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Original Research Article

Antimicrobial Resistance Patterns of Enteric Bacteria Isolated from Diarrhoeic Patients Attending Selected Hospitals in Akure, Nigeria

Rofiat Abidemi Adeleke[1], Anthony Kayode Onifade[1*]

[1] Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria.

Correspondence should be addressed to: adelekerofiat8@gmail.com

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Abstract: Diarrhoea remains a major public health challenge worldwide, particularly in developing countries where poor sanitation, unsafe water and inadequate hygiene contribute significantly to disease transmission. The increasing emergence of antimicrobial-resistant enteric bacteria, especially extended-spectrum β-Lactamase (ESBL) producing organisms, has complicated the treatment and management of diarrhoeal infections. This study aimed to evaluate the occurrence of ESBL-production among antibiotic-resistant bacterial isolates obtained from diarrhoeic patients. A total of 367 stool samples were collected from patients attending selected hospitals in Akure, Ondo State, Nigeria, comprising 17 diarrhoeic and 350 non-diarrhoeic samples. Standard microbiological techniques were employed for the isolation and identification of bacterial pathogens, while antibiotic susceptibility testing was performed using standard antimicrobial sensitivity methods. ESBL production was phenotypically detected using appropriate confirmatory methods on multidrug-resistant bacterial isolates. The study identified several enteric bacterial pathogens associated with diarrhoeal infections, including Escherichia coli, Proteus mirabilis, and the species of Salmonella, Shigella, Klebsiella and Enterobacter. Antibiotic susceptibility testing revealed varying degrees of resistance to commonly used antibiotics, with several isolates demonstrating multidrug resistance. 6.7% of isolates were resistant to ofloxacin, 37.8 % to augmentine, 18.9% to peflacine, 45.9% to ceftazidime, 13.5% to gentamycin, 59.46% to ciprofloxacin, 17.5% to ceporex, 40.5% to ceftriaxone, 9.4% to streptomycin and 32.4% to cefuroxime. ESBL-producing isolates were detected among the bacterial pathogens, suggesting the circulation of resistant enteric bacteria within the study population. The findings of this study highlight the growing threat of ESBL-producing enteric bacteria in diarrhoea diseases and emphasize the need for continuous surveillance, effective infection prevention measures and rational antibiotic use to reduce the spread of antimicrobial resistance.

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Keywords: Diarrhoea; Antibiotic resistance; Multiple antibiotic resistance index; Extended-spectrum β-lactamase

Introduction

Diarrhoea is an illness caused by bacterial, viral and parasitic organisms which spread mostly through contaminated food and drinking water characterized by three or more stools within 24hours. It is the third leading cause of death in children, which is mostly caused by enteric bacterial pathogens like *E. coli*, *Klebsiella pneumoniae,* Enterobacteriaceae and the species of *Camphylobacter, Salmonella* and *Shigella* (World Health Organization, 2024). These pathogens often require antibiotics for treatment.

However, the rise in antimicrobial resistance (AMR), especially due to misuse of antibiotics is making treatment difficult (WHO, 2021; Murray *et al*., 2022). A key issue is the emergence of Extended-Spectrum Beta-Lactamases (ESBLs)-enzymes produced by bacteria that render many beta-lactam antibiotics ineffective by hydrolysing third-generation cephalosporins (Paterson & Bonomo, 2005). ESBLs are common in Enterobacteriaceae and often associated with multiple antibiotic resistance. The management of diarrhoea is increasingly complicated by the global surge in antimicrobial resistance (AMR). This crisis threatens to render common infections untreatable, increasing healthcare costs, prolonging illness and increasing mortality rates.

Among the most clinically significant resistance mechanisms are those conferring resistance to beta-lactam antibiotics, a cornerstone class of antimicrobials. Beta-lactam antibiotics, which include cephalosporins, penicillins, carbapenems, and monobactams are one of the most important classes of antimicrobial agents used in different clinical practices. Their antibacterial activity is attributed to the presence of beta-lactam ring, this enables them to inhibit penicillin-binding proteins (PBPs). These proteins are very essential for the synthesis of peptidoglycan, which is an important component of the bacterial cell wall. Disruption of this process ultimately leads to the bacteria cell wall instability and the death of such bacterial (Bush & Bradford, 2020; Tooke *et al.,* 2021). Due to their broad-spectrum activity, clinical effectiveness and favourable safety profile, they are widely utilized in the treatment of bacterial infections worldwide.

Diarrhoea caused by ESBL-producing Enterobacteriaceae represents an emerging public health challenge, most especially in developing regions. The presence of these resistant pathogens in diarrheal diseases may lead to treatment failure when commonly prescribed empirical therapies are administered, thereby worsening patient outcomes and increasing the risk of disease transmission (WHO, 2021). Given the increasing burden of antimicrobial resistance, there is a critical need for continuous surveillance of ESBL-producing organisms, most especially in specific clinical contexts such as diarrhoea. Such information is vital for informing local treatment guidelines, guiding infection prevention and control measures and contributing to public health interventions aimed at curbing antimicrobial resistance (Logan & Weinstein, 2021).

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Methodology

Study Area

This study was conducted at Akure, the capital of Ondo State, Nigeria. Ethical approval was obtained from the Research Ethics Committee of the Ondo State Ministry of Health Akure, with assigned number NHREC/Ondo SMH-HREC/02/01/2025 and protocol number OSHREC/02/07/2025/1027 and the Ethics Committee of Federal University of Akure Teaching Hospital (FUTATH) prior to sample collection.

Sample Size Estimation

The sample size for this study was determined using the formula for estimating a single population proportion as described by Cochran (1977):

$$n = Z^2p(1-p)/d^2$$

Where:

n = minimum required sample size
Z = standard normal deviation at a 95% confidence interval (1.96)
p = estimated prevalence of ESBL-producing bacteria from a previous study
d = margin of error (0.05)

A previous study by Musa *et al.,* (2020) reported an ESBL-producing bacteria prevalence of 34.62%, which was used as the estimated prevalence for this study. Therefore:

$$n = (1.96)^2 \times 0.3462(1-0.3462)/ (0.05)^2$$

$$n = 3.8416 \times 0.2263/0.0025$$

$$n = 0.869/0.0025$$

$$n = 348$$

Thus, the minimum sample size required for this study was approximately 348 participants/samples.

To compensate for possible sample loss, contamination, or incomplete data, an additional allowance of 5% was added: 348 + (5%×348) = 365

Sample collection

A total of three hundred and sixty-seven (367) stool samples were collected for this study. Seventeen (17) were diarrhoeic, while three hundred and fifty 350 were non-diarrhoeic from three selected hospitals in Akure, Ondo State. The hospitals included FUTA Teaching Hospital, FUTA Health Centre and Nigeria Police Force Directorate of Medical Service (Police Clinic), all in Akure. Stool samples were classified as diarrhoeic or non-diarrhoeic based on the administered questionnaires. Written informed consent was obtained from all participants, and confidentiality of information was maintained throughout the study. The questionnaires provided information on the demographic, socioeconomic and clinical characteristics. Stool samples were collected using sterile stool

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collection bottles which were transferred to an airtight bag, sealed, labelled and transferred in an ice pack to the Microbiology laboratory, Federal University of Technology, Akure for further analysis within 1hr of collection. Summary is presented on Tables 2, 3 and 4.

Table 2: Socio-demographic, Environmental Profile, Food Hygiene and Behavioural Risk Factors

Variable	**Category**	**Frequency (n)**	**Percentage (%)**
Gender	Male	179	48.8
	Female	188	51.2
Age Group	0 – 5 years	126	34.3
	6 – 15 years	57	15.5
	16 – 25 years	170	46.3
	26+ years	14	3.9
Primary Water Source	Tap	234	63.8
	Well	95	25.9
	Borehole	26	7.1
	Boiled/Sachet	12	3.2
Food Storage Practice	No (Eat Fresh Only)	354	96.5
	Yes (Cold Storage)	13	3.5
Method of Storage	Refrigerator	8	2.2
	Freezer	5	1.4
Street Food Consumption	Yes	192	52.3
	No	175	47.7

(*N*=367, %=100%)

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Table 3: Health Status and Clinical Presentation

Variable	Category	Frequency (n)	Valid Percent (%)
Current Diarrhoea Status	Yes	17	4.6
	No	350	95.4
Antibiotic Use	Yes	244	66.5
	No	123	33.5
Morbidity Impact	Unable to perform activities	201	54.8
	Unable to eat	114	30.9
Comorbid Headache	Yes	105	28.6

Table 4: Relationship between Street Food Consumption and Diarrhoea

	Diarrhoea: YES n (%)	**Diarrhoea: NO n (%)**	**Total (N)**	**χ^2**	**p-value**
Street Food					
Yes	12 (6.3%)	180 (93.8%)	192	**2.386**	**0.122**
No	5 (2.9%)	170 (97.1%)	175		
Total	**17 (4.6%)**	**350 (95.4%)**	**367**		

Bacterial culture and isolation

The stool samples collected were processed under aseptic conditions in the laboratory. Serial dilution was carried out to reduce the microbial load by transferring 1ml of stool samples into 9ml of sterile normal saline in a test tube. 1ml aliquot of each of dilution 10^{-4} and 10^{-6} was introduced into petri dishes using sterile pipette, the media after cooling were aseptically poured in petri dishes under laminar flow using pour plate techniques and allowed to solidify. The plates were incubated for 24hr at 37^{o} C after which growth was examined. Distinct colonies were selected, subcultured and incubated for 24hr

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at 37 ° C. The pure culture of isolates where inoculated on agar slants for preservation and subsequent analyses at 4° C.

Biochemical characterization

Individual colonies were subjected to Gram staining and identified by biochemical and morphological techniques using Bergey's manual of Determinative Bacteriology (Garrity, 2001–2012), District laboratory practice in Tropical countries (Cheesbrough, 2010) and Laboratory Manual of Microbiology (Fawole & Oso, 2007). The various biochemical tests carried out included Indole reaction, Motility test, Sugar fermentation test, Methyl Red Test, Voges-Proskaure Test, Citrate Utilisation Test, Hydrogen sulphide production, Nitrate Reduction Test, and Catalase Test (Slide Method). Biochemical characteristics of bacteria isolates are presented in Tables 5 while the percentage occurrence of the bacterial isolates are presented in Table 6.

Table 5: Biochemical Characteristics of Bacterial Isolates

S/N	Mannitol	Catalase	Gas	H_2S	Lactose	Sucrose	Glucose	MR	VP	Sorbitol	Nitrate reduction	Motility	Indole	Citrate	Xylose	Malt	Manose	Glycerol	Fructose	Probable Organism
1	+	+	+	-	+	+/-	+	+	-	+	+	+	+	-	+	+	+	+	+	*Escherichia coli*
2	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	*Klebsiella pneumoniae*
3	+	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	+/-	-	+/-	*Shigella dysenteriae*
4	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	+	+	+/-	+	*Salmonella enterica*

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5	+/-	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	*Enterobacter* sp
6	-	+	+	+	-	+/-	+	+	_	+ /-	+	+	+ /-	+ /-	+ /-	+ /-	+	+/-	+	*Proteus mirabilis*
7	+	+	-	-		+/-	+			+ /-	+	-	-	-	+ /-	+	+	+/-	+/-	+	*Staphylococcus* sp

KEYS: + = Positive, - = Negative, +/-= Variable, **MR**= Methyl Red, **VP** = Voges-Proskauer

Table 6: Percentage occurrence of Bacterial Isolates

Isolates	**Frequency**	**Percentage occurrence (%)**
Escherichia coli	31	**34.07**
Klebsiella sp	20	**21.98**
Shigella sp	10	**10.99**
Salmonella sp	14	**15.38**
Enterobacter sp	8	**8.79**
Proteus sp	5	**5.49**
Staphylococcus sp	3	**3.29**
TOTAL	**91**	**100%**

Standardization of Inoculum

A fresh pure culture of the test organism was used for the preparation of inoculum for standardization. Using a sterile inoculating loop, 3–5 well-isolated colonies were picked and suspended in sterile test tubes containing sterile normal saline. The turbidity was adjusted, to increase turbidity more bacterial colonies were added while more sterile saline was added to decrease turbidity. The suspension was mixed thoroughly until a uniform turbidity was obtained. McFarland standards were checked using a spectrophotometer with a 1-cm light path. A 0.5 McFarland Standard corresponds to an absorbance reading of 0.08 to 0.1 at 625-nm. The standardized inoculum was used immediately for the antibiotic susceptibility testing to ensure consistency in bacterial cell density between isolates.

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Antibiotic susceptibility testing

Antibiotic susceptibility tests was done in accordance with Kirby-Bauer antibiotic testing (KB test or disc diffusion antibiotic sensitivity testing). A standardized bacterial suspension equivalent to 0.5McFarland was streaked aseptically on Mueller-Hinton agar plates to form a bacterial lawn. This was done in a direction to form uniform growth. The plate was rotated at 90^0 and streaked again in that direction. This rotation was repeated thrice and the plate was allowed to dry for 5minutes. A sterile forceps was used to dispense disc containing ofloxacin 10µg, augmentin 30µg, peflacine 10µg, ceftazidime 30µg, gentamycin 10µg, ciprofloxacin 10µg, ceporex 10µg, ceftriaxone 30µg, streptomycin 30µg and cefuroxime 30µg. The set-up was done in triplicate for each isolate, with a control plate containing no antibiotic disc. These were incubated for 18-24 h at 37°C, after which the diameter of the zone of inhibition was measured and the results were interpreted as resistance, susceptible and intermediate using standard interpretative charts as recommended by the Clinical Laboratory Science Institute (CLSI, 2020). In this study, multiple antibiotic resistance was indicated by resistance to a minimum of three different antibiotics. Antibiotic sensitivity patterns of bacteria isolates is presented on Table 7.

Table 7: Antibiotic sensitivity patterns of bacterial isolates

Antibiotic	No. Resistant	Resistant (%)	No. Intermediate	Intermediate (%)	No. Susceptible	Resistant (%)
Ofx	5	6.76	9	12.17	60	80.18
Au	28	37.83	5	6.76	41	55.41
Pef	14	18.92	41	55.41	19	25.67
Ctz	34	45.94	24	32.43	16	21.63
Cn	10	13.51	4	5.40	60	81.08
Cpx	44	59.46	21	28.38	9	12.16
Cep	13	17.57	31	41.89	30	40.54
Trx	30	40.54	34	45.95	10	13.51
S	7	9.46	3	4.05	64	84.49
Cef	24	32.43	10	13.51	40	54.05

Keys: Ofx= Ofloxacin, **Au=** Augmentin, **Pef=** Peflacine, **Ctz=** Ceftazidime, **Cn=** Gentamycin, **Cpx=** Ciprofloxacin, **Cep=** Ceporex, **Trx=** Ceftriaxone, **S=** Streptomycin, **Cef=** Cefuroxime

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Multidrug Resistance determination

Isolates resistant to at least one agent in three or more antimicrobial classes were classified as multidrug-resistant (MDR). The Multiple Antibiotic Resistance Index (MARI) was calculated for each isolate as $\frac{a}{b}$, where a is the number of antibiotics to which the isolate was resistant and b is the total number of antibiotics tested. The distribution of MARI values are presented as seen on Table 8.

Table 8: Distribution of Multiple Antibiotic Resistance Index (MARI) values among resistant bacterial isolates

MARI range	Interpretation	Number of isolates (n)	Percentage (%)
0.3–0.4	Low	6	24.00
0.5–0.6	Moderate	8	32.00
0.7–0.8	High	7	28.00
0.9	Very high	4	16.00
Total		25	**100.0**

ESBL screening and confirmation

Isolates showing reduced susceptibility or resistance to any third-generation cephalosporins and also identified as Multidrug-resistant isolates were suspected to be ESBL producers. The phenotypic confirmation of ESBL production was carried out using the Double Disk Synergy Test (DDST) according to standard microbiological procedures. A bacterial suspension equivalent to 0.5 McFarland standard was prepared from overnight cultures and inoculated uniformly onto Mueller–Hinton agar plates using a sterile swab stick. An amoxicillin-clavulanic acid disc (20/10 µg) was placed at the center of the agar plate, while discs of third-generation cephalosporins such as ceftazidime (30 µg) and cefotaxime (30 µg) were placed at a distance of 15mm (center to center) from the amoxicillin-clavulanic acid disc (Salihu *et al*., 2020). The plates were incubated at 37°C for 18–24 hours. Enhancement of the inhibition zone of any cephalosporin disc towards the amoxicillin-clavulanic acid disc or keyhole effect indicated synergy and was interpreted as positive for ESBL production. This summary of the screening is represented in Figure 1. Plate 1 is showing a positive keyhole effect.

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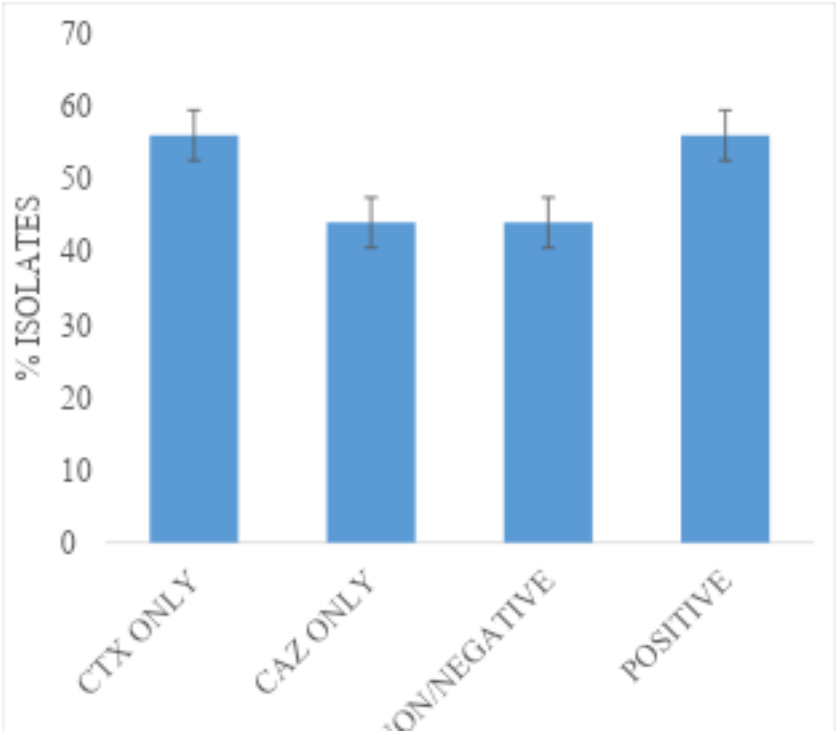


Figure 1: Synergistic effect between Ceftazidime (CAZ) and Cefotaxime (CTX) with Amoxicillin-clavulanic acid discs



Plate 1: Photograph showing a positive ESBL effect.

KEY: A = Ceftazidime, **B** = Amoxicillin-clavulanic acid, **C** = Cefotaxime

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Molecular Identification, Sequencing and Phylogenetic Analysis

Genomic DNA was extracted from representative isolates using the QIAamp mini extraction kit. Species-specific singleplex PCR assays were performed using primers targeting the uidA gene (*Escherichia coli*), gyrA gene *(Klebsiella* sp.) and invA gene (*Salmonella* sp.) (Table 1), with amplification comprising an initial denaturation (95°C, 5 min), 35 cycles of denaturation (95°C, 30 s), primer-specific annealing (30 s) and extension (72°C, 45 s), and a final extension (72°C, 7 min). PCR products were resolved on 1.5% agarose gel and visualised under UV transillumination. Amplicons of the 16S rRNA gene were purified, sequenced by the Sanger method (Inqaba Biotec, Ibadan) and identities confirmed by BLAST search against the NCBI database; a neighbour-joining phylogenetic tree was constructed to establish evolutionary relationships with reference strains. Table 1 gives a breakdown of all primers used in the molecular identification while figures 2, 3 and 4 shows the phylogenic tree of all the isolates identified.

Table 1: List of the Specific Primers used for the Bacterial Isolates

Bacteria	**Target genes**	**Primer sequence (5'-3')**	**Product Size (bp)**
E. coli	uidA	F:5'-TATGGAATTCGCCGATTTT-3' R:5'-TGTTTGCCTCCCTGCTGCGG-3'	166
Klebsiella sp.	gyrA	F:5'-GCTGGCGGTACACGCCAGCCCG-3' R:5'-AGAAGCATCCTGCTGTGCG-3'	441
Salmonella sp.	invA	F:5'-CGGTGGTTTTAAGCGTACTCTT-3' R:5'-CGAATATGCTCCACAAGGTTA-3'	796

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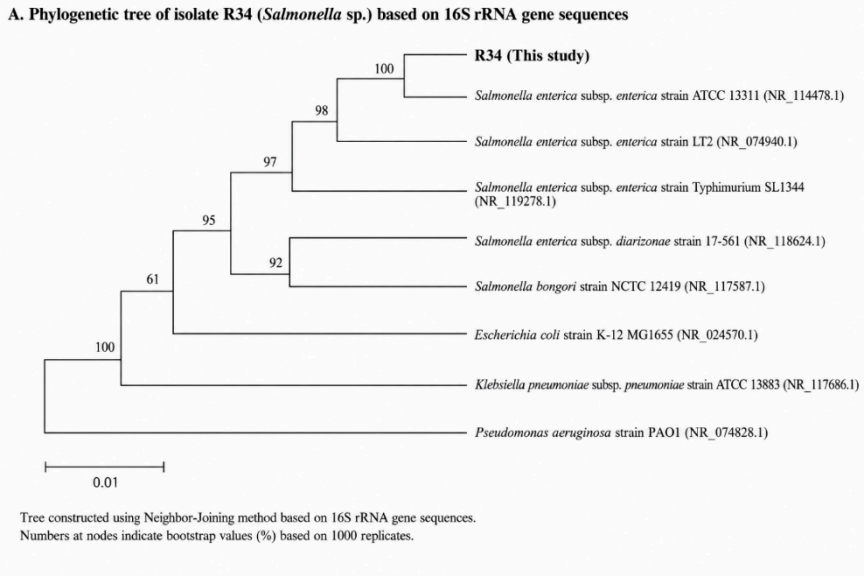


Figure 2: Phylogenetic tree of isolate identified as *Salmonella enterica*

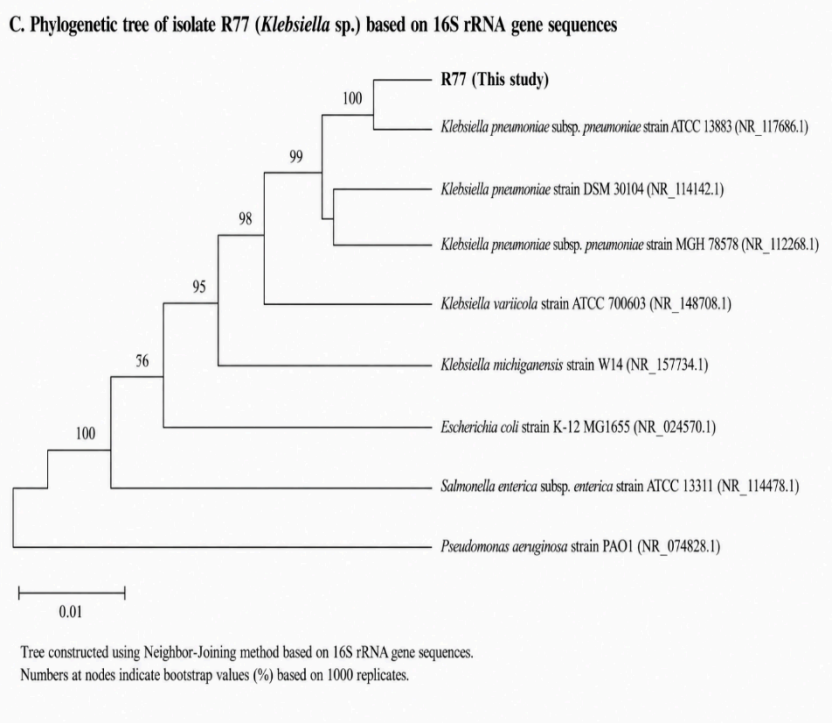


Figure 3: Phylogenetic tree of isolate identified as *Klebsiella spp.*

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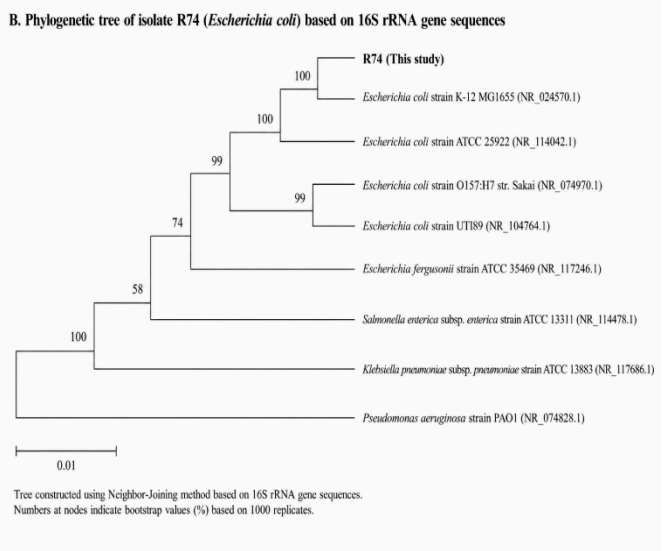


Figure 4: Phylogenetic tree of isolate identified as *Escherichia coli*

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Statistical Analysis of Data

All experiments were carried out in triplicates and data obtained from the study were subjected to analysis of variance, and treatment means were compared using Duncan's New Multiple Range Test (DNMRT) at 5% level of significance with the aid of Statistical Package for Social Science (SPSS) version 20.

Discussion

The study showed that diarrhoeal disease predominantly affected children under five and young adults. High antibiotic use, despite a low prevalence of diarrhoea suggests widespread self-medication, which may contribute to antimicrobial resistance. Food storage practices were significantly associated with diarrhoea. Refrigerator users had the highest prevalence of diarrhoeal infection, likely due to frequent power interruptions causing temperature fluctuations that encourage bacterial growth. Immediate consumption of freshly prepared food appeared to be the safest practice. Age was significantly associated with diarrhoea. Children under five remained highly vulnerable because of immature immunity, while young adults were at increased risk due to greater exposure to contaminated food and frequent antibiotic self-medication. *Escherichia coli* accounted for the highest proportion of isolates recovered from diarrhoeic stool samples followed by *Klebsiella* spp. The predominance of *E. coli* from this study suggests that it remains one of the leading bacterial pathogens associated with diarrhoeal disease which agrees with studies by Odonkor and Addo (2021), who reported that diarrhoeagenic *E. coli* was the most frequently isolated enteric pathogen in patients presenting with acute diarrhoea. Although many strains of *E. coli* are normal intestinal flora, pathogenic strains possess virulence

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factors which enable them to cause diarrhoea through toxin production, epithelial invasion or intestinal colonization.

The isolates exhibited high levels of multidrug resistance, particularly to ciprofloxacin, ceftazidime and ceftriaxone. Similar findings were reported by Tadesse *et al.* (2022), who observed increasing ciprofloxacin resistance among clinical isolates recovered from patients with diarrhoeal diseases. Resistance patterns strongly suggested ESBL production. The predominance of high MARI values therefore suggests continuous exposure of bacterial populations to antimicrobial agents within the study area. Such exposure may result from inappropriate prescriptions, self-medication, incomplete treatment courses and indiscriminate use of antibiotics in both human and animal health.

Only a proportion of multidrug-resistant isolates were confirmed as ESBL producers by the Double Disc Synergy Test, demonstrating that multidrug resistance can arise from mechanisms other than ESBL production. The positive DDST results obtained for some isolates indicated the presence of clavulanic acid-inhibitable β-lactamases capable of hydrolysing ceftazidime and cefotaxime. During the DDST, the enhancement of the inhibition zone (keyhole effect) between any of the cephalosporin disc and the amoxicillin-clavulanic acid disc demonstrates inhibition of the ESBL enzyme by clavulanic acid, thereby restoring the antibacterial activity of the cephalosporin. This characteristic "keyhole" or synergistic effect remains one of the classical phenotypic indicators of ESBL production and is recommended for routine laboratory confirmation. The phenotypic confirmation of ESBL-producing isolates in this study correlates well with the high levels of resistance observed against ceftazidime (45.9%) and ceftriaxone (40.5%) during antimicrobial susceptibility testing. PCR and sequencing confirmed representative isolates as *Salmonella enterica*, *Escherichia coli* and *Klebsiella pneumoniae*.

Conclusion

This study investigated antimicrobial resistance profiles of ESBL-producing enteric bacterial pathogens isolated from diarrhoeal patients. The findings demonstrated that diarrhoeal infections remain an important public health problem, especially among younger individuals, with behavioural and environmental factors such as food storage practices and antibiotic use contributing to the occurrence of this disease and antimicrobial resistance. The microbiological analysis showed that *Escherichia coli* was predominant among the bacterial isolate, followed by *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., *Proteus* spp., and *Staphylococcus* spp. The predominance of members of the family *Enterobacteriaceae* highlights their continued importance as causative agents of diarrhoeal diseases within the study area. These findings highlight the need for improved antimicrobial stewardship, routine surveillance, and strengthened infection prevention and control measures.

Antimicrobial susceptibility testing revealed high resistance to several commonly prescribed antibiotics indicating the increasing burden of multidrug-resistant enteric bacteria. The Multiple Antibiotic Resistance Index (MARI) values ranged from 0.3 to 0.9,

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with all isolates exhibiting values greater than 0.2, suggesting that these organisms from the environment are characterized by frequent and indiscriminate antibiotic exposure.

Phenotypic screening confirmed the presence of ESBL-producing isolates, while molecular characterization accurately identified representative isolates as *Salmonella enterica*, *Escherichia coli*, and *Klebsiella pneumoniae*.

Recommendation

i. Perform routine antimicrobial susceptibility testing before antibiotic prescription.
ii. Implement routine ESBL screening and PCR-based detection in diagnostic laboratories.
iii. Strengthen antimicrobial stewardship programmes and regulate over-the-counter antibiotic sales.
iv. Promote public education on appropriate antibiotic use, hygiene, food safety, and sanitation.
v. Establish continuous antimicrobial resistance surveillance.

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