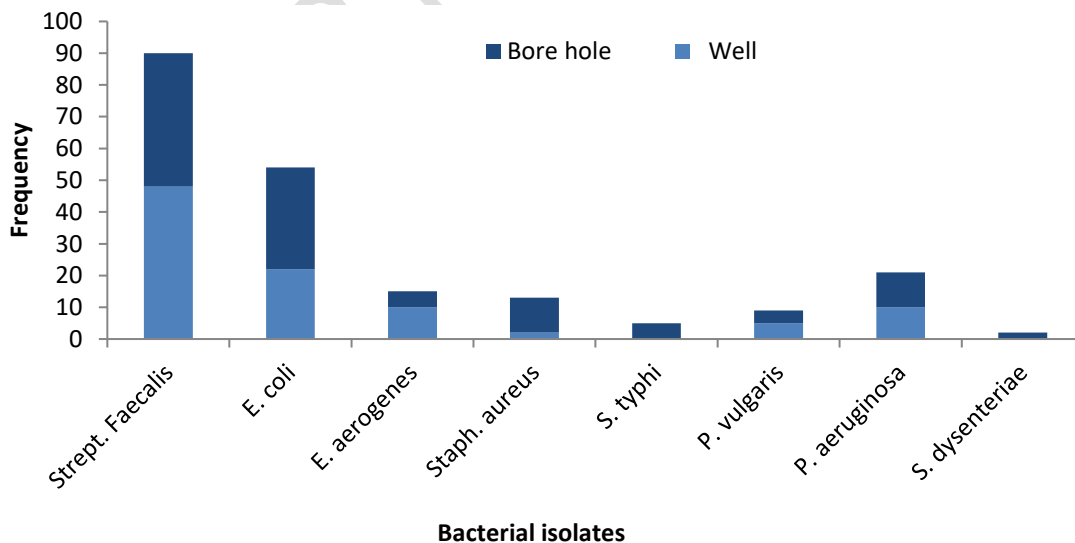


Fig. 1. Bacteria isolated from the biofilms of well water



**Fig. 2. Bacteria isolated from biofilms of borehole water samples**

A total of 97 bacteria were isolated from the biofilms of well water; *S. faecalis* had the highest occurrence (49.5%) while *Staphylococcus aureus* occurred least (2.1%). A total of 112 bacteria were isolated from the biofilms of borehole water with *S. faecalis* having the highest occurrence (37.5%) and *Shigella dysenteriae* having the lowest occurrence (1.8%) (Fig. 3). This may be due to the fact that *Strept. faecalis* and *E. coli* are the major indicator organisms and they have the ability to inhabit any part of the environment most especially water. Biofilm samples from borehole water had the highest mean total bacterial count of  $11.1 \times 10^4$  cfu/ml whereas the biofilms from well water had the least mean total bacterial count of  $7.8 \times 10^4$  cfu/ml (Table 1). This result showed that the total bacterial count from the biofilms of borehole water was high which implies high rate of contamination which may be due to irregular cleaning of the stored tanks and running taps and lack of treatment of the water from the borehole. This result is in line with Sunday *et al.* [15] where the authors obtained a high level of bacterial counts in borehole water samples from Abakaliki area of Abia State, Nigeria.



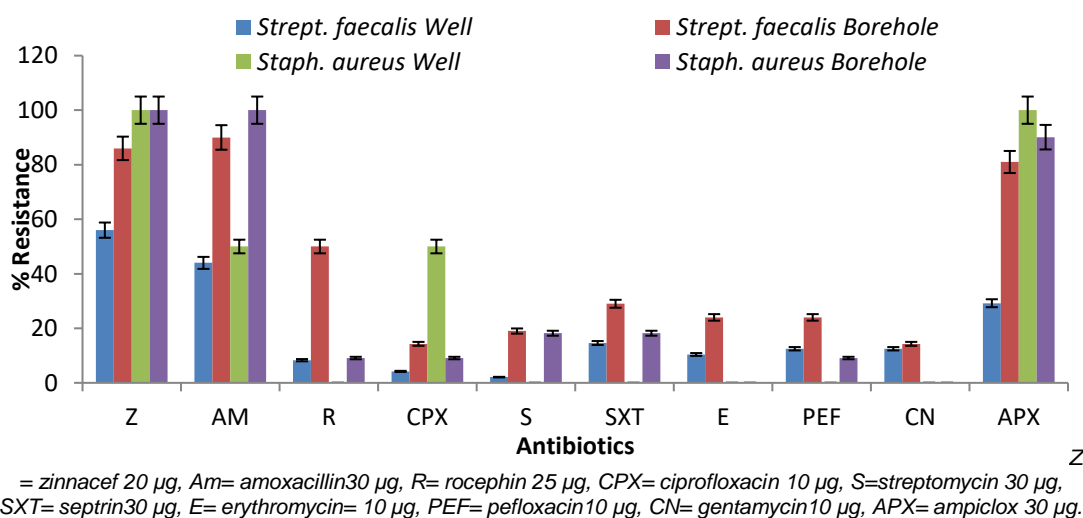
**Fig. 3. Frequency of occurrence of bacterial isolates from biofilms of the water samples**

**Table 1. The mean total bacterial count of bacterial isolates**

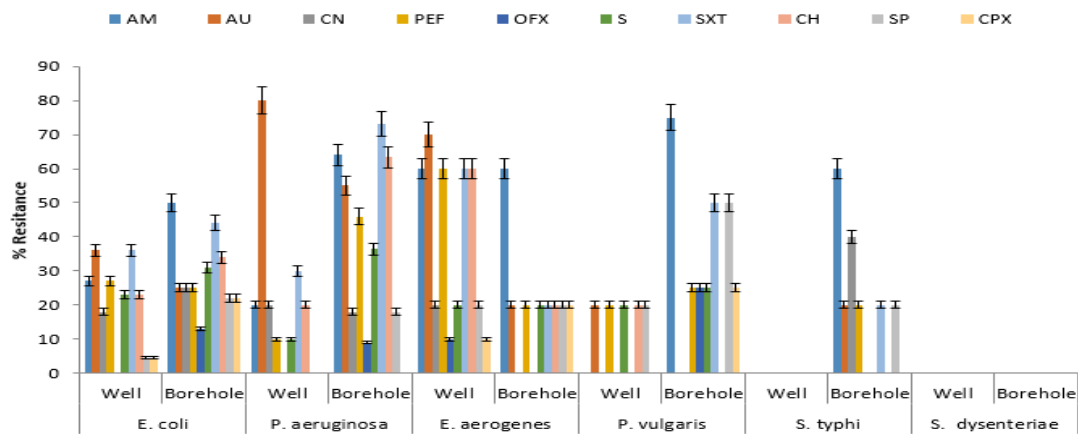
Drinking water sources (n= 50 )	Total bacterial count (Cfu/ml)
Well	$7.80 \times 10^3$
Bore hole	$11.10 \times 10^3$

Key: n = number of samples

The Gram positive and the Gram negative bacterial isolates showed considerable resistance to the antibiotics. Some of the isolates were resistant while some were susceptible to the antibiotics, for instance, the gram positive bacteria (*Strept. faecalis* and *Staph. aureus*) from borehole showed high resistance to zinnacef (Z), amoxicillin (AM) and ampiclox (AM) and low resistance to the remaining antibiotics (Fig. 4). Also, Gram negative bacteria isolated from the biofilms of the two drinking water sources revealed that nearly all the isolates were resistant to pefloxacin, septrin, chloramphenicol and augumentin and high resistance was also observed with the remaining antibiotics (Figure 5). Results also showed that the bacterial isolates from the biofilms of borehole were more resistant than the isolates from well water biofilms to the various antibiotics, resistance could contribute to the spread and persistence of antibiotic resistant bacteria. This result suggests that bacteria from biofilms are resistant to antibiotics than their planktonic counterpart and this is in agreement with Gilbert *et al.* [16] who observed that bacterial cells in biofilms exhibit 10 to 1000 times less susceptibility to specific antimicrobial agents than their planktonic counterparts. The resistance observed in the bacterial isolates may be due to the ability of the bacteria from biofilms of the drinking water to synthesize enzymes that can neutralization the antibiotics [2]. Some of the bacteria may even possess adaptive mechanisms such as the possession of efflux pump which can remove the antibiotics and possession of antibiotic resistant gene [17]. This result is in agreement with the work of Okafor *et al.* [6] who revealed that bacteria isolated from the biofilms of borehole water were completely resistant to multiple antibiotics including ciprofloxacin, tetracycline, norfloxacin, ofloxacin, cefuroxime and gentamycin. Hawa *et al.* [18] also observed that *Pseudomonas aeruginosa* and *E. coli* isolated from the biofilms of borehole, well and tap water from greater Accra region in Ghana were resistant to cefuroxime, trimethoprim and amoxillin-clavulanate



**Fig. 4: Antibiotic resistance of Gram positive bacterial isolates from the biofilms of the water samples**



AM = amoxicillin (30 µg), AU = augumentin (30 µg), CN = gentamycin (10 µg), PEF = pefloxacin (30 µg), OFX = tarivid (10 µg), S = streptomycin (30 µg), SXT = septrin (30 µg), CH = chloramphenicol (10 µg), SP = sparfloxacina (10 µg), CPX = ciprofloxacina (10 µg)

**Fig. 5. Antibiotics resistance of Gram negative bacterial isolates**

The percentage occurrence of MAR bacterial isolates from the biofilms of borehole and well water showed that out of the 209 bacterial isolates, 106 (52.5%) were MAR isolates with the highest percentage (63.4%) from the biofilms of borehole water, indicating a high prevalence of MAR in this study (Table 2). This finding agrees with Okafor *et al.* [6] who isolated MAR isolates which were resistant to at least seven commonly used antibiotics. The high percentages of MAR isolates found in the biofilms of the drinking water most especially borehole indicated that water is a major reservoir of antibiotic resistant bacteria. It could also be a reflection of misuse or abuse of antibiotics in the environment. A total of 16 bacterial isolates out of the 209 isolates had MAR index of 0.1, 17 isolates had MAR index of 0.2 and 106 of the isolates had MAR index greater than 0.2.

**Table 2: Percentage occurrence of MAR bacterial isolates from biofilms of borehole and well water**

Sources	No of isolates	No of multiple antibiotics resistant isolates (%)
Well	97	35 (36.1)
Borehole	112	71 (63.4)
Total	209	106 (52.5)

The multiple antibiotics resistant index ranged from 0.1 to 0.8, with MAR index 0.3 having the highest percentage, followed by MAR index of 0.2 having 13.6% and MAR index of 0.1 having 11.3%, while the lowest percentage MAR index of 0.6 had 4.5 % (Table 3). The MAR indexes of the majority of the bacterial isolates were above 0.2. This revealed a high prevalence of MAR indexes which indicates high risk source of contamination in the study area. The high MAR index values may be due to the widespread use of antibiotics and the continuous use of a single antibiotic over a period of time which select bacteria that are resistant to different kind of antibiotics. This work is in accordance with Oluyeye *et al.* [10] who isolated bacteria with high MAR indexes from drinking water.

**Table 3: Multiple antibiotic resistance index of bacterial isolates**

Sources isolates	0.1	0.2	0.3	0.4	0.5	0.6	0.7 and above
Well (n = 97)	8	12	14	8	5	5	3
Borehole (n = 112)	8	5	20	14	17	7	13
Total (209)	16	17	34	22	22	12	16

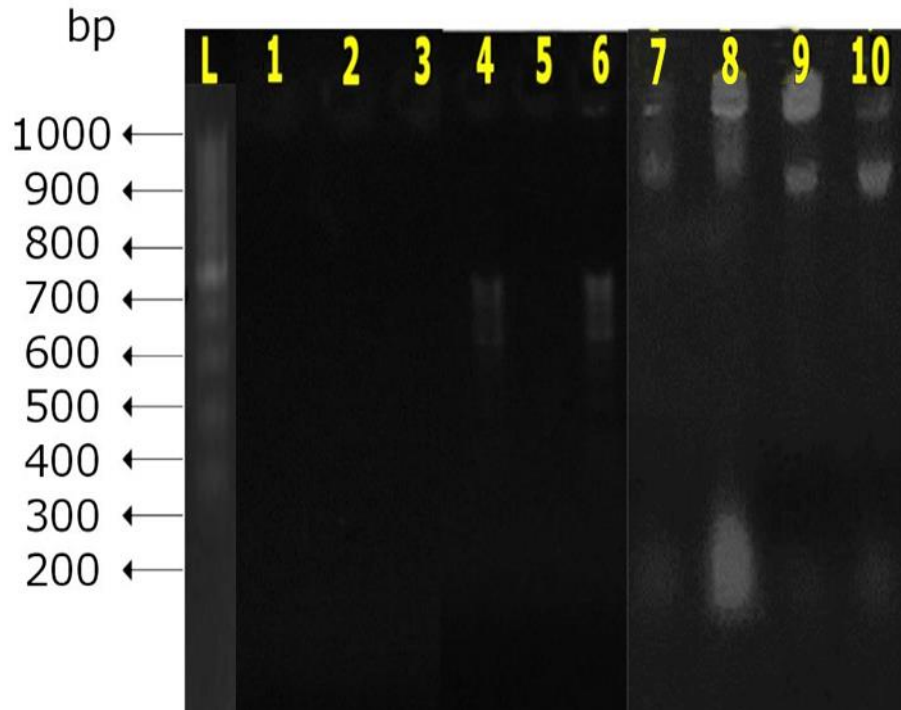
Of the 10 MAR isolates selected and examined for plasmid analysis, four isolates from the biofilms of borehole harboured plasmid; *Strept. faecalis* B25 (No 10) harboured a plasmid of 900 bp, two isolates *Proteus vulgaris* B11 (No 4) and *Salmonella typhi* B4 (No 6) harboured a plasmid of 750 bp while the fourth isolate *E. coli* B37 (No 8) harboured a dual- plasmid of 300 bp and 900 bp. Two isolates from the biofilms of well water harboured plasmid, with *E. coli* W21 (No 7) harbouring a plasmid of 950 bp while the other one *Enterobacter aerogenes* W25 (No 9) harboured a plasmid of 900 bp. No plasmid was detected in the remaining 4 isolates (Plate 1). It was discovered that only six isolates harbored plasmids with molecular size range of 300- 950 bp. These findings are consistent with the work of Kroll *et al.* [19] who reported that plasmids may be present in an individual cell in varying number and sizes, ranging from one to several hundreds. Two isolates (*Proteus vulgaris* B11 and *Salmonella typhi* B4) showed the same plasmid size of 750 bp, another two isolates *Enterobacter aerogenes* W25 and *Strept. faecalis* B25 also harbored the same plasmid size of 900 bp, but these isolates that had the same plasmid size did not have the same resistant phenotype (Table 4), possession of plasmid of the same molecular weight or size may suggest common origin or source. *E.coli* W21 harbored a plasmid of 950 bp and the eighth isolates *E. coli* B37 harbored a dual plasmid of 300 and 900 bp, this isolate was resistant to all the antibiotics, this may likely be the reason for the possession of double plasmid. The presence of plasmids in the six isolates showed that their resistance to antibiotics were mediated by plasmids, it implies that plasmids can be transferred to susceptible bacteria in the environment through horizontal gene transfer. This result is in accordance with the work of Mbim *et al.* [20] who observed that the presence of resistant genes in the plasmids of bacteria explains to a large extent the antibiotics resistance among the isolates. Similarly, Falegan *et al.* [21] demonstrated that multiple resistance genes are harbored on resistance plasmids (R-plasmids), some of which are conjugative. In this study, 4 of the 10 MAR representative isolates examined for plasmid DNA showed a negative result indicating the absence of plasmid in the isolates. Thus, suggesting that the resistance in these isolates may not be mediated by plasmids and may likely be chromosomal borne.

**Table 4: Antibiotic resistant phenotype and plasmid analysis of the representative isolates**

Isolates/ identification No	Sources	Antibiotic resistant phenotype	No of plasmid detected	Estimated size in bp
<i>Strept. faecalis</i> W27(1)	Well water	CN, APX, Z, AM, E	-	-
<i>Staph. aureus</i> B3 (2)	Borehole	APX, Z, AM, S, SXT	-	-
<i>Ps. aeruginosa</i> B7 (3)	Borehole	CH, AM, AU, SXT	-	-
<i>Pr vulgaris</i> B11 (4)	Borehole	PEF, S, SP, CPX, AM	1	750
<i>Enter. aerogenes</i> W23 (5)	Well water	PEF, S, CH, CPX, AU, SXT	-	-
<i>Sal. typhi</i> B4 (6)	Borehole	PEF, CH, AM, AU, CN, SXT	1	750
<i>E. coli</i> W21 (7)	Well water	PEF, CH, SP,CPX, AM, AU, SXT	1	950
<i>E. coli</i> B37 (8)	Borehole	PEF, OFX, S, CH, SP, CPX, AM AU, CN, SXT	2	300, 900
<i>Enter. aerogenes</i> W25(9)	Well water	PEF, OFX, AM, AU	1	900
<i>Strept. faecalis</i> B25 (10)	Borehole	APX, Z, AM, R	1	900

AM = amoxicillin (30 µg), AU = augumentin (30 µg), CN = gentamycin (10 µg), PEF = pefloxacin (30 µg), OFX = tarivid (10 µg), S = streptomycin (30 µg), SXT = septrin (30 µg), CH = chloramphenicol (10 µg), SP = sparfloxacin

(10 µg), CPX = ciprofloxacin (10 µg), APX = ampiclox (30 µg), Z = zinnacef (20 µg), R = rocephin (25 µg), E = erythromycin (10 µg)



L = Molecular weight ladder (200 to 1000bp) Lane 1 = *Strept. faecalis* W 27, lane 2 = *Staph. aureus* B3, lane 3 = *Pseudomonas aeruginosa* B7, lane 4 = *Proteus vulgaris* B11, lane 5 = *Enterobacter aerogenes* W23, lane 6 = *Salmonella typhi* B4, lane 7 = *E. coli* W21, lane 8 = *E. coli* B37, lane 9 = *Enterobacter aerogenes* W25, lane 10 = *Strept. faecalis* B25

**Plate 1: Plasmid profile of bacterial isolates from the biofilms of drinking water.**

Plasmid curing and antibiotic sensitivity test after curing showed that curing of plasmids was effective in four isolates (*P. vulgaris* B11, *E. coli* W21, *Enterobacter aerogenes* W25 and *Strept. faecalis* B25) and partially effective in two isolates (*Salmonella typhi* B4 and *E. coli* B37) (Table 5). The isolates were no longer resistant to the antibiotics after the plasmid curing procedure, this is an indication that the isolates have been cured of their plasmids, and therefore the resistance was plasmid mediated. However, plasmid curing was not effective in the other isolates indicating that their resistance were chromosomal mediated. This result is in line with Lavanya *et al.* [22] who observed that most resistant isolates from fermented milk were cured of their plasmid, that is, their resistance were plasmid-borne and few of the isolates were resistant against tested antibiotics after curing (chromosomal-borne) as compared with the initial resistant pattern before curing (pre-curing).

**Table 5: Antibiotic resistant phenotype of the cured bacterial isolates**

Isolates	Sources	Antibiotics resistant phenotype
<i>Strept. Faecalis</i> W 27 (1)	Well water	CN, APX, Z, AM,
<i>Staph. aureus</i> B 3 (2)	Borehole	S, SXT
<i>Pseudomonas aeruginosa</i> B7 (3)	Borehole	CH, AM, SXT
<i>Proteus vulgaris</i> B11 (4)	Borehole	-
<i>Enterobacter aerogenes</i> W23 (5)	Well water	CH, S, AU

<i>Salmonella typhi</i> B4	(6)	Borehole	AU
<i>E. coli</i> W 21	(7)	Well water	-
<i>E. coli</i> B 37	(8)	Borehole	CN
<i>Enterobacter aerogenes</i> W25	(9)	Well water	-
<i>Strept. faecalis</i> B 25	(10)	Borehole	-

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*S* = streptomycin (30 µg), *AU* = augumentin (30 µg), *SXT* = septrin (30 µg), *CH*= chloramphenicol (10 µg), *CN* = gentamycin (10 µg), *APX* = ampiclox (30 µg), *Z* = zinnacef (20 µg), *AM* = amoxicillin (30 µg), *R* = rocephin(25 µg), *PEF* = pefloxacin (30 µg).

#### 4. CONCLUSION

*Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Staphylococcus aureus* *Escherichia coli*, *Enterobacter aerogenes*, and *Proteus vulgaris* were isolated from the biofilms of the water samples from well and borehole. High level of contamination of bacterial isolates was revealed indicating that most of the water supplies were unfit for human consumption if kept for long and do not meet the World Health Organization (WHO) drinking water standards. Consumption of these drinking water supplies may result in public health hazard. A high level of antibiotic resistance was observed among the bacterial isolates as results demonstrated that 139 of 209 bacterial isolates were resistant to one or more antibiotics and the percentage of multiple antibiotics resistant isolates (MAR) was 106 (52.5)%. The presence of plasmids in six isolates out of the 10 representative MAR isolates and the ability of these isolates to be cured of their plasmids indicated that their resistance to antibiotics were mediated by plasmids, while the resistance of the remaining isolates were chromosomal based.

Findings from this study suggests that the well and borehole water must be treated at the point of use and water Stored tanks or water storage vessels) must be washed regularly. Well and borehole must be sited far away from septic tanks and there should be public enlightenment on indiscriminate use of antibiotics, over-counter or self-prescription and over usage of antibiotics in order to eradicate the incidence of antibiotic resistance.

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