

Original Research Article

ISOLATION AND CHARACTERIZATION OF PLASMID-BEARING MULTIPLE ANTIBIOTIC RESISTANCE BACTERIA FROM DIFFERENT AQUATIC SOURCES IN AKURE, NIGERIA.

ABSTRACT

Aims: This study was designed to investigate the plasmid bearing multiple antibiotic resistance bacteria from different aquatic sources.

Place and Duration of Study: This research work was carried out in Akure south area of Ondo state, Nigeria between January, 2018 and June, 2018.

Methodology: The pathogenic bacteria associated with water samples collected from different sources in Akure, Nigeria were isolated and characterized. A total of 521 water samples were collected between January and June, 2018 from sources such as wells, taps, streams, rivers, boreholes and rain. All the samples were subjected to presumptive, confirmed and completed tests to evaluate their microbiological quality. The microbial types in the samples were determined using standard microbiological techniques. All isolates obtained in this study were subjected to antibiotic sensitivity analysis and screened for Beta-lactamase production (ESBL). Plasmid-gene profile analysis of the resistance isolates were carried out using standard method. Furthermore, post curing of the plasmid mediated antibiotic resistance isolates were carried out and data obtained were analyzed and presented using analysis of variance.

Results: Bacterial isolates such as *Acinetobacter baumannii*, *Citrobacter freundii*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella typhimurum*, *Salmonella paratyphi*, *Shigella dysenteriae*, *Serratia marcescens*, *Proteus vulgaris* and *Vibrio cholerae* were identified from the water samples. The isolate *E. coli* had the highest percentage distribution of 24.10% in well water and 26.19% in stream water while *Salmonella species* had the highest occurrence of 53.85% in rain water. The Beta-lactamase producing (ESBL) isolates were resistant to multiple antibiotics except Ciprofloxacin, Gentamycin and Pefloxacin that conferred antibacterial effect. Plasmid-gene profile analysis of the isolates revealed that *S. typhimurum*, *K. pneumoniae*, *P. aeruginosa* and *P. vulgaris* possess single plasmid each while only *E. coli* contain two

Comment [DJ1]: How did you arrive at pathogenicity criterion

Comment [DJ2]: Not clear....It better referred to as Plasmid profiling, what plasmid are looking out for resistance plasmids or what plasmid

29 plasmid bands. The post plasmid-curing antibiotic sensitivity test of the isolates revealed that the initial
30 antibiotic resistance of the bacterial isolates were plasmid mediated.

31 **Conclusion:** Findings from this study suggest the purification of water from these sources before
32 consumption as most microbes found in these samples are potential pathogens that are capable of
33 causing infectious diseases with multiple antibiotic resistant features.

Comment [DJ3]: Is critical or important

34 **Keywords:** Isolation, Characterization, Antibiotic Resistant Bacteria; Aquatic sources, Plasmid profile
35 analysis; Beta-lactamase production.

36 1. INTRODUCTION

37 Africa faces huge challenges with multiple issues that adversely affect public health, one of which is the
38 ability for both rural and urban Africans to access a clean water supply. According to the WHO [1], only
39 59% of the world's population had access to adequate sanitation systems, and efforts to achieve the
40 Millennium Development Goal, which is aiming for 75% by the year 2015, has fallen short by nearly half a
41 billion people. The potable water sources most accessible to inhabitants in rural African are largely dams,
42 wells, rivers, streams, ponds, which might harbor pathogen that cause diseases such as diarrhea,
43 cholera, typhoid fever, river blindness, Schistosomiasis among others. Antibiotic resistance is a form of
44 drug resistance whereby some sub-populations of microorganisms, usually bacterial species, are able to
45 survive after exposure to one or more antibiotic [2]. Antibiotic resistance may result from antibiotic
46 resistance genes residing on transmissible plasmids thus facilitating their transferor antibiotic drug misuse
47 by respondents. Therefore, this study aims at isolating and identifying plasmid-bearing multiple antibiotics
48 resistant gram negative bacteria from different water sources in Akure south local government.

50 2. MATERIALS AND METHODS

51 2.1 Description of Study Location

52 This research work was carried out between January, 2018 and June, 2018 in Akure south area of Ondo
53 state, Nigeria. Akure covers an area of 14,798.8 ,993.7 square kilometers and lies at latitude 7°15'0"N, 70
54 11' N 5°11'42"E and longitude 5°11'42'E, 5°35'E [3].

Comment [DJ4]: Kindly place this coordinates into a map

55 2.2 Sample Collection and Processing

56 A total of 521 water samples were collected into sterile containers from different areas in Akure metropolis
57 between February 2018 and June 2018. The water samples were collected aseptically from different

58 sources such as tap, borehole, and well, stream, rain and swimming pool. All samples obtained were
59 analyzed microbiologically within 4 hours of collection.

60 **2.3 Test for water quality**

61 The test for quality of the water samples were carried out as described by Cheesbrough [4]. Lactose broth
62 containing Durham tubes were prepared in test tubes. These tubes were inoculated with 1 ml of water
63 sample each and incubated at 37°C for 24 hours. Thereafter, lactose broth was examined for change in
64 colour (fermentation) and gas production. Also, plates of Levine Eosine Methylene blue were streaked
65 with the isolates that were able to ferment lactose and subsequently incubated at 37°C for 24 hours.
66 Production of Greenish metallic sheen on the plate after the incubation time indicated the presence of
67 *Escherichia coli* while the presence of nucleated colonies (large dark centre) indicated the presence of
68 Gram-negative lactose fermenter (coliform). The isolated microbes were kept and maintained on nutrient
69 agar slants prepared. A Gram-stained slide was made from the slant, and the slide was examined under
70 oil immersion optics. If the organism proves to be a Gram-negative, non-spore-forming rod that ferments
71 lactose, the presence of coliforms was confirmed in the tested water sample.

72 **2.4 Isolation and Identification of bacteria**

73 The pour plate technique was employed in the isolation of bacteria from the water samples as described
74 by Olutiola [5]. 1 mL each of the water samples were pour-plated and incubated at 37°C for 24 hours.
75 The media used for the isolation include: Salmonella Shigella Agar, Eosine Methylene Blue Agar and
76 Nutrient Agar. Distinct colonies were then subcultured to obtain pure cultures on which Gram staining and
77 other biochemical tests [Sugar fermentation (glucose, sucrose, lactose, mannitol and triple salt iron),
78 Methyl Red/Voges-Proskauer, Indole, Nitrate reduction, Oxidase, Coagulase, Citrate, Urease, Motility and
79 Catalase tests] were carried out. The methods described by Willey [6] were adopted for characterization
80 of isolated bacteria. The isolates were further identified with reference to the Bergey's manual of
81 systematic bacteriology [7].
82

83 **2.5 Standardization of Bacterial inoculum for Sensitivity Test**

84 The McFarland's standard of a one percent (1%v/v) sulphuric acid solution was prepared with one per
85 cent (1%w/v) solution of Barium Chloride (BaCl₂.2H₂O). The turbid solution formed was transferred into a
86 test tube containing 2.0 mL of normal saline until the suspension matches the turbidity of the standard
87 (1% barium sulphate).

88 **2.6 Antibiotic Sensitivity Test of Bacterial Isolates**

89 The Kirby-Bauer test was used to determine the effect of standard antibiotics on bacterial isolates on
90 Mueller Hinton agar. The agar was seeded with 18 hold pure broth cultures of each isolates [8]. Aseptic

Comment [DJ5]: Lower case O AND
SUPERSCRIPT is appropriate

Comment [DJ6]: Please pour plate method is
inappropriate for isolation. I regard this flaw worthy
of note.

91 swabs of the identified bacteria isolates were made on solidified Mueller Hinton Agar. The discs were
92 applied unto the surface of plates and incubated for 24 h at 37°C with control as sterile distilled water [9].
93 The bacterial isolates were tested against a wide range of antibiotics namely; Ofloxacin (5µg),
94 Amoxicillin (25µg), Ciprofloxacin (10µg), Tetracycline (30µg), Pefloxacin (5µg). Thereafter, a ruler
95 caliberated in millimeter (mm) was used to measure the diameter of the clear zones of inhibition observed
96 on the plates and this was noted as degree of antibiotic resistance as described by [9]. The isolates'
97 zones of inhibition was classified into susceptible (17mm and above), intermediate (13mm-17mm), and
98 resistant (0-12mm) based on the specified standard of mean zone of inhibition for pathogenic gram
99 positive and gram negative bacteria respectively [9].

Comment [DJ7]: Wrong spelling

100 2.7 Molecular Characterization of Multiple Antibiotic Resistant Bacteria via Plasmid 101 Profile

102 Plasmid profile analysis of the multiple antibiotic resistant bacteria isolates were carried out using
103 protocols described by Chan [10] and Matsui [11]. Thereafter, a 1% SDS-PAGE gel was prepared and
104 loaded into electrophoresis chamber containing between 4 wells; this was buffered with 20 mM sodium
105 acetate, 2mM EDTA and then adjusted to pH 7.8 with acetic acid. The sample buffer contained 25%
106 sucrose, 5mM sodium acetate, 0.05% bromophenol blue and 0.1% SDS. Electrophoresis was allowed to
107 proceed at room temperature. After electrophoresis, gels were stained with ethidium bromide (1µl/ml) and
108 observed with UV transillumination. The molecular marker used was the bacteriophage Hind III digest.
109 The multiple antibiotic resistant isolates were cured of their plasmid afterwards by exposing overnight
110 grown bacterial cultures at 37 °C with 10mg/mL of ethidium bromide by adopting the methods described
111 in Birnboim and Dolly [12] as well as Brown [13].

Comment [DJ8]: I thought ethidium bromide has been decommissioned for use in laboratory use.

112 2.8 Antibiotic Sensitivity Test after Plasmid Curing

113 The characterized multiple antibiotic resistant bacterial isolates were subjected to antibiotic sensitivity test
114 post plasmid curing using broad spectrum antibiotics by adopting the method described in Matsui [11].
115

Comment [DJ9]: You never mentioned the primers you employed is for Mec A gene or what.....????????????????????

116 2.9 Data Analysis

117 Analyzed sample treatments were in triplicates and data means obtained were subjected to a 2-way
118 analysis of variance. The treatment means were separated using Duncan's New Multiple Range test at
119 $P \leq 0.05$ levels of significance.
120

121 3. RESULTS AND DISCUSSION

122 3.1 Water Samples Quality and Percentage Frequency Distribution of Bacteria Isolates

123 The quality of water and the frequency of distribution of bacteria isolated from the different water sources
124 are presented in Table 1.

125 3.2 Microscopic and Biochemical Characteristics of Bacterial Isolates from Water 126 Samples

127 The characterization of the bacterial isolates obtained from the water samples across the different
128 locations are presented in Table 2. The Gram negative bacteria isolates include: *Escherichia coli*,
129 *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Citrobacter freundii*,
130 *Enterobacter aerogenes*, *Salmonella typhi*, *Salmonella typhimurum*, *Salmonella paratyphi*, *Shigella*
131 *dysenteriae*, *Serratia marcescens*, *Proteus vulgaris* and *Vibrio cholerae*.
132

133 **Table 1: water quality and percentage distribution of the bacteria isolated from different water sources**

T.I	Gram st	Glu	Suc	Lac	Ma	TSI	O/C	Mt	Ci	Ur	Ni	In	Mr	VP	COT	NA	EMB
Sources	Water Quality				No of Isolates / Frequency of Distribution (%)												
	PT	CFT	CT	(NO/F.D)	AB	CF	EA	EC	KP	PV	PA	SS	SM	SD	VC	TOTAL	
RAIN	+ve	GMT	CC	NO	-	-	-	36	12	-	24	84	-	-	-	-	156
				F.D	-	-	-	23.07	7.69	-	15.38	53.85	-	-	-	100	
STREAM	+ve	GMT	CC	NO	12	18	60	132	60	24	48	72	36	36	-	-	504
				F.D	2.38	4.76	11.90	26.10	11.90	4.76	9.52	14.28	7.14	7.14	-	-	100
S.P	-ve	-ve	-ve	NO	-	-	-	-	-	-	24	-	-	12	-	-	36
				F.D	-	-	-	-	-	-	66.67	-	-	33.3	-	-	100
TAP	+ve	GMT	CC	NO	12	-	36	84	48	-	156	216	-	84	-	-	636
				F.D	1.89	-	5.67	13.21	7.55	-	24.52	33.16	-	13.20	-	-	100
WELL	+ve	GMT	CC	NO	24	36	108	564	108	60	540	444	48	408	-	-	2340
				F.D	1.02	1.54	4.62	24.10	4.62	2.56	23.00	18.97	2.05	17.44	-	-	100
B.H	+ve	GMT	CC	NO	6	-	18	40	20	10	22	50	19	12	-	-	97
				F.D	3.04	-	9.13	20.30	10.15	5.07	11.06	25.38	9.64	6.09	-	-	100

134

135 Keys: AB= *Acinetobacter baumannii*, CF= *Citrobacter freundii*, EA= *Enterobacter aerogenes*, EC= *Escherichia coli*, KP= *Klebsiella pneumonia*,
 136 PA= *Pseudomonas aeruginosa*, PV= *Proteus vulgaris*, SP= *Salmonella paratyphi*, ST= *Salmonella typhi*, STY= *Salmonella typhimurum*, SM=
 137 *Serratia marcescens*, SD= *Shigella dysenteriae*, VC= *Vibrio cholerae*, PT= Presumptive Test, CFT= Confirmed test, CT= Completed Test, NO=
 138 Number of Isolates, F.D= Frequency of Distribution(%), GMT= Green metallic Sheen , CC=Coliform Confirmed.

139

140

141

142

A.B	-ve	+ve	-ve	+ve	+ve	-ve/+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	Blue/R	+ve(Blue)	143	Table 2: Biochemical Characteristics of Bacteria Isolates from Water Sample s
C.F	-ve	+ve	+ve	+ve	+ve	-ve/+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	Grey/F	+ve	144	
E.A	-ve	+ve	+ve	+ve	-ve	-ve/+ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	Cream/R	+ve(BDC)	145	
E.C	-ve	+ve	+ve	+ve	+ve	-ve/+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	Cream/R	+ve(GMS)	146	
K.P	-ve	+ve	+ve	+ve	+ve	-ve/+ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve	Cream/R	+ve(BDC)	147	
P.A	-ve	-ve	-ve	-ve	-ve	-ve/+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	Cream/R	+ve(Pink)	148	
P.V	-ve	+ve	+ve	-ve	-ve	-ve/+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	Blue/F	+ve(Pink)	149	
S.P	-ve	+ve	-ve	-ve	+ve	-ve/+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	Cream/F	+ve	150	
S.T	-ve	+ve	+ve	+ve	+ve	-ve/+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	Black/F	+ve	151	
S.TY	-ve	+ve	-ve	-ve	+ve	-ve/+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	Cream/F	+ve	152	
S.M	-ve	+ve	-ve	-ve	-ve	-ve/+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve	Red/F	+ve(Red)	153	
S.D	-ve	+ve	-ve	-ve	+ve	-ve/+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	Pink/R	+ve	154	
V.C	-ve	+ve	+ve	-ve	+ve	-ve	+ve/+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	Yellow/R	+ve(Yellow)	155	

156

157 Keys: AB-*Acinetobacter baumannii*, CF-*Citrobacter freundii*, EA-*Enterobacter aerogenes*, EC-*Escherichia coli*, KP-*Klebsiella pneumonia*, PA-
158 *Pseudomonas aeruginosa*, PV-*Proteus vulgaris*, SP-*Salmonella paratyphi*, ST- *Salmonella typhi*, STY-*Salmonella typhimurum*, SM-*Serratia*
159 *marcescens*, SD-*Shigella dysenteriae*, VC-*Vibrio cholerae*. +ve-positive, -ve - negative, Gl-Glucose, Su-Sucrose, La-Lactose, Ma-Mannitol, TSI-
160 Triple Sugar Iron test, O/C-Oxidase/catalase, Mt-Motility test, Ci- Citrate, Ur- Urease, Ni-Nitrate test, IN- Indole, MR- Methyl Red, VP- Voges-
161 proskaus, COT- Coagulase test, NA-Nutrient Agar, EMB-Eosine Methylene Blue agar, BDC-Black Dark centre, GMS- Greenish Metallic Sheen,
162 T.I.- Tentative Identity.

163 **3.3 Antibiotics Sensitivity Pattern of the Bacterial Isolates**

164 The results of the antibiotics sensitivity pattern of the bacterial isolates before plasmid curing based on
165 their zones of inhibitions subjected to statistical analysis at $p \leq 0.05$ levels of significance are presented in
166 table 3, while their deduced antibiotic resistance patterns of the multiple antibiotic resistant bacteria are
167 presented in table 4. The resistance patterns were denoted by comparison of analyzed data with
168 accepted standards for Gram negative bacteria. The zones of inhibition ranges from 10.00 ± 0.577 mm to
169 24.67 ± 0.577 mm with septrin being the least effective on *Acinetobacter baumannii* and Ciprofloxacin being
170 the highest on *Escherichia coli*

171 The antibiotic resistance patterns in Tables 4 were all denoted as either Susceptible (S) at ≤ 16.00 mm
172 and above, Intermediate (I) at $\leq 12.00 - 15.00$ mm or Resistant (R) at ≤ 11.00 .

173 **3.4 Plasmid Profiles of Multiple Antibiotic Resistant Bacterial Isolates**

174 The Gram negative bacterial isolates which were resistant to more than two antibiotics were termed
175 multiple antibiotic resistant isolates (MDRIs) and were subsequently profiled for plasmid analysis as
176 represented in the electropherogram (Plate 1).

177 From this plate, isolate in lane 2, 6, 7, 8 and 11, all have plasmid band with isolate in lane 11 showing
178 double bands. All of these isolates have plasmid band ranging from 1567bp to 2027bp

179 The electropherogram depicts the different plasmid sizes of the profiled Gram negative producing MDRIs
180 and their magnitudes.

181 **Table 3: Antibiotics Sensitivity pattern of the Isolated bacteria**

1	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	S
AB	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	15.67±0.577 ^d	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a
CF	13.67±0.577 ^c	15.67±0.577 ^c	14.67±0.577 ^d	14.67±0.577 ^d	11.67±0.577 ^b	10.00±0.577 ^a	13.67±0.577 ^c	15.67±0.577 ^c	18.67±0.577 ^e	13.67±0.577 ^c
EA	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^E	10.00±0.577 ^a	10.00±0.577 ^a	15.67±0.577 ^d	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a
EC	17.67±0.577 ^d	10.00±0.577 ^a	16.67±0.577 ^c	24.67±0.577 ^f	10.00±0.577 ^a	10.00±0.577 ^a	24.67±0.577 ^f	24.67±0.577 ^f	23.67±0.577 ^e	11.67±0.577 ^b
KP	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	14.67±0.577 ^d	10.00±0.577 ^a	10.00±0.577 ^a	14.67±0.577 ^d	10.00±0.577 ^a	10.00±0.577 ^E	10.00±0.577 ^a
PA	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	23.67±0.577 ^d	10.00±0.577 ^a	10.00±0.577 ^a	12.67±0.577 ^b	10.00±0.577 ^a	10.00±0.577 ^E	13.67±0.577 ^c
PV	10.00±0.577	12.67±0.577 ^c	19.67±0.577	19.67±0.577	14.67±0.577 ^d	15.67±0.577 ^d	19.67±0.577 ^b	22.67±0.577	14.67±0.577 ^c	10.00±0.577 ^a
SP	11.67±0.577 ^b	13.67±0.577 ^c	12.67±0.577 ^c	15.67±0.577 ^d	11.67±0.577 ^b	10.00±0.577 ^a	16.67±0.577 ^d	17.67±0.577 ^d	16.67±0.577 ^f	13.67±0.577 ^c
ST	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	12.33±0.577 ^c	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	12.33±0.577 ^b	10.00±0.577 ^a
STY	10.00±0.577 ^a	10.00±0.577 ^a	10.00±0.577 ^a	23.67±0.577 ^e	10.00±0.577 ^a	10.00±0.577 ^a	20.46±0.57 ^b	24.67±0.57	22.67±0.577 ^c	20.00±0.000 ^a
SM	17.67±0.577 ^d	15.67±0.577 ^d	21.67±0.577 ^b	19.67±0.577 ^e	10.00±0.577 ^a	10.00±0.577 ^a	17.67±0.577 ^d	21.67±0.577	20.67±0.577 ^g	14.67±0.577 ^d
SD	10.00±0.577 ^a	10.00±0.577 ^a	12.33±4.041 ^b	14.33±0.577 ^d	10.00±0.577 ^a	10.00±0.577 ^a	22.67±0.577 ^c	14.67±0.577 ^d	19.67±0.577 ^e	10.00±0.577 ^a
VC	10.00±0.577 ^E	10.00±0.577 ^E	10.00±0.577 ^E	17.67±0.577 ^d	10.00±0.577 ^E	10.00±0.577 ^E	17.67±0.577 ^d	15.67±0.577 ^d	13.67±0.577 ^c	10.00±0.577 ^E

182 Means followed by the same letter(s) within the group along the same column are not significantly different at $p \leq 0.05$ levels of significance using Duncan's new multiple range test.

183

184 Keys: 1-Isolates, SXT-Septrin, CH-Chloramphenicol, SP-Sparfloxacin, CPX-Ciprofloxacin, AM-Amoxacillin, AU-Augmentin, CN-Gentamycin, PEF-Pefloxacin, OFX-Ofloxacin, S-

185 Streptomycin, AB-Acinetobacter baumannii, CF-Citrobacter freundii, EA-Enterobacter aerogenes, EC-Escherichia coli, KP-Klebsiella pneumoniae, PA-Pseudomonas aeruginosa, PV-

186 Proteus vulgaris, SP-Salmonella paratyphi, ST- Salmonella typhi, STY-Salmonella typhimurum, SM-Serratia marcescens, SD-Shigella dysenteriae, VC-Vibrio cholerae

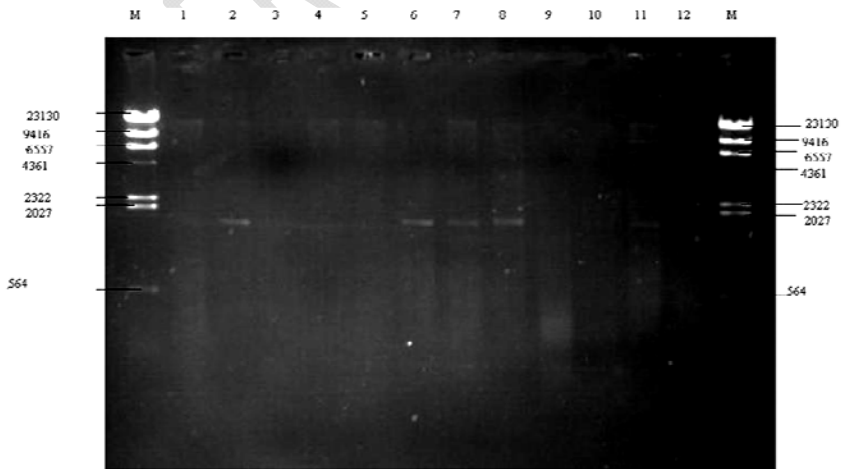
187

Comment [DJ10]: Please how did you arrive at the same standard error values for the entire content.....I also perceive it is inaccurate, see a good statistician

188 Table 4: Deduced Antibiotics Sensitivity pattern of Multiple Antibiotic Resistant Bacteria

AT	AB	CF	EA	EC	KP	PA	PV	SP	ST	STY	SM	SD	VC
SXT	R	I	R	S	R	R	R	R	R	R	R	R	R
CH	R	I	R	R	R	R	R	R	R	R	R	R	R
SP	R	I	R	I	R	R	R	R	R	R	R	R	R
CPX	R	I	R	S	I	S	R	R	R	R	R	R	R
AM	R	R	R	R	R	R	R	R	R	R	R	R	R
AU	R	R	R	R	R	R	R	R	R	R	R	R	R
CN	I	I	S	S	I	I	R	R	R	R	R	I	R
PEF	R	I	R	S	R	R	S	R	R	I	I	R	R
OFX	R	S	R	S	R	R	I	S	I	S	S	S	I
S	R	I	R	R	R	I	R	S	R	S	S	R	R

189 Keys: R-Resistance, S-Susceptible, I-Intermediate, AT- Antibiotic, SXT-Septin, CH-Chloramphenicol, SP-
190 Sparfloxacin, CPX-Ciprofloxacin, AM-Amoxicillin, AU-Augmentin, CN-Gentamycin, PEF-Pefloxacin, OFX-
191 Ofloxacin, S-Streptomycin, AB-*Acinetobacter baumannii*, CF-*Citrobacter freundii*, EA-*Enterobacter*
192 *aerogenes*, EC-*Escherichia coli*, KP-*Klebsiella pneumoniae*, PA-*Pseudomonas aeruginosa*, PV-*Proteus*
193 *vulgaris*, SP-*Salmonella paratyphi*, ST- *Salmonella typhi*, STY-*Salmonella typhimurum*, SM-*Serratia*
194 *marcescens*, SD-*Shigella dysenteriae*, VC-*Vibrio cholerae*



195 Plate 1: Electropherogram of multiple antibiotic resistant bacteria plasmids of screened isolates

196 3.5 Post-Curing Antibiotics Sensitivity Analysis of Cured MDRI Bacterial Isolates

197 The five multiple antibiotic resistant bacterial isolates that possess plasmid bands were cured of their
198 plasmids. They were then re-subjected to antibiotics sensitivity test to elucidate their resistance pattern.
199 The antibiotic sensitivity pattern of the MDRI bacterial isolates after plasmid curing are presented in
200 Table 5 while their deduced antibiotic resistant patterns are contained in Table 6. The multiple antibiotic
201 resistant isolates (MDRI) were screened out of the Gram negative bacterial isolates using sensitivity
202 discs containing Septrin, Chloramphenicol, Sparfloxacin, Ciprofloxacin, Amoxicillin, Augmentin,
203 Gentamycin, Pefloxacin, Ofloxacin, and Streptomycin. The zones of inhibition ranges from From
204 10.67 ± 0.577 mm to 27.667 ± 0.577 mm with chloramphenicol being the least effective on *P. vulgaris* and
205 Ciprofloxacin being the highest on *Escherichia coli*. From the table, it can be deduced that *E. coli* was
206 resistant to Amoxicillin, Augmentin, Chloramphenicol, while it was susceptible to Ciprofloxacin,
207 Gentamycin, Septrin and Pefloxacin. *Enterobacter aerogenes* was resistant to Septrin, Chloramphenicol,
208 Sparfloxacin, Ciprofloxacin, Amoxicillin, Augmentin, Pefloxacin, Ofloxacin, and Streptomycin and they
209 are all significantly different from Gentamycin which had the effect of inhibiting it. Also, *Klebsiella*
210 *pneumoniae* was resistant to Septrin, Chloramphenicol, Amoxicillin, Augmentin, Pefloxacin, Tarivid,
211 Streptomycin but it was susceptible to Ciprofloxacin and Gentamycin.

212 *Salmonella paratyphi* was resistant to Augmentin, Amoxicillin and Septrin while it was susceptible to
213 Pefloxacin, Gentamycin and Ofloxacin, Pefloxacin, *Salmonella typhi* was resistant to Septrin,
214 Chloramphenicol, Sparfloxacin, Amoxicillin, Augmentin, Gentamycin, Pefloxacin, and Streptomycin but
215 was inhibited and by Ciprofloxacin and Ofloxacin. Also, *Pseudomonas aeruginosa* was resistant to
216 Septrin, Chloramphenicol, Sparfloxacin, Amoxicillin, Augmentin, Pefloxacin, Ofloxacin while it was
217 susceptible to Ciprofloxacin, Gentamycin and Streptomycin. Augmentin had no effect on *C. freundii* while
218 Septrin, Gentamycin, Streptomycin were all having the same effect on *C. freundii* and they were
219 significantly different from others which was capable of inhibiting *C. freundii*.

220 *Salmonella typhimurum* was resistant to Amoxicillin, Augmentin, Sparfloxacin, Chloramphenicol, Septrin
221 and Streptomycin while susceptible to Gentamycin, Ofloxacin, Ciprofloxacin and Pefloxacin. Only
222 Gentamycin was capable of inhibiting *Acinetobacter baumannii* while it was resistant to the other
223 antibiotics.

224

225 **Table 5: Post-Curing Antibiotics sensitivity analysis of cured Bacteria Isolates**

I	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	S
E.	21.667±0.577	19.667±0.577	18.667±0.577	27.667±0.577	21.667±0.577	20.667±0.577	25.667±0.577	25.667±0.577	23.667±0.577	19.667±0.577 ^d
C	^d	^d	^c	^f	^d	^d	^f	^f	^e	
K.	15.667±0.57 ^b	15.667±0.57 ^b	19.667±0.57 ^d	25.667±0.57 ^f	14.667±0.57 ^b	17.667±0.577	24.667±0.57 ^c	19.667±0.57 ^d	16.667±0.577	16.667±0.577 ^c
P						^c			^c	
P.	17.667±0.577	15.667±0.577	19.667±0.577	20.667±0.57 ^d	13.667±0.57 ^b	11.667±0.577	22.667±0.57 ^c	18.667±0.57 ^c	23.667±0.577	17.67±0.577 ^c
A	^c	^c	^d			^a			^e	
P.	11.667±0.577	10.67±0.577 ^a	19.667±0.577	19.667±0.57 ^d	14.667±0.577	15.667±0.577	19.667±0.57 ^d	22.667±0.57 ^e	14.667±0.57 ^b	10.67±0.57 ^a
V	^a		^d		^b	^b				
S.T	21.667±0.577	19.667±0.577	18.667±0.577	27.667±0.577	21.667±0.577	20.667±0.577	25.667±0.577	25.667±0.577	23.667±0.577	19.667±0.577 ^d
	^d	^d	^c	^f	^d	^d	^f	^f	^e	

Comment [DJ11]: These standard error values are inaccurate

226 Keys: R-Resistance, S-Susceptible, I-Intermediate, SXT-Septrin, CH-Chloramphenicol, SP-Sparfloxacin, CPX-Ciprofloxacin, AM-Amoxacillin, AU-Augmentin, CN-
 227 Gentamycin, PEF-Pefloxacin, OFX-Ofloxacin, S-Streptomycin, E.C- *Escherichia coli*, K.P- *Klebsiella pneumoniae*, P.A- *Pseudomonas aeruginosa*, P.V- *Proteus*
 228 *vulgaris*, S.T-*Salmonella typhi*, 1- Isolates.

230 **Table 6: Deduced Antibiotics Sensitivity Patterns of Multiple Antibiotics Bacteria after Plasmid curing**

1	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	S
E.C	S	S	S	S	S	S	S	S	S	S
K.P	I	I	S	S	I	S	S	S	I	I
P.A	I	I	I	I	I	I	S	I	S	I
P.V	I	R	S	S	I	I	S	S	I	R
S.T	S	S	S	S	S	S	S	S	S	S

231 Keys: R-Resistance, S-Susceptible, I-Intermediate, SXT-Septrin, CH-Chloramphenicol, SP-Sparfloxacin, CPX-Ciprofloxacin, AM-Amoxacillin, AU-
232 Augmentin, CN-Gentamycin, PEF-Pefloxacin, OFX-Ofloxacin, S-Streptomycin, E.C- *Escherichia coli*, K.P- *Klebsiella pneumoniae*, P.A-
233 *Pseudomonas aeruginosa*, P.V- *Proteus vulgaris*, S.T-Salmonella *typhi*, 1- Isolates.

234 The water sources with the exception were positive for presumptive and confirmatory tests, which
235 indicates that these water sources contain coliforms especially *Escherichia coli*. This is in agreement with
236 the research of Odeyemi [14] which presented *E. coli* as a common encounter in different water sources;
237 be it rivers, streams, rain water, well water, underground water and even pipe borne water. The stream
238 water also ranked higher in microbial contamination compared to other sources of water. This could be
239 based on the fact that it is categorized as a surface water hence subject to influx of bacteria isolates. This
240 study has been able to establish the correlation between ESBL production and the MDRI screened out
241 by the antibiotic sensitivity test. All the MDRI were implicated for ESBL production as it was observed in
242 *A. baumannii*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, *S. typhi*, *S. typhimurum*
243 and *S. dysenteriae*. According to Souha and Zeina [15], the production of Beta-lactamase enzymes by
244 bacteria is one of the most common causes of bacterial resistance to the Beta-lactam antibiotics and
245 Gram negative bacteria producing these enzymes are formidable adversaries to microbiologists and
246 researchers [15]. The antibiotic resistance patterns in this present study is in agreement with the findings
247 of Odeyemi [16] which reported most of the tested isolates to be least resistance to Ofloxacin.

248
249 The isolates were resistant to Amoxicillin, which corroborated the findings of Rahal [17] confirming that
250 most strains of *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia*, *Salmonella*, *E. coli* and
251 indole positive *Proteus* species are resistant to Ampicillin. Incidence of multiple antibiotic resistant
252 bacteria (MDRI), and especially that they possess plasmids in this study is in agreement with the study
253 of Akinyemi [18]. Five (5) out of twelve (12) isolates on which plasmid analysis were carried out contained
254 plasmid band whose molecular weight ranged from 1564bp to 2027bp. This might be responsible for the
255 initial antibiotic resistance exhibited by the isolates before plasmid analysis in this study while the
256 resistance observed in other isolates might have been chromosomal mediated and this is in agreement
257 with the findings of Kroll [19] who submitted that plasmid have encoded genes that provide resistance to
258 occurring antibiotic in competitive environmental niche.

259
260 The post-curing antibiotics sensitivity analysis carried out on the MDRI bacterial isolates revealed that
261 the test isolates were susceptible to those antibiotics that they were previously resistant to. This implies
262 that the presence of the plasmids in the five isolates were responsible for the multiple antibiotic resistance
263 pattern exhibited by the isolates initially. This finding agrees with the work done by Afolami [20] who
264 reported that plasmid-mediated mechanisms might increase like-hood of horizontal spread of antibiotic
265 resistances in bacteria. More so, efflux pump mechanisms or other factors like mutation of gene encoding
266 ribosomal protein, which decreases permeability of the cell envelope in enteric bacteria might also be
267 responsible for antibiotic resistances [21].

Comment [DJ12]: Do you mean likelihood or any other thing?

271 **4. CONCLUSION**

272 Shortage of water for drinking and household use has been a major concern to public health in
273 developing countries; this work has been able to characterize Gram negative bacteria associated with
274 different water sources in Akure south local government area of Ondo State, Nigeria. Worrisome is the
275 fact that majority of these water samples contain Gram negative bacteria that are resistant to antibiotics.
276 This work further confirms the emergence of resistance of microorganism to current antibacterial. More
277 worrisome is the fact that some of these Gram negative bacteria contain plasmid(s) which ease the
278 transfer of resistant genes to other members of the population. Thus, there is a need for an inexpensive
279 medium of purification of water prior to human intake to avoid deleterious effect of these pathogens in the
280 area of study.

281

282 **COMPETING INTERESTS**

283 No competing interest exist

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286 rural challenge of the decade. The World Health Report. Geneva, Switzerland: ISBN 97892 4
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