Widespread occurrence of antibiotic resistance genes in bacterial isolates from River Ala in Akure, Nigeria

ABSTRACT

Antibiotic resistance genes can be naturally occurring in bacteria or can be acquired through horizontal gene transfer. This study investigated the antibiotic resistance genes in bacterial isolates from River Ala. Water samples were collected bi-weekly over a period of 24 weeks from three representative points in River Ala. The load and identities of bacteria in the water samples were determined using standard microbiological methods. Antibiotic susceptibility pattern of the isolates were determined using disc diffusion method. Antibiotic resistance genes (ARGs) were detected using molecular methods. Results showed that the total plate count ranged from 7.0×10^1 to 1.3×10^4 cfu/100 ml and total enterobacteriaceae count ranged from 9.2×10^2 to 4.2×10^4 cfu/ 100 ml. The bacterial isolates detected in the samples were *Escherichia coli, Salmonella enterica*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Enterobacter cloacae* Antibiotic susceptibility pattern of the isolates revealed that all the bacterial isolates had 100% resistance to penicillin, while 92.86% of the isolates were resistant to nalidixic acid and ceporex. ARGs such as blaNDM, blaTEM, blaSHV and tetA were detected in *Escherichia coli, Salmonella enterica* and *Enterobacter cloacae*. There were positive correlations between *Escherichia coli, Salmonella enterica* and *Enterobacter cloacae*, ARGs such as blaNDM, blaTEM, blaSHV and tetA. The findings of this study demonstrated the presence of ARGs such as blaNDM, blaTEM, blaSHV and tetA in *Escherichia coli, Salmonella enterica* and *Enterobacter cloacae* in water samples from River Ala. Understanding the presence and dynamics of these genes in aquatic environments is essential for managing public health risks.

Keywords: Antibiotic resistance genes, antibiotic susceptibility pattern, enterobacteriaceae.

INTRODUCTION

Antibiotics are one of the most transformative discoveries in the history of medicine, revolutionizing the strategies for combating bacterial infections (Jamal *et al*., 2023). It has saved many lives and significantly extended human life expectancy. Resistance occurs when bacteria evolve mechanisms to withstand antibiotics designed to inhibit or eliminate their cells (Amente *et al*., 2023). This natural evolutionary process has been exacerbated by the widespread and often indiscriminate use of antibiotics in healthcare, agriculture, and veterinary medicine (Tiedje *et al*., 2023). Antibiotic resistance genes can be naturally

33 occurring in bacteria or can be acquired through horizontal gene transfer (Kavya *et al*., 2023; Mancuso *et*
34 *al*., 2023).
35
36 The direct selection pressure exerted by antibiotics on organisms carrying antibiotic resistance genes
37 (ARGs) suggests that the routes taken by antibiotic-resistant microorganisms and their ARGs are likely
38 similar to those of the antibiotics themselves. Additionally, it is likely that ARGs remain present in these
39 pathways, as they are frequently retained within microbial populations even after the antibiotic selection
40 pressure is removed (Li *et al*., 2023). Aquatic environments are known to be a reservoir for antibiotic-
41 resistant bacteria (ARB), which are a major global public health concern due to their prevalence and
42 dissemination (Muteeb *et al.*, 2023).
43
44 Antibiotics found in sewage and agricultural runoff, which result from the widespread and increased use of
45 antibiotics, select for and enrich naturally occurring antibiotic-resistant genes (ARGs) and ARBs in the
46 aquatic environment (Manaia *et al*., 2024). Many of these bacteria are hosts of antibiotic resistance genes,
47 water ecosystems are seriously contaminated by these antibiotic-resistant microorganisms (Mounzer *et al.*,
48 2024). Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and multi-
49 resistant pseudomonads are threat to ecological public health (Bai *et al*., 2024). The use of antibiotics in
50 medical settings, by veterinarians, and in animal husbandry may contribute to the promotion of antibiotic
51 resistance and its genetic material transmission in bacteria (Duwor *et al.*, 2024).
52
53 The degree of simplicity of the DNA that makes an organism resistant to antibiotics and the ease with which
54 it can obtain DNA from other microbes are often linked to the development of antibiotic resistance (Muteeb
55 *et al.,* 2023). Two essential components must come together for antibiotic resistance to form: an antibiotic
56 that can inhibit most of the bacteria in a colony and a heterogeneous colony of bacteria, where at least one
57 bacterium has a genetic determinant that can express antibiotic resistance (Tran *et al*., 2024). Susceptible
58 bacteria in the colony are inhibited, while resistant strains survive. The genetic determinants of the surviving
59 bacteria specify the kind and level of resistance that the bacterial cell will express (Pepi and Focardi, 2021).
60 Antibiotic resistance can occur naturally (intrinsic) or be acquired, and it can spread both vertically and
61 horizontally (Liang *et al.*, 2022). Antimicrobial resistance genes can be transferred from other bacteria to
62 susceptible bacteria, or they can be acquired genetically (Djordjevic *et al*., 2024). The study was aimed at
63 determining the antibiotic resistance genes in bacterial isolates from River Ala.
64
65
66 **METHODS**

Study area

The study area is the upper region of River Ala catchment in Akure, Ondo State, Nigeria. The catchment lies between Latitudes 7° 14’ N, and 7° 17’N, and Longitudes 5° 8’ E and 5° 16’ E covering a total area of 55 km² (Figure 1). The River Ala and its tributaries is one of the main tributaries of River Ogbese in Southwestern, Nigeria. River Ala has a total length of about 57 km of which 14.8 km traverses the thickly populated built up area of Akure Township. The river takes its source from northwestern part of Akure town and flow southeastern direction of the town. The study area experiences an intermittent rain fall between February and July, with the heaviest rainfall in July. The river was selected due to its close proximity to sources of faecal contamination, its use for recreational activities as well as the use of the water for irrigation.

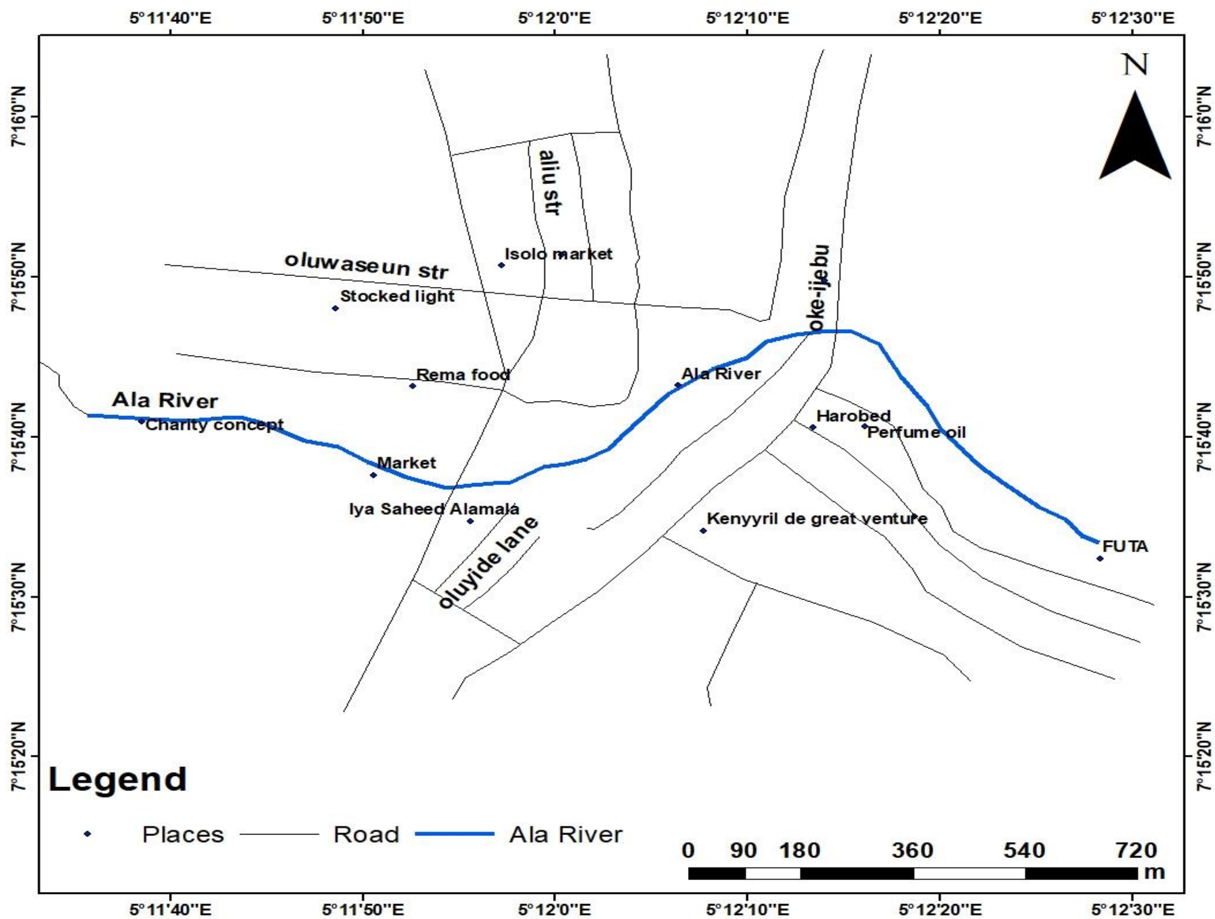

Figure 1: The location of River Ala in Akure, Ondo State, Nigeria

83 **Sample collection**

84 Water samples were collected during wet and dry season from the three sampling points. Sample collection
85 was done at the three different sampling points bi-weekly for 24 weeks, 36 samples were collected in total
86 for the study. Sterile bottles of 500 ml were used for sample collection. Samples for microbial analysis were
87 collected aseptically, labelled and stored in ice packed plastic coolers and transported to the laboratory in
88 the Department of Microbiology at the Federal University of Technology, Akure, Nigeria where analysis
89 was done within one hour of collection.

90

91 **Determination of total plate count and total enterobacteriaceae count in the water samples**

92 Serial dilutions of the water samples were carried out aseptically up to 10^{-4} dilution in order to obtain
93 countable bacteria colonies on the agar plate. Dilution 10^{-3} and 10^{-4} were plated on different media (Mac
94 agar, SSA, MFCA and MLSA). Colonies with different morphologies were observed on the plates and
95 streaked out on Nutrient Agar plate for purification. Colonies were later stored at 4ºC on Nutrient Agar
96 (NA) slant.

97

98 **Biochemical identification of bacterial isolates from water samples from River Ala**

99 Biochemical tests such as Gram staining, catalase, oxidase, sugar fermentation, citrate, indole etc. were
100 carried out on the bacterial isolates from the water samples (Dimri *et al*., 2020).

101

102 **Enumeration of faecal coliforms in water samples from River Ala**

103 The concentrations of *Escherichia coli*, faecal coliforms, *Salmonella* and *Shigella* in the water samples
104 were determined using standard microbiological methods. Using membrane filters (0.45 µm), the
105 concentrations of the bacteria were determined by placing the filters on freshly prepared selective media:
106 M-lauryl sulphate agar (MLSA), eosin methylene blue (EMB), membrane faecal coliform agar (m-FC agar)
107 and *Salmonella–Shigella* agar (SSA). Agar plates were incubated at 37°C for 24 h (MLSA, EMB, SSA)
108 and 44°C for 24 h (m-FC). Colonies were counted recorded and expressed as colony forming unit (CFU)
109 per 100 ml of water utilizing a colony counter.

110

111 **Determination of antibiotic susceptibility pattern of the isolates**

112 The antibiotics susceptibility of the isolates was determined by the Kirby-Bauer disk diffusion method on
113 Mueller-Hinton agar. The isolates were tested against ten antibiotic discs (Mast Diagnostics, UK) which
114 comprised of ampicillin (PN), septrin (SXT) streptomycin (S), erythromycin (E), ciprofloxacin (CPX),
115 ceporex (CEP), nalidixic acid (NA), azithromycin (AZM), augmentin (AU), and Gentamycin (CN). The
116 inoculums were standardized by adjusting their densities to the turbidity of a Barium sulphate (BaSO4) (0.5

McFarland turbidity standard). 100 µl of bacterial suspension were spread-plated on Mueller-Hinton agar plates, the antibiotic discs were placed on the plates and plates were incubated for 24 hours at 37°C. After incubation, zones of inhibition (mm) were measured and results obtained were used to classify isolates as resistant, intermediate resistant, or susceptible using standard reference values according to Clinical and Laboratory Standards Institute (CLSI) (Ardila *et al*., 2023).

Detection of antibiotic resistant genes (ARGs) in bacterial isolates

Primer sequences described by Ismaeel and Nasser (2017) were adopted. Reaction cocktail used for all PCR per primer set included (Reagent Volume µl) - 5X PCR SYBR green buffer (2.5), MgCl2 (0.75), 10pM DNTP (0.25), 10pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06) and made up to 10.5 with sterile distilled water to which 2 µl template was added. Buffer control was also added to eliminate any probability of false amplification Table below shows the primer sequence and PCR profile used in amplifying each fragment. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair (Table 1).

Table 1: Primers used for detection of antibiotic resistance genes

Multiplex	Gene	Primer	Primer sequence 5'-3'	Profile
Multiplex1	blaVIM	VIM F	TCGTTTGAAGAAGTTAACG	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s, 50ºC for 40s 72ºC for 40s and terminate at 72ºC for 10min
		VIM R	ATGTAAGTTTCAAGAGTGATGC	
	blaNDM	NDM F	GGTGTTTGGTCGCATATCGCAA	
		NDM R	ATTCAGCCAGATCGGCATCGGC	
Multiplex2	blaIMP	IMP F	GGTTTGGCGATCTGGTTTTC	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s, 47ºC for 40s and 72ºC for 30s. and terminate at 72ºC for 10mins
		IMP R	CGGAATGGCTCATCACGATC	
	blaKPC	KPCF	CATTCAAGGGCTTTCTTGCTGC	
		KPCR	ACGACGGCATAGTCATTTGC	
	blaOXA	OXA R	TTCTGTTGTTTGGGTTTCGC	
		OXA R	ACGCAGGAATTGAATTTGTT	
Multiplex3	blaTem	Tem F	GTCGCCGCATACACTATTCTCA	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s, 49ºC for 40s 72ºC for 35s and terminate at 72ºC for 10min
		Tem R	CGCTCGTCGTTTGGTATGG	
	blaSHV	SHV F	GCCTTGACCGCTGGGAAAC	
		SHV R	GGCGTATCCCGCAGATAAAT	
Multiplex4	tet(A)	tet(A)F	GGTTCACTCGAACGACGTCA	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s,
		tet(A)R	CTGTCCGACAAGTTGCATGA	
	tet(B)	tet(B)F	CCTCAGCTTCTCAACGCGTG	

		tet(B)R	GCACCTTGCTCATGACTCT	48ºC for 40s 72ºC for 40s and terminate at 72ºC for 10min
Multiplex5	VanA	VanAF	TCTGCAATAGAGATAGCCGC	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s, 50ºC for 30s 72ºC for 60s and terminate at 72ºC for 10min
		VanAR	GGAGTAGCTATCCCAGCATT	
	VanB	VanBF	ATGGGAAGCCGATAGTC	
		VanBR	GATTTCGTTCCTCGACC	

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136 **Statistical analysis**

137 All data obtained from the study were subjected to descriptive statistics. Two-way Analysis of Variance
138 (ANOVA) were carried out using SPSS version 22 (IBM, NY) and means were separated using Duncan's
139 New Multiple Range Test at 95% confidence interval. The relationship between the pollution levels and
140 ARGs were determined using Pearson correlation coefficients.

141

142 **RESULTS**

143 **Total plate count and total enterobacteriaceae count in water samples from River Ala**

144 The total plate count (TPC) ranged from 7.0×10^1 cfu/100 ml to 1.67×10^4 cfu/100 ml. Sample 9 had the
145 highest TPC of 1.67×10^4 cfu/100 ml, while sample 3 had the lowest TPC of 7.0×10^1 cfu/100 ml and the
146 total enterobacteriaceae count (TEC) ranged from 9.2×10^2 cfu/100 ml to 4.17×10^4 cfu/100 ml. Sample
147 12 had the highest TEC of 4.17×10^4 cfu/100 ml, while Sample 1 exhibited the lowest TEC of 9.2×10^2
148 cfu/100 ml (Table 2).

149

150 **Morphological and biochemical characteristics of bacterial isolates in water samples from River Ala**

151 The morphological characteristics of the bacterial isolates from the water samples such as size, shape,
152 texture, opacity, margin, colour and elevation were determined while the biochemical tests carried out on
153 the bacterial isolates revealed the presence of *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumonia*,
154 *Proteus mirabilis* and *Enterobacter cloacae* in the water samples (Table 3 and Table 4).

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160 **Table 2: Total plate count and total enterobacteriaceae count in water samples from River Ala**

Samples	Total plate count (cfu/100 ml)	Total enterobacteriaceae count (cfu/100 ml)
1	5.3×10^2	9.2×10^2
2	2.0×10^3	4.9×10^3
3	7.0×10^1	2.1×10^3
4	3.9×10^3	9.5×10^3
5	3.9×10^3	9.7×10^3
6	3.7×10^3	1.9×10^4
7	1.0×10^2	9.9×10^2
8	2.5×10^3	8.8×10^3
9	1.7×10^4	2.7×10^4
10	5.0×10^2	3.3×10^3
11	9.8×10^3	3.2×10^4
12	1.3×10^4	4.2×10^4

161
162
163 **Table 3: Morphological characteristics of bacterial isolates in water from River Ala**

Morphological Tests							
Sample	Size	Shape	Texture	Opacity	Margin	Colour	Elevation
A1	medium	circular	shiny	translucent	entire	pink	flat
A2	medium	rhizoid	dry	transparent	irregular	cream	flat
A5	small	circular	dry	translucent	entire	black	flat
A6	small	circular	shiny	opaque	entire	cream	flat
A9	small	circular	dry	opaque	irregular	cream	flat
A11	large	irregular	dry	opaque	irregular	cream	flat
A23	small	irregular	dry	opaque	irregular	cream	flat
A24	small	circular	dry	opaque	entire	cream	flat
A26	medium	rhizoid	dry	transparent	irregular	cream	flat
A29	large	irregular	dry	opaque	irregular	cream	flat
A31	medium	circular	shiny	translucent	entire	cream	flat
A32	medium	circular	shiny	transparent	entire	cream	raised
A35	small	circular	shiny	transluscent	entire	pink	flat
A36	small	irregular	shiny	transluscent	irregular	pink	flat
A37	small	irregular	shiny	transluscent	irregular	pink	flat
A38	small	irregular	shiny	opaque	entire	cream	raised
A39	small	circular	shiny	opaque	entire	cream	raised
A40	small	circular	dry	opaque	entire	cream	flat
A41	small	circular	dry	opaque	entire	cream	flat

164 **Table 4: Biochemical characteristics of bacterial isolates in water from River Ala**

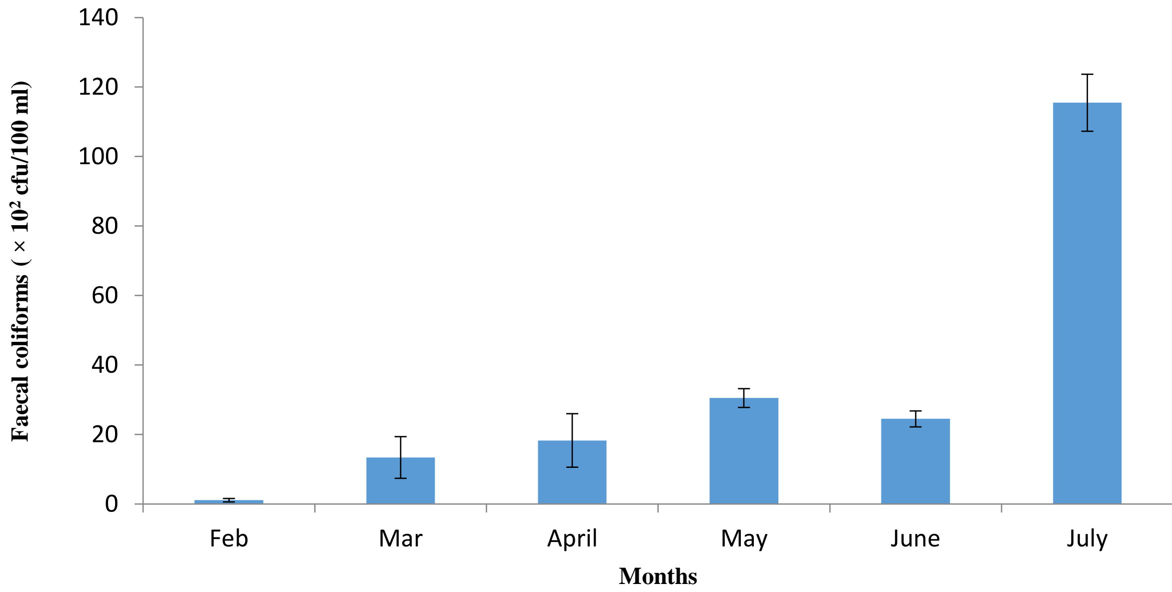
Sample	Shape	G-RXT	Motility	Catalase	Oxidase	Citrate	Indole	Glucose	Lactose	Sucrose	MR	VP	Probable Organisms
		Biochemical Tests											
A1	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A2	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A5	rod	-ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	*Samonella enterica*
A6	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A9	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A11	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A23	rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Enterobacter cloacae*
A24	rod	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	*Proteus mirabilis*
A26	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Klebsiella pneumonia*
A29	rod	-ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	*Samonella enterica*
A31	rod	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	*Klebsiella pnuemonia*
A32	rod	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	*Proteus mirabilis*
A35	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A36	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A37	rod	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	*Escherichia coli*
A38	rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Enterobacter cloacae*
A39	rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Enterobacter cloacae*
A40	rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Enterobacter cloacae*
A41	rod	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Enterobacter cloacae*

165 **Keys:** G-RXT- Gram reaction, MR- Methyl red, VP- Vogues-Proskauer

166

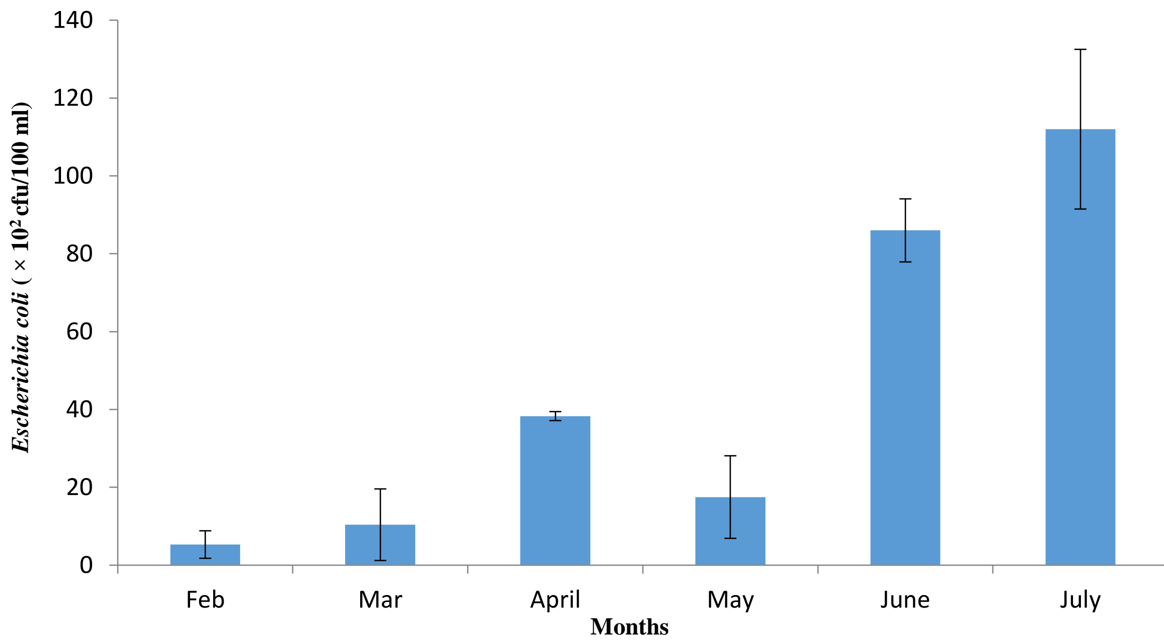
167 **Load of bacteria in water samples from River Ala**

168 The mean load of faecal coliforms ranged from 1.1 x 10^2 to 1.2 x 10^4 cfu/100 ml. The highest was recorded
169 in July (1.2 x 10^4 cfu/100 ml. while the least was recorded in February (1.1 x 10^2 cfu/100 ml) (Figure 2).
170 The mean load of *E. coli* ranged from 5.3 x 10^2 to 1.1 x 10^4 cfu/100 ml. The highest was recorded in July
171 (1.1 x 10^4 cfu/100 ml), while the least was recorded in February (5.3 x 10^2 cfu/100 ml) (Figure 3). The
172 mean load of *Salmonella* spp. ranged from 2 x 10^2 to 1.3 x 10^4 cfu/100 ml. The highest was recorded in
173 July (1.3 x 10^4 cfu/100 ml), while the least was recorded in February (2 x 10^2 cfu/100 ml) (Figure 4). The
174 mean load of *Shigella* spp. ranged from 0.8 x 10^2 to 1.3 x 10^3 cfu/100 ml. The highest was recorded in July
175 (1.3 x 10^3 cfu/100 ml), while the least was recorded in February (0.8 x 10^2 cfu/100 ml) (Figure 5).



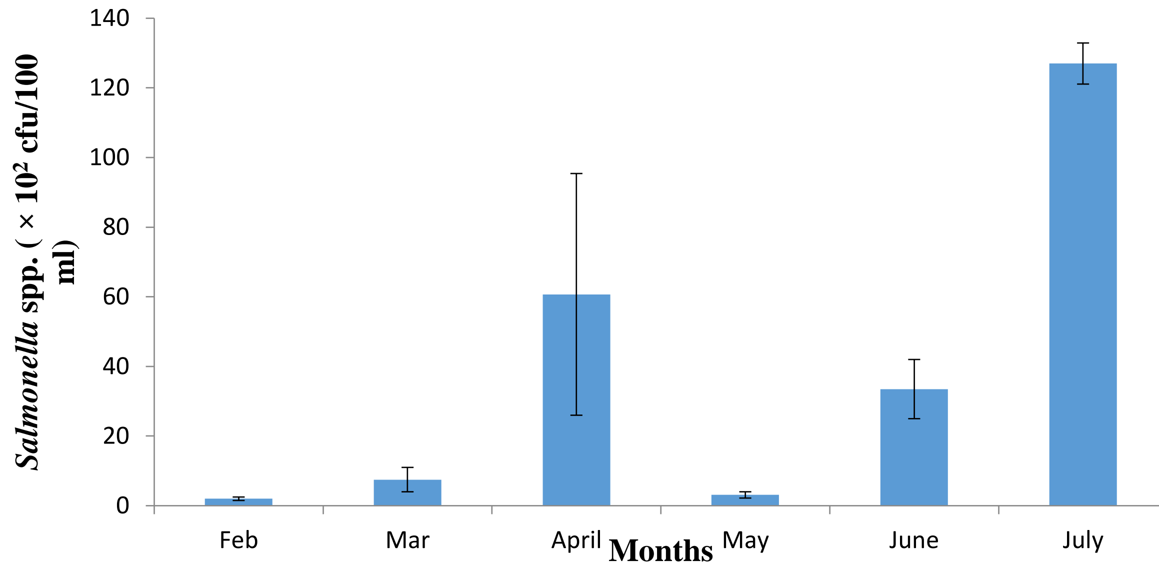
176

177 **Figure 2:** Mean load of faecal coliforms in water samples from River Ala (n=36)



178

179 **Figure 3:** Mean load of *Escherichia coli* in water samples from River Ala (n=36)



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181 **Figure 4:** Mean load of *Salmonella* spp. in water samples from River Ala (n=36)

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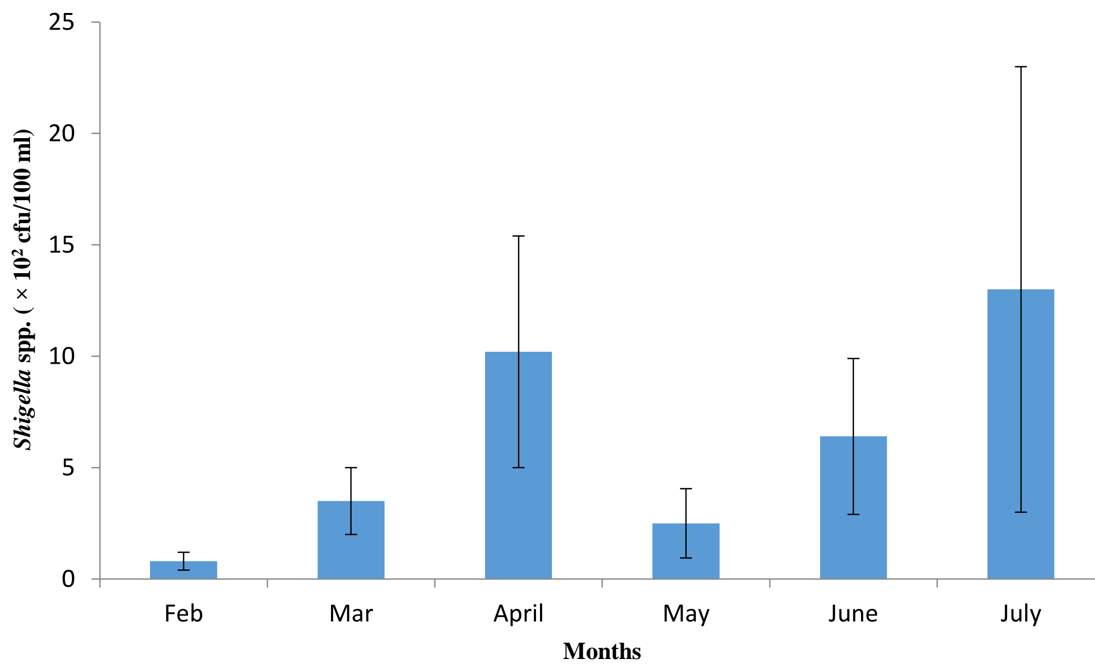


184

185 **Figure 5:** Mean load of *Shigella* spp. in water samples from River Ala (n=36)

186

Antibiotic resistance pattern of bacterial isolates from River Ala

The antibiotic resistance patterns observed in bacterial isolates from River Ala against ten (10) different antibiotics with varying resistance rates to commonly used antibiotics such as penicillin (100%), ceporex (92.86%), nalidixic acid (92.86%), augmentin (85.71%) (Figure 6).

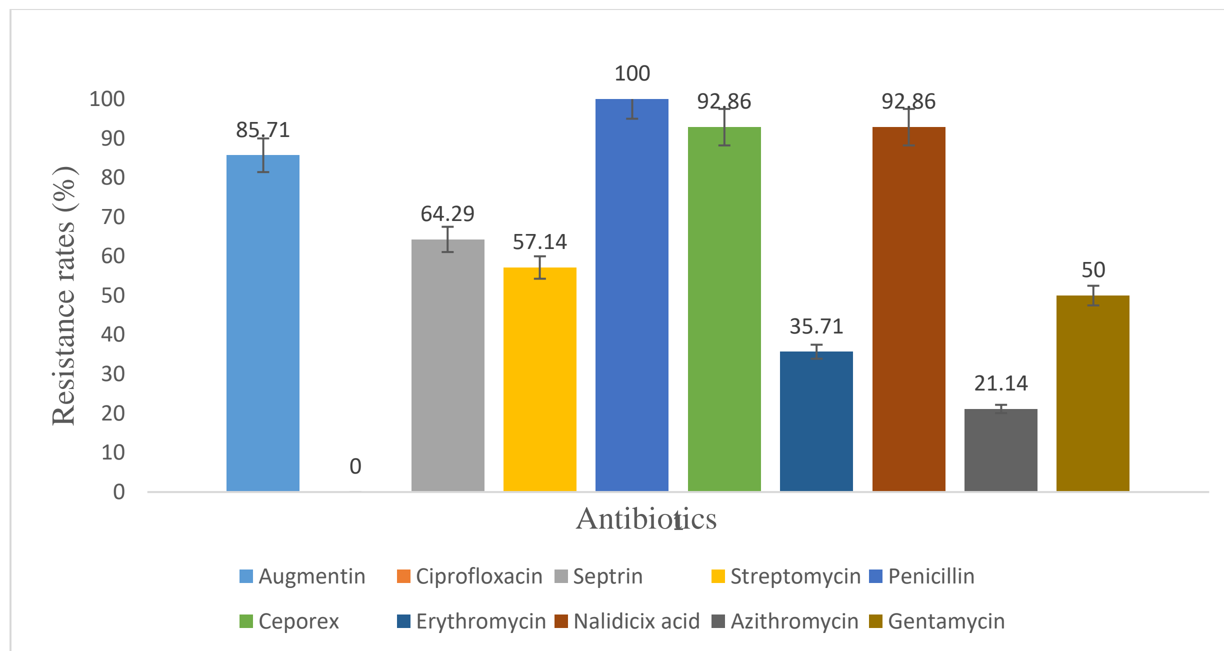


Figure 6: Antibiotic resistance pattern of the bacterial isolates against 10 different antibiotics

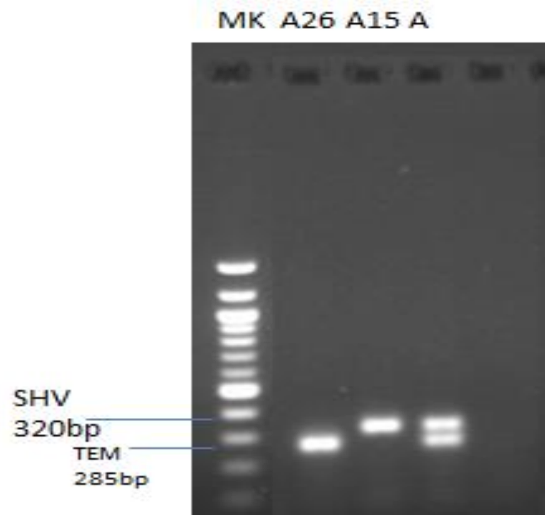
ARGs in bacterial isolates in water samples from River Ala

The antibiotic resistant genes tetA, tetB, blaVIM, blaNDM, blaTEM, blaSHV, vanA and vanB were detected in bacteria isolated from River Ala (Table 5). The gel electrophoresis of the PCR products showed positive amplification of the ESBL TEM gene in samples A26 and A and the SHV gene in samples A15 and A by the presence of specific DNA bands at the expected sizes (Plate 1). This confirms the presence of these resistance genes in the respective bacterial isolates. The agarose gel electrophoresis shows positive amplification of the ESBL NDM gene (624 bp) in samples A26 and A, indicating the presence of this resistance gene in these isolates. The absence of a 502 bp band in all samples indicates that the VIM gene is not present in any of the tested bacterial isolates (Plate 2).

The agarose gel electrophoresis shows positive amplification of the ESBL CTX-M gene (598 bp) in samples A and A15, indicating the presence of this resistance gene in these isolates. No band is present in sample A26, indicating it is negative for the CTX-M gene (Plate 3). The agarose gel electrophoresis shows positive amplification of the tetracycline resistance gene tetA (577 bp) in samples A15 and A, indicating the presence of this resistance gene in these isolates (Plate 4). No band at 634 bp indicates that the tetB gene is

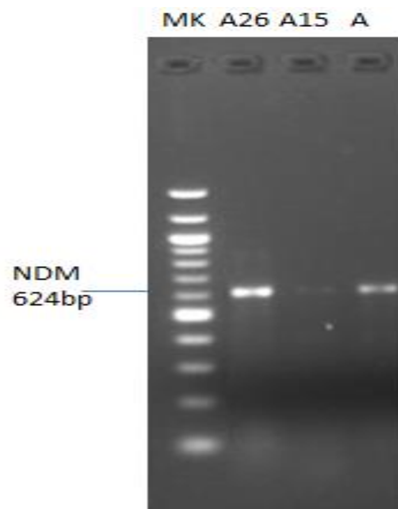
208 absent in all tested samples. The multiplex electrophoresis of the PCR products shows no bands at 400 bp
209 or 740 bp, indicating that neither the vanA gene (400 bp) nor the vanB gene (740 bp) was amplified in any
210 of the bacterial isolates (Plate 5). This shows the absence of both vanA and vanB resistance genes in the
211 tested samples.

212



213

214 **Plate 1:** Gel image indicates a positive of TEM in sample A26 and A and SHV in A15 and A. (**A**-
215 *Escherichia coli*, **A26**- *Salmonella enterica* and **A15**- *Enterobacter cloacae*)



216

217 **Plate 2:** Gel image indicates a positive of NDM in samples A26 and A while VIM absent in all samples.
218 (**A**- *Escherichia coli*, **A26**- *Salmonella enterica* and **A15**- *Enterobacter cloacae*)

219

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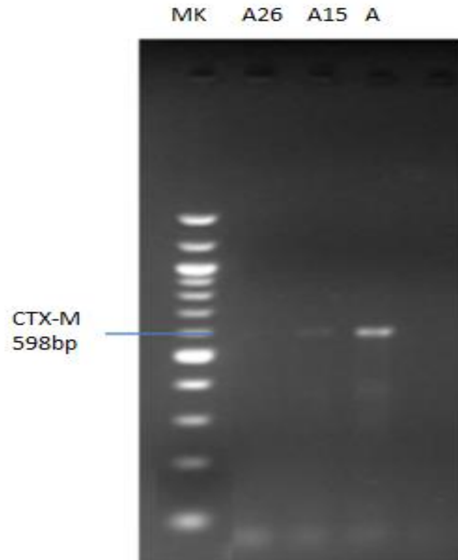
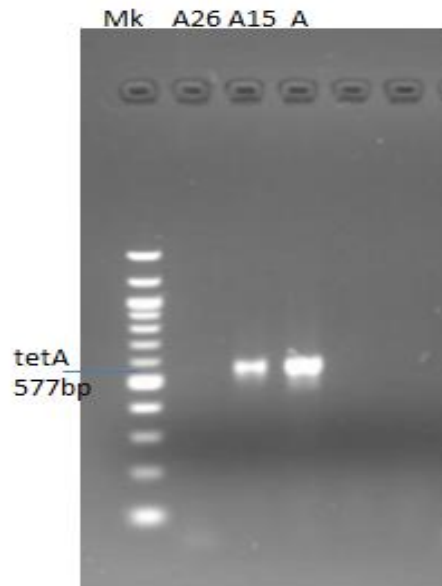
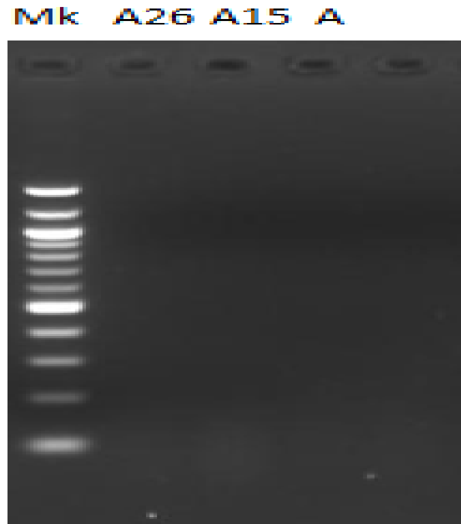


221 **Plate 3:** Gel image indicates a positive of CTX-M in only sample A and A15 but negative in A26. (**A**-
222 *Escherichia coli*, **A26**- *Salmonella enterica* and **A15**- *Enterobacter cloacae*)



223

224 **Plate 4:** Gel image indicates a positive of tetA in only sample A15 and A and tetB gene in none of the
225 sample. (**A**- *Escherichia coli*, **A26**- *Salmonella enterica* and **A15**- *Enterobacter cloacae*)



226

227 **Plate 5:** Gel image indicates no amplification for vanA and vanB gene. (**A**- *Escherichia coli*, **A26**-
228 *Salmonella enterica* and **A15**- *Enterobacter cloacae*)

229

230

231 **Table 5: ARGs in bacterial isolates in water samples from River Ala**

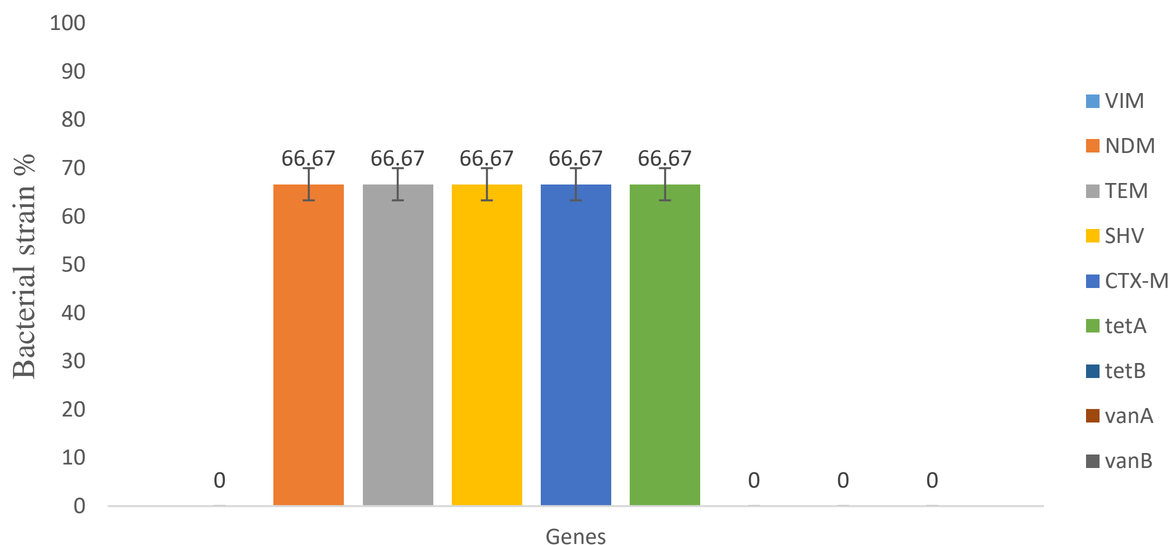
Bacterial/strain ID	bla_{VIM}	bla_{NDM}	bla_{TEM}	bla_{SHV}	bla_{CTX-M}	tetA	tetB	vanA	vanB
Escherichia coli (A)	-	+	+	+	+	+	-	-	-
Salmonella enterica (A26)	-	+	+	-	-	-	-	-	-
Enterobacter cloacae (A15)	-	-	-	+	+	+	-	-	-

232 **Keys:** Present (+), Absent (-) Note: Only isolates that gave a positive signal in at least one PCR experiment
233 were included in this table.

234

235 ## ARGs and enteric bacterial isolated from River Ala

236 The bacterial strains isolated from River Ala exhibited a high prevalence of antibiotic resistance genes. The
237 antibiotic resistant genes tetA, blaVIM, blaNDM, blaTEM and blaSHV were detected in 66.67% of the
238 bacterial isolates (Figure 7). blaNDM showed negative correlation with *Escherichia coli* and
239 *Salmonella* spp. blaTEM showed a significant positive correlation with *Escherichia coli*,
240 *Salmonella* spp. and *Enterobacter cloacae*. blaCTX and tetA showed a significant positive
241 correlation with *Escherichia coli* and *Enterobacter cloacae* (Table 6).



242

243 **Figure 7**: Percentage of bacterial strain in ARGs.

244

245

246 **Table 6: Correlation between the loads of enteric bacteria and ARGs in water samples from River**
247 **Ala**

	blaNDM	blaTEM	blaSHV	blaCTX	tetA
Escherichia coli	**-0.56****	**0.71****	0.39	**0.58****	**0.93****
Salmonella spp.	**-0.55****	**0.63****	0.19	0.38	-0.11
Shigella spp.	-0.21	0.48*	0.34	-0.28	0.31
Faecal coliform	0.25	0.32	0.23	-0.44	-0.36
Enterobacter cloacae	0.32	**0.58****	0.37	**0.62****	**0.81****

248 **Keys:** **Correlation is significant at the 0.01 level (2-tailed)
249 *Correlation is significant at the 0.05 level (2-tailed)

250

251 **DISCUSSION**

252 High total plate counts and total enterobacteriaceae counts suggests bacterial contamination. This is in
253 agreement with Kilonzo-Nthenge *et al*. (2018) where the authors reported high bacterial loads to poor
254 sanitary conditions and faecal pollution in river. Morphological characteristics and biochemical tests,
255 confirmed the presence of *Escherichia coli*, *Salmonella enterica*, *Proteus mirabilis*, *Klebsiella pneumonia*
256 and *Enterobacter cloacae*. The result of this study is in line with Hanna *et al*. (2020), who reported a

257 significant presence of *E. coli*, *Salmonella*, *Klebsiella* and *Enterobacter* in river samples due to higher
258 occurrence of thermotolerant (faecal) coliform in temperate environments, contrasting with the infrequent
259 occurrence of *Escherichia coli*. The presence of *E. coli* and faecal coliforms in the water samples is in line
260 with Hanna *et al*. (2020), who reported a significant presence of *E. coli* (faecal coliform) in river water
261 samples.
262
263 High faecal coliforms counts in water samples was as a result of the weather change in Nigeria characterized
264 by intermittent rain. This observation is in agreement with Adenola *et al*. (2021) and Egberongbe *et al*.
265 (2021) where the authors reported high faecal coliform counts in surface waters during periods of heavy
266 rainfall. High mean load of *Escherichia coli* in water samples indicates indiscriminate discharge of sewage.
267 Borja-Serrano *et al*. (2020) reported that the presence of higher load of *Escherichia coli* was attributed to
268 the polluted water conditions. The high mean loads of *Salmonella* spp. observed in the water samples
269 suggests direct discharge of waste water. Akrong *et al*. (2019) reported that high loads of *Salmonella* was
270 as a result of heavy rainfall, land run-off and direct discharge of untreated or partially treated wastewater
271 into the water sources.
272
273 Antibiotic susceptibility pattern of the isolates revealed that all bacterial isolates were 100% resistance to
274 penicillin. This is in agreement with Tesfaye *et al*. (2019) where authors reported high *E.coli, Salmonella*
275 *and Enterobacter* were resistance penicillin, while 92.86% of the isolates were resistant to nalidixic acid
276 and ceporex. 85.71% of the isolates were resistant to augmentin, 64.29% of the isolates were resistant to
277 septrin, and 57.14% of the isolates were resistant to streptomycin, while 50% of the isolates were resistant
278 to gentamycin. Also, 35.71% of the bacterial isolates were resistant to erythromycin and the 21.14% of the
279 isolates were resistant to azithromycin which is in line with Bamidele *et al*. (2022) who reported a low rate
280 of resistance.
281
282 Resistance to tetracyclines is often associated with the presence of the tetA and tetB genes, which encode
283 efflux pumps that actively expel tetracycline from bacterial cells, the moderate resistance rates to other
284 antibiotics like streptomycin (57.14%) and septrin (64.29%) suggest the potential presence of these genes.
285 Li *et al*. (2024), reported a high prevalence of tetA and tetB in environmental bacterial isolates from water
286 bodies contaminated by agricultural runoff and sewage. The authors noted that the wide usage of
287 tetracyclines in animal farming had led to the enrichment of tet genes in aquatic environments. Sung *et al*.
288 (2024) further corroborated these findings, showing over 70% of bacterial isolates from rivers in

289 agricultural areas harboring tetA or tetB, with corresponding high levels of tetracycline resistance (80-
290 90%).

291 Beta-lactam resistance genes, including blaVIM, blaNDM, blaTEM, and blaSHV, play critical roles in the
292 high resistance observed to penicillin (100%), ceporex (92.86%), and augmentin (85.71%). These genes
293 encode beta-lactamases, enzymes that hydrolyze beta-lactam antibiotics and render them ineffective.
294 blaTEM and blaSHV genes are commonly associated with resistance to penicillins and first-generation
295 cephalosporins, explaining the near-complete resistance observed in this study. Taha *et al*. (2023) reported
296 a 90% prevalence of blaTEM in bacterial isolates from hospital wastewater, which correlated with over
297 85% resistance to beta-lactam antibiotics, including penicillin and amoxicillin. Similarly, Rana *et al*. (2024)
298 found that blaTEM and blaSHV were prevalent in over 80% of environmental isolates from wastewater
299 treatment plants, contributing to the widespread beta-lactam resistance observed in aquatic environments.

300 blaNDM (New Delhi metallo-beta-lactamase) and blaVIM (Verona integron-encoded metallo-beta-
301 lactamase) genes are primarily linked to carbapenem resistance but are often co-located with other
302 resistance genes. Javid and Ahmed (2024) found a rising occurrence of blaNDM in bacteria isolated from
303 rivers near healthcare facilities, with co-resistance to multiple beta-lactams, including cephalosporins like
304 ceporex. This supports the high resistance rates observed in this study, where ceporex resistance was at
305 92.86%. Zhang *et al*. (2024) also observed the co-occurrence of blaVIM and blaNDM in bacterial isolates
306 from rivers in urban areas of China, contributing to multidrug resistance and posing a significant threat to
307 public health.

308

309 ARGs such as blaNDM, blaTEM, blaSHV, blaCTX and tetA were detected in *Escherichia coli, Salmonella*
310 *enterica* and *Enterobacter cloacae*. Li *et al*. (2024) highlighted the role of these elements in horizontal gene
311 transfer, facilitating the spread of antibiotic resistance. *Escherichia coli, Salmonella enterica* and
312 *Enterobacter* cloacae that showed positive correlation with blaNDM, blaTEM, blaCTX and tetA may be as
313 a result of waste disposed in river water (An *et al*., 2024). In contrast, *Shigella* spp. and faecal coliforms
314 exhibit weaker correlations between ARGs and MGEs, particularly for blaNDM and blaCTX. Zhao *et al*.
315 (2021) noted that the environment plays a significant role in the maintenance and dissemination of ARGs,
316 with factors such as microbial community composition and the presence of antibiotics in the environment
317 influencing the dynamics of ARG transfer. Zhao *et al*. (2020) suggested that the presence of ARGs in
318 environmental bacteria is often linked to the local selective pressures, such as the concentration of
319 antibiotics in the environment, which can vary significantly.

320

321 Recent studies consistently highlight the interplay between human activities e.g., agriculture, healthcare,
322 and wastewater discharge and the dissemination of ARGs in aquatic environments. The results of this study
323 align with these findings, particularly the widespread resistance to beta-lactams, aminoglycosides, and other
324 antibiotics, suggesting that River Ala, like many other water bodies, serves as a reservoir for ARGs. Beshiru
325 *et al*. (2024) demonstrated that the presence of ARGs, particularly blaNDM and tetA, in bacterial isolates
326 from rivers in Nigeria correlates with high levels of multidrug resistance. The authors linked this trend to
327 the poor regulation of antibiotic usage in healthcare and agriculture, which contaminates water bodies with
328 antibiotic residues and resistant bacteria. Blanco-Peña *et al*. (2024) reported similar findings in their
329 assessment of ARG prevalence in river water used for irrigation in Latin America, where blaTEM, blaSHV,
330 and tetB were among the most common ARGs detected. Their study found over 90% resistance to first-
331 generation cephalosporins, in line with the high resistance levels to ceporex observed in River Ala. Barathan
332 *et al*. (2024) also noted the spread of ARGs like blaNDM and blaVIM in water bodies near industrial areas
333 in Southeast Asia, underscoring the global nature of the ARG problem. The authors emphasized the
334 importance of monitoring water quality and implementing stricter antibiotic regulations to curb the
335 environmental spread of ARGs.

336 The negative correlation observed between blaNDM and *E. coli* and *Salmonella spp.* suggests that these
337 bacterial species are less likely to harbor this particular carbapenem resistance gene in this environment.
338 This finding is consistent with Chen *et al*. (2023), who reported that *blaNDM* is prevalent in environmental
339 and clinical settings. Similarly, Khaledi *et al*. (2024) found that in Nigerian rivers, *Salmonella spp.* were
340 less likely to carry blaNDM, suggesting environmental factors or species-specific genetic barriers limiting
341 the spread of this gene in these bacterial populations.

342 The negative correlation may also indicate selective pressures where other beta-lactamase genes like
343 blaTEM and blaCTX are more dominant in *E. coli* and *Salmonella spp.*, reducing the likelihood of co-
344 occurrence with blaNDM in these species. This supports the theory that different bacterial species may
345 prefer certain resistance genes based on ecological and evolutionary factors, as observed by Zhang *et al*.
346 (2023), who noted that *E. coli* strains often prioritize extended-spectrum beta-lactamase (ESBL) genes over
347 metallo-beta-lactamases like blaNDM.

348 The significant positive correlation of blaTEM with *E. coli*, *Salmonella spp.*, and *Enterobacter cloacae* is
349 consistent with a growing body of evidence. Aslan *et al*. (2024) reported that blaTEM was highly prevalent
350 in *E. coli* and *Enterobacter cloacae* from wastewater and river systems, the widespread distribution of
351 blaTEM is linked to the ability of *E. coli* and *Salmonella* to efficiently acquire and disseminate plasmids
352 carrying this gene.

353 The positive association with *Enterobacter cloacae* underscores the role of this gene in promoting
354 resistance across various Enterobacteriaceae species. According to Molina *et al*. (2024), blaTEM is often
355 found in plasmids or integrons that are highly mobile among *E. coli*, *Salmonella spp.*, and *Enterobacter*
356 *spp.*, contributing to the rapid spread of resistance in both clinical and environmental settings.

357 The blaCTX gene showed a significant positive correlation with *E. coli* and *Enterobacter cloacae*,
358 indicating its widespread presence in these bacteria. This finding aligns with Bhattacharya *et al*. (2024),
359 who found that blaCTX was highly prevalent in *E. coli* and *Enterobacter spp.* isolated from rivers
360 contaminated with hospital and industrial effluents. The positive correlation suggests that both bacterial
361 species serve as major reservoirs for blaCTX, facilitating the dissemination of ESBLs in aquatic
362 environments.

363 Similarly, the positive correlation of tetA with *E. coli* and *Enterobacter cloacae* indicates a strong
364 association with tetracycline resistance. The tetA gene encodes an efflux pump that confers resistance to
365 tetracyclines, and its presence in both bacterial species is consistent with findings from Ramírez-Castillo *et*
366 *al*. (2023), who reported high levels of tetA in Enterobacteriaceae isolated from agricultural runoff and
367 polluted rivers. The combination of blaCTX and tetA in *E. coli* and *Enterobacter* points to their co-selection
368 in environments where both beta-lactam and tetracycline antibiotics are present, driving multidrug
369 resistance in these species.

370

371

372 **CONCLUSION**

373 The findings of this study demonstrated high total plate count and total enterobacteriaceae count in the
374 water samples from River Ala. *Escherichia coli, Salmonella enterica* and *Enterobacter cloacae, Proteus*
375 *mirabilis* and *Klebsiella pneumonia* were the predominant bacterial genera. All bacterial isolates exhibited
376 marked resistance to pencillin. blaNDM, blaTEM, blaSHV, tetA were the antibiotic resistance genes
377 detected in the isolates. *Escherichia coli, Salmonella enterica* and *Enterobacter cloacae* had positive
378 correlation with antibiotic resistance genes.

379

380 **HIGHLIGHTS**

381 1. Widespread of antibiotic resistance genes (ARGs) were investigated.

382 2. Load and identities of bacteria in the water samples were determined.

383 3. Antibiotic susceptibility pattern of the isolates were determined using disc diffusion method.

384 4. The relationships between pollution levels in the river and ARGs in the bacterial isolates were
385 determined.
386 5. River Ala is a potential environmental reservoir for antibiotic resistant bacteria.
387
388
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392 equipment and laboratory used for the study.

393 **Conflict of interest statement**

394 No conflict of interest declared.
395

396 **Data availability statement**

397 The data that support the findings of this study are available from the corresponding author upon reasonable
398 request.

399 Disclaimer (Artificial intelligence)

400 Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT,
401 COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this
402 manuscript.
403

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