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ESCHERICHIA COLI ENCODING EXTENDED-SPECTRUM BETA-LACTAMASES ISOLATED FROM DIARRHEIC PATIENTS IN MINNA, NIGERIA

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ABSTRACT

Escherichia coli remains one of the most isolated etiological agents of diarrhea, accounting for more than 1 million deaths and about 4% of the total global disability-adjusted life-years (DALYS) per year across all age groups according to the global disease burden. This study evaluated multidrug-resistant *Escherichia coli* encoding extended spectrum beta-lactamases isolated from diarrheic patients in Minna, Nigeria using standard microbiological methods. A prevalence of 37.7% of Diarrheagenic *E. coli* (DEC) was obtained from the stool samples evaluated. Within the environment sampled, age group 21 – 30 years had the highest *E. coli* isolation rate (27.8%) while age group ≥71years had the least *E. coli* isolation rate (2.6%). Females (64.9%) were the most affected compared to males (35.1%). The isolates were significantly resistant to most of the beta-lactams tested especially to 3rd generation cephalosporins [Cefotaxime (98.2%), cefuroxime (93%), ceftazidime (84.2%), Augmentin (70.2%), Amoxicillin (59.6%)]. Resistance to other classes of antibiotics was also observed in varying percentages. A high percentage (98.2% and 87.7%) of the diarrheagenic *E. coli* had a multiple resistant index (MARI) ≥ of 0.3. The isolates had varying patterns of resistance with 47.6% resistant to more than 5 classes of antibiotics tested and produced ESBL characteristics phenotypically. Molecular evaluation showed that 40%, 50%, and 90% of the isolates harbored the OXA, CTX-M and TEM genes respectively while 50% harbored VEB and PER genes. This study isolated *E. coli* from diarrheic patients with multidrug resistance profiles and encodes more than one type of ESBL gene within Minna, Nigeria.

Contribution/Originality: This study established the prevalence and multidrug resistance profile patterns of *Escherichia coli* encoding ESBL genes from diarrheic patients in Minna, Nigeria. The findings also revealed a genetic diversity of ESBL genes responsible for significant resistance of Diarrheagenic *E. coli* to most of the beta-lactams and other classes of antibiotics.

1. INTRODUCTION

Escherichia coli (E. coli) are a vital commensal Enterobacteriaceae in the gastrointestinal tract of humans and animals. Nevertheless, some pathogenic strains have emerged with multidrug-resistant (MDR) and virulent characteristics that consequently result in significant public health diseases including diarrhea (Margulieux et al., 2018). One of such strains mostly implicated in diarrhea is the Enterotoxigenic *E. coli* (ETEC) that produces 1–3 enterotoxins, which spontaneously results in acute diarrhea in infants and traveler's disease worldwide (Kantele et

al., 2020; Pandey et al., 2011). This strain operates by attaching itself to the small intestinal wall along the epithelial surface and colonizes the cells using its colonization factors (CFs) (Vipin & Sakellaris, 2015). This results in the production of heat-stable enterotoxins (STh or STP) and/or heat-labile enterotoxins (LT) which interfere with crucial cellular mechanisms resulting in abdominal cramps and watery stool (Isidean, Riddle, Savarino, & Porter, 2011). In addition, the other strains of Diarrheagenic *E. coli (DEC)*, notably O157:H7, O104:H4, O121, O26, O103, O111, O145, and O104:H21 have been reported to elicit potential lethal toxins resulting in diarrheic diseases (Shridhar et al., 2018). Other serotypes also associated with diarrhea are Enterohemorrhagic *E. coli* (EHEC) Enteroaggregative *E. coli* (EAEC) Enteroinvasive *E. coli* (EIEC) and Enteropathogenic *E. coli* (EPEC) (Croxen et al., 2013). ETEC remains the most prevalent enteropathogenic *E.coli* common in developing countries, resulting in about 210 million diarrhea cases and nearly 380,000 deaths (Archana, O`Keefe, Cuthill, Varman, & Kumar, 2019). Clinical manifestation of EIEC includes crampy abdominal pain, bloody mucoid diarrhea with fever, and tenesmus in infected individuals (Archana et al., 2019). About Ten percent (10%) of patients with EHEC frequently come down with hemolytic uremic syndrome (HUS) as a result of the serotype 0157:H7. HUS is mostly accompanied by acute renal failure, hemolytic anemia and thrombocytopenia. According to the Centre for Disease Control and Prevention (2013), these classes of *E. coli* are established as prominent international public health threats, and transmission of virulence properties is through ingestion of pathogen infected food or water, or contact with diseased animals or infected persons. Most worrisome is the endemic nature of these strains and serotypes in developing countries including Nigeria as they contribute to high rates of mortality and morbidity especially in neonates (Ghorbani-Dalini, Kargar, Doosti, Abbasi, & Sarshar, 2015). Statistical evaluation of various diseases has shown that diarrhea is the second primary cause of death in children under 5 years of age and over 525000 children die annually (Mandal et al., 2017). Diarrhea caused by *E.coli* can be avoided by maintaining good hygienic practices and breast feeding of infants in ETEC endemic regions. The diarrhea is best managed through administering of oral fluid and electrolyte replacement (intravenous in severe cases) to infected individuals. Use of antibiotics is discouraged as it can lead to spread of antibiotic-resistant pathogenic *E coli* and other deadly enteropathogens (Todar, 2020). Interestingly, diarrheagenic *E. coli* (DEC) has been revealed to possess resistance to some antimicrobial agents such as extended spectrum beta-lactamases (ESBL) encoding genes (Ogefere, Ibadin, Omoregie, & Ilerhunwa, 2016). DEC encoding ESBLs causes resistance to the commonly prescribed beta-lactam antibiotics, for example, the 3rd and 4th generations' cephalosporins such as ceftriaxone, ceftazidime, cefotaxime, cefuroxime, and cefixime. This, however, degenerates to failure in therapeutic outcomes in human and animal medicine (Lee & Yeh, 2017). Interestingly, Bushand and Jacoby (2010) have reported the capability of clavulanic acid, sulbactam, and tazobactam to inhibit ESBLs. The most commonly isolated ESBL genes include TEM, SHV, and CTX-M (Igwe et al., 2014). A study by Gniadkowski (2008) reported that all CTX-M-types are enzymatic, while the TEM and SHV types occur as a result of a point mutation at specific residues from the natural TEM-1/TEM-2 and SHV-1 β-lactamase respectively. Studies by Igwe et al. (2014); Pitout and Laupland (2008) have shown that ESBLs are plasmid-mediated and these plasmids also bear genes that encode resistance to other classes of antimicrobials, for example fluoroquinolones and aminoglycosides. Therefore, this is to determine the prevalence of ESBL producing *E. coli* among diarrheic patients in Minna metropolis.

2. MATERIALS AND METHODS

2.1. Study Area

Stool samples were obtained from the diagnostic laboratories of four (4) different hospitals in Minna metropolis. The hospitals include Standard Hospital, Old airport road, Top Medical Clinic, Tunga, Ibrahim Badamosi Babangida (IBB) Specialist Hospital, Chachanga, and General Hospital, Minna. Stool samples were also obtained from healthy individuals which served as control. Figure 1 is the Map showing the locations where samples were collected within Minna Metropolis, Niger State as captured by Google (2020) is shown below:

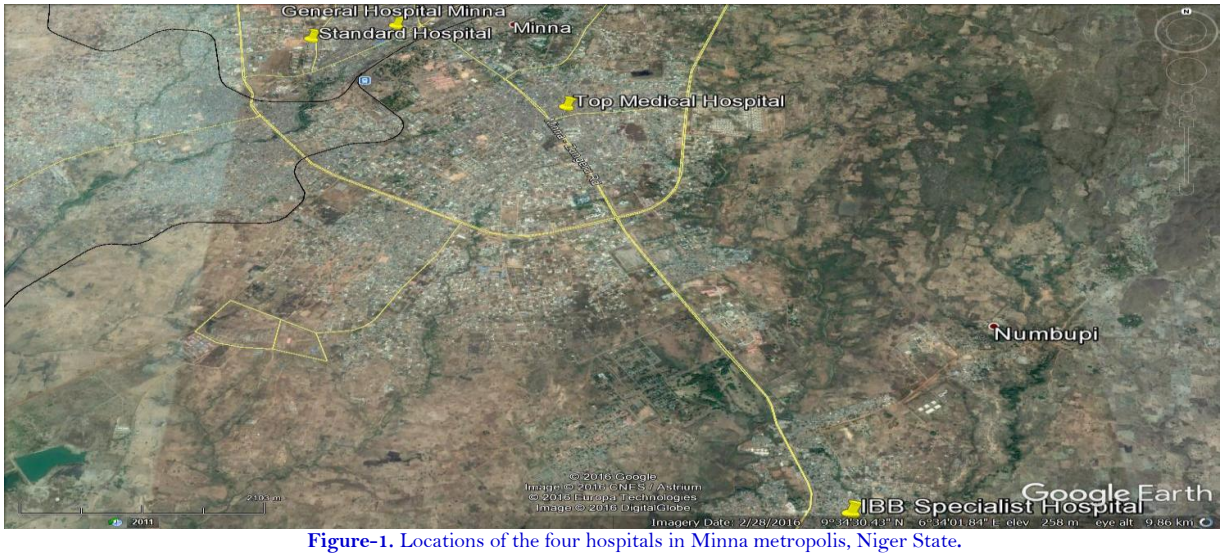

Figure-1. Locations of the four hospitals in Minna metropolis, Niger State.

2.2. Determination of Sample Size

The single proportion method was used to determine the sample size with a 6.9% prevalence rate as described by Onanuga, Igbeneghu, and Lamikanra (2014).

$$n = \frac{Z^2 \quad P \quad (1-P)}{D^2}$$

Where n= size of the sample
Z = 1.96 for Confidence level at 95%
P = rate of prevalence at 6.9%
D = 0.05 for Marginal error at 5%
The sample size was calculated as 98.7, rounded up to 120.

2.3. Ethical Clearance

Ethical authorization for this study was issued by the Research Ethics Committee, General Hospital, Minna, Niger State with the registration number HMB/GHM/STA/136/VOL.II/350, dated 29th October 2015.

2.4. Specimen Collection and Processing

A total of 120 samples of loose or watery stool passed by diarrheic patients and submitted to the Microbiology Laboratory of the four hospitals in Minna metropolis were randomly obtained within 5 months i.e. May - September 2015. The samples were carefully transported to the laboratory of the Microbiology Department, Federal University of Technology (F.U.T), Minna in an ice pack within 40 minutes of collection for further analysis.

2.5. Isolation and Identification of Escherichia coli

Using a sterile wire loop, a loopful of each stool sample was picked and streaked onto sterilized Eosin methylene blue (EMB) agar and MacConkey agar (MCA) followed by incubation at 37°C for 24 hours. Identification of suspected isolates of *E. coli* was done based on morphological and cultural characteristics, sugar fermentation, Gram's reaction, and series of biochemical tests such as lysine decarboxylase, motility, triple sugar iron, citrate utilization, urease production, Voges-Proskauer, methyl red, and indole test were all carried out as described by Cheesbrough (2006).

2.6. Antibiotic Susceptibility Test

The confirmed *E.coli* isolates were screened using 13 frequently prescribed antibiotics used for the treatment of *E. coli* related infections. This test technique was carried out according to Cheesbrough (2006) and results were interpreted using CLSI (2016) as shown in Table 1.

Table-1. Clinical laboratory science index interpretation for selected antibiotics.

S/N	Beta-Lactam Antibiotics	Zone of Inhibition (mm)		
		Susceptible	Intermediate	Resistance
1	Ceftazidime (30µg)	≥18	15- 17	≤14
2	Cefotaxime (30µg)	≥23	15-22	≤14
3	Cefuroxime (30µg)	≥18	15-17	≤14
4	Amoxicillin (30µg)	≥17	14-16	≤13
5	Augmentin (10/20µg)	≥18	14-17	≤13
6	Cotrimoxazole (7/23µg)	≥16	11-15	≤10
7	Chloramphenicol (30µg)	≥18	13-17	≤12
8	Tetracycline (30µg)	≥15	12-14	≤11
9	Ciprofloxacin (5µg)	≥21	16-20	≤15
10	Gentamicin (10µg)	≥15	14-15	≤12
11	Streptomycin (30µg)	≥15	12-14	≤11
12	Pefloxacin (10µg)	≥21	16-20	≤15
13	Ofloxacin (10µg)	≥16	13-15	≤12

2.7. Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR of each isolated *E.coli* was determined utilizing the method of Krumperman (1983).

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which isolate is resistant to}}{\text{Total number of antibiotics tested}}$$

2.8. Presumptive Test for Extended-Spectrum Beta-Lactamase (ESBL) Production

The double disc synergy test as described by Igwe et al. (2014) was adopted to confirm the production of ESBL by the *E. coli* isolates. The confirmed *E. coli* isolates which exhibited multidrug-resistant features were standardized to 0.5 McFarland turbidity standard using phosphate buffered saline. Each confirmed isolate was first suspended in broth culture and incubated overnight. The inoculum of each isolate was taken from each broth culture and inoculated uniformly on nutrient agar then incubated at 37° C for 24h. A 0.5 McFarland turbidity standard was prepared for the standardization of inoculums (1-2 x 10^5 cfu/ml). Well isolated colonies of *E.coli* were picked from nutrient agar and emulsified in test tubes containing 5ml sterile saline. Under good lighting, the turbidity of the test suspension was compared with 0.5 McFarland turbidity standard. Using swab sticks, the standardized cultures were uniformly streaked on sterile Mueller Hinton Agar (MHA) plates and allowed to dry at room temperature for 5minutes. Sterile forceps were used to carefully place antibiotic discs containing amoxicillin-clavulanate (20µg/10µg) at the center of the plate, and each disc of ceftazidime (30µg), cefotaxime (30µg), and cefuroxime (30µg) were positioned 20 mm (center to center) from the amoxicillin-clavulanate disc. The MHA plates were incubated at 37°C for 24 h. Control used was *E. coli* ATCC 25922, authorized by the Clinical and Laboratory Standard Institute (CLSI) as a reference strain for detection of ESBL production.

The positive results obtained for the production of ESBL by test isolates were interpreted following CLSI procedures. Positive results were interpreted as isolates that exhibited a distinct shape/size with potentiation towards amoxicillin + clavulanic disc with a ≥5 mm increase in zone diameter for either antimicrobial agent compared to its zone when tested alone. Additionally, any isolate that had the zone of inhibition around the test antibiotic disc extended towards the center disc of amoxicillin-clavulanic acid is presumed positive.

2.9. Molecular Characterization of Extended-Spectrum Beta-Lactamase Resistant Escherichia Coli

2.9.1. Bacterial Cell Preparation

The bacteria cells were prepared for molecular analysis using the procedure described by Igwe et al. (2014). Luria and Bertani broth medium were prepared by dissolving 10g of peptone water, 5g of NaCl, 1ml of 1N NaOH, 5g of yeast extract in 1litre of distilled water. NaOH was used in adjusting the pH of the solution to 7.0 before

sterilization at 121°C for 15mins. A loopful of each multidrug-resistant isolate of *E. coli* was inoculated in 5 mL of Luria and Bertani (LB) broth and incubated at 37°C for 24 hours.

2.10. DNA Extraction

The extraction of DNA was accomplished following the manufacturer's instructions as described on the Bioneer Accuprep® GMO DNA Extraction Kit. The resistant *E.coli* isolates suspended in Luria broth were centrifuged for 1 minute at 6000rpm before harvesting the cell pellets. The harvested cell pellets were again suspended in 200 µl of phosphate-buffered saline (PBS) and subsequently transferred to a falcon tube comprising 20 µl of Protinease K and vortexed for 10secs. The cells were lysed by placing the falcon tubes in a water bath for 10mins at 55°C. 100 µl of absolute ethanol was dispensed into the falcon tubes and placed on a rack to stand for 2mins. A micropipette was used to transfer 600 µl of cell lysate into a binding column and centrifuged for 1min at 8000rpm. After centrifugation, the binding column was transferred into a new binding column, and the supernatant was discarded. 500 µl of washing buffer 1 was added into the new binding column and centrifuged for 2 minutes at 8000 rpm. Thereafter, the binding column was transferred into another binding column and supernatant discarded, 500 µl of washing buffer 2 was added into the new binding column and centrifuged for 1 minute at 8000rpm. The binding column was taken out and the supernatant discarded. To remove excess water and ethanol, the column was centrifuged for 1minute at 13000rpm. To the binding column, 50 µl elution buffer was added and placed on a rack to stand for 1 minute before centrifuging for 2 minutes at 10000 rpm to enable the dissolved DNA to settle at the base of the binding column. For analysis and preservation, extracted DNA was suspended in elution buffer (Bioneer, 2004).

2.11. Detection and Characterization of Resistant Genes Using Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify the target genes from extracted plasmid DNA. Table 2 constitutes specific gene primers used for the amplification of specific gene fragments responsible for coding beta-lactamase resistance in *E. coli*. This process was performed utilizing Dream Taq™ PCR master mix (2x), thawed, vortexed, and centrifuged at 10000 rpm for 30 seconds. To constitute 50µl volume reaction; 10.0µl of template DNA (plasmid DNA), 1.0µl of the forward primer, 1.0 µl of the reverse primer, 13µl nuclease-free water and 25µl of Dream Taq™ PCR master mix were aliquoted into an Eppendorf tube, vortexed and spun. PCR conditions utilized are stated in Table 3.

Table-2. Extended-spectrum beta-lactamase primers.

Below is the nucleotide sequence of primers and size of each ESBL Gene respectively.

Gene	Sequence	Amplicon size (bp)	Reference
TEM	F: 5'ATTCTTGAAGACGAAAGGGCCTC3' R: 5'TTGGTCTGACAGTTACCAATGC3'	931	Igwe et al. (2014)
SHV	F: 5'GCCGGGTTATTCTTATTTGTCCG3' R:5' ATGCCGCCGCCAGTCA3'	868	Igwe et al. (2014)
CTX-M	F: 5'CGCTTTGCGATGTGCAG3' R: 5'ACCGCGATATCGTTGGT3'	593	Monstein et al. (2007)
OXA	F:5'AAGAAACGCTACTCGCCTGC3' R: 5'CCACTCAACCCATCCTACCC3'	478	Igwe et al. (2014)
PER	F: 5'GCAACTGCTGCAATACTCGG3' R: 5'ATGTGCGACCACAGTACCAG3'	716	Guessennd et al. (2008)
VEB	F:5'CGACTTCCATTTCCCGATGC3' R:5'GGACTCTGCAACAAATACGC3'	542	Guessennd et al. (2008)

Key: TEM = Temoneira, SHV = Sulfhydryl variable, VEB = Vietnamese extended beta- lactamase, PER = Pseudomonas extended resistant, OXA = Oxacillin hydrolyzing, CTX-M = Cefotaxime hydrolyzing, G = Guanine, T = Thymine, C = Cytosine, A = Adenine, bp = base pair.

Table-3. PCR thermal cycling conditions used.

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30sec	35 cycles
Annealing	68 (SHV) 55 (TEM) 60(CTX-M) 50(OXA) 65(PER) 65(VEB)	1mins	1
Extension	72	1mins	1
Final extension	72	7mins	1

Key: SHV = Sulfhydryl variable, TEM = Temoneira, CTX-M = Cefotaxime hydrolyzing, OXA = Oxacillin hydrolyzing, PER = Pseudomonas extended resistant, VEB = Vietnamese extended beta- lactamase.

2.12. Agarose Gel Electrophoresis of Extracted Plasmid

The plasmid isolated from *E. coli* was resolved using 1% agarose gel. The gel was constituted by dissolving 1g of agarose in 90ml distilled water in a 250ml beaker, the beaker was placed in a microwave for 2minutes to completely dissolve the agarose. To the agarose gel solution, 2.5ml ethidium bromide (EtBr) was added and swirled gently to mix. The gel solution was poured into a mini horizontal electrophoresis tank with well combs in place and left for 30 minutes to completely solidify. The well combs were carefully removed after the gel had solidified. 1X TAE (TBE) electrophoresis buffer was added to the electrophoresis tank until the agarose gel was covered.

5µl of bromophenol blue (tracking dye) was added to 15µl of each genomic DNA isolate and mixed gently. The wells were carefully loaded with 20µl, the electrophoresis tank was closed and the electrodes plugged in, the gel ran for 45miutes at 100mV. At the end of the run time, power was turned off, electrodes disconnected and the gel was removed from the gel tank and visualized using a Transilluminator at 302 nm. The DNA fragments (bands) were documented employing an electrophoresis gel documentation system.

3. RESULTS

A prevalence of 37.7% (14%) DEC was observed in the stool samples evaluated while a 53.3% isolation rate was observed in healthy subjects. ST hospital (60%) had the highest *E. coli* isolation percentage while the least *E. coli* yield was in GH (34.3%) Table 4.

Table-4. Distribution of diarrheic stool samples evaluated in Minna, Nigeria.

Hospital	No. of stool samples	*E. coli* isolated	% *E. coli* per Source
ST	20	12	60
IBB	24	13	54.2
GH	70	17	24.3
TMED	22	7	31.8
H	15	8	53.3
Total	**151**	**57**	**37.7**

Key: ST = Standard hospital, IBB = Ibrahim Badamosi Babangida Hospital, GH = General Hospital, Time Medical Hospital, H = Healthy subjects.

Table-5. Age distribution of diarrheic stool sampled in Minna, Nigeria.

Age Group	ST	IBB	GH	TMH	H	Total (%)
0 – 10	1	0	2	0	2	5 (3.3)
11 – 20	5	1	6	1	2	15 (9.9)
21 – 30	4	8	20	7	3	42 (27.8)
31 – 40	2	5	18	7	5	37 (24.5)
41 – 50	3	2	12	4	1	22 (14.6)
51 – 60	2	5	5	3	2	17 (11.3)
61 – 70	3	3	3	0	0	9 (6)
≥71	0	0	4	0	0	4 (2.6)
Total	**20**	**24**	**70**	**22**	**15**	**151 (100)**

Key: ST = Standard hospital, IBB = Ibrahim Badamosi Babangida Hospital, GH = General Hospital, Top Medical Hospital, H = Healthy subjects.

Within the environment evaluated, age groups 21 – 30 (27.8%) and 31 – 40 (24.5%) had the highest *E. coli* isolation rate. Age groups ≥71 (2.6%) and 0-10 (3.3%) had the least *E. coli* isolation rate Table 5. Females (64.9%) were the most affected with diarrhea in Minna compared to their male counterparts (35.1%) Table 6.

Table-6. Gender distribution of diarrhea stool samples evaluated in Minna, Nigeria.

Gender	ST	IBB	GH	TMH	H	Total (%)
Male	8	10	23	6	6	53 (35.1)
Female	12	14	47	16	9	98 (64.9)
Total	**20**	**24**	**70**	**22**	**15**	**151 (100)**

Key: ST = Standard hospital, IBB = Ibrahim Badamosi Babangida Hospital, GH = General Hospital, Top Medical Hospital, H = Healthy subjects.

3.1. Antibiotics Susceptibility Profile of Diarrheagenic E. coli

The *E.coli* isolates were significantly resistant to most of the beta-lactams tested especially 3rd generation cephalosporins [Cefotaxime (98.2%), cefuroxime (93%), ceftazidime (84.2%), Augmentin (70.2%), Amoxicillin (59.6%)]. Also observed was resistance to other classes of antibiotics such as quinolones [pefloxacine (56.1), ciprofloxacin (54.4%), and ofloxacin (45.6)], chloramphenicol (61.4%), cotrimoxazole (61.4%). The isolates were observed to show high percentage susceptibility to tetracycline (77.2%), streptomycin (56.1%), ofloxacin (54.4%), and gentamicin (52.6%) as shown in Figure 2.

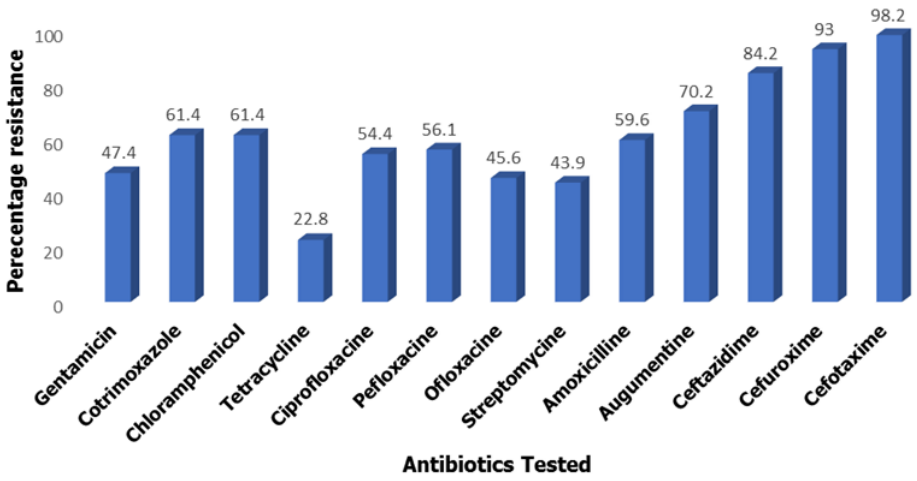

Figure-2. Antibiotics Susceptibility Profile of Diarrheagenic *E. coli*.

The graph below showed that 98.2% of the diarrheagenic *E. coli* had MARI ≥ 0.3 Figure 3.

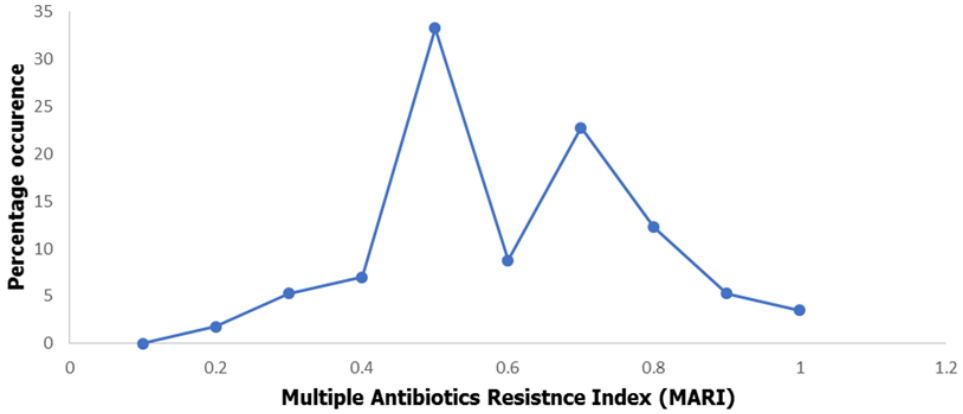

Figure-3. Multiple Antibiotics Resistance Index (MARI).

Table-7. Resistant pattern and percentage multidrug resistance of *E. coli* Isolated from diarrheic patients in Minna, Nigeria

S/N	Isolate	Resistant Pattern	NART	CART	LR
1	S52	CN, TE, C, SXT, CPX, S, OFX, PEF, AM, AU, CXM, CTX, CAZ	13	7	MDR
2	S73	AM, CAZ, CTX, CXM	4	1	NMDR
3	S44	CN, SXT, C, PEF, OFX, S, AM, AU, CAZ, CTX, CXM	11	6	MDR
4	S42	CN, SXT, C, AU, CTX, CXM	6	4	MDR
5	S46	CN, SXT, TE, CPX, OFX, S, AM, AU, CAZ, CTX, CXM	11	6	MDR
6	S79	SXT, OFX, S, AM, AU, CTX, CXM	7	4	MDR
7	S1	CN, TE, C, SXT, CPX, S, OFX, PEF, AM, AU, CXM, CTX, CAZ	13	7	MDR
8	S81	CN, SXT, AM, AU, CTX, CXM	6	3	MDR
9	HS1	CN, C, TE, AM, CAZ, CTX, CXM	7	4	MDR
10	HS3	CN, SXT, C, OFX, S, AU, CAZ, CTX, CXM	9	6	MDR
11	HS7	CN, SXT, C, TE, PEF, OFX, S, AM, AU, CAZ, CTX, CXM	12	7	MDR
12	S80	CN, CPX, PEF, OFX, AM, AU, CAZ, CTX, CXM	9	3	MDR
13	HS10	CN, PEF, OFX, S, AM, AU, CTX	7	4	MDR
14	S22	C, TE, CPX, CAZ, CTX, CXM	6	4	MDR
15	S5	C, CPX, PEF, S, AM, AU, CAZ, CTX, CXM	9	4	MDR
16	S21	C, TE, CPX, S, OFX, PEF, AM, CXM, CTX, CAZ	6	5	MDR
17	S4	TE, C, SXT, CPX, S, OFX, PEF, AM, AU, CXM, CTX, CAZ	12	6	MDR
18	S31	CXM, CTX, CAZ, TE, C	5	3	MDR
19	HS9	CXM, AM, CAZ, CTX, CPX, C, SXT	7	4	MDR
20	S8	CN, C, S, AU, CAZ, CTX, CXM	7	4	MDR
21	S7	CN, SXT, S, AM, AU, CAZ, CTX, CXM	8	4	MDR
22	S12	SXT, C, CPX, PEF, S, AM, CAZ, CTX, CXM	9	4	MDR
23	S24	CN, SXT, S, AM, CAZ, CTX, CXM	7	4	MDR
24	S40	AU, CAZ, CTX, CXM	4	1	NMDR
25	S114	SXT, C, TE, CPX, PEF, S, OFX, AM, CXM, CTX, CAZ	11	6	MDR
26	S3	CN, C, SXT, CPX, PEF, S, OFX, CXM, CTX, CAZ	10	6	MDR
27	S37	CN, C, PEF, OFX, AU, CAZ, CTX, CXM	8	4	MDR
28	S28	SXT, CPX, OFX, CTX, CXM	5	3	MDR
29	S32	CN, SXT, TE, AM, AU, CXM	6	4	MDR
30	S115	CN, C, PEF, S, AM, AU, CAZ, CTX, CXM	9	5	MDR
31	S113	CN, SXT, C, S, AM, AU, CAZ, CTX, CXM	9	5	MDR
32	HS11	CN, C, PEF, OFX, AM, AU, CAZ, CTX, CXM	9	4	MDR
33	S38	SXT, PEF, AU, CAZ, CTX, CXM	6	3	MDR
34	HS6	CN, CPX, TE, C, SXT, C, S, AU, AM, CXM	10	7	MDR
35	S118	CXM, CTX, CAZ, AU, AM, OFX, PEF, SXT, CN	9	4	MDR
36	S117	CXM, CTX, CAZ, AU, AM, TE, C, SXT	8	4	MDR
37	S55	CN, SXT, PEF, AU, CAZ, CTX, CXM	7	4	MDR
38	S51	SXT, AM, AU, CAZ, CTX, CXM	6	2	NMDR
39	S54	CPX, CAZ, CTX, CXM	4	2	NMDR
40	S50	SXT, C, PEF, OFX, AU, CAZ, CTX, CXM	8	4	MDR
41	S61	CXM, CTX, CAZ, AU, S, PEF, CPX	7	3	MDR
42	S41	CXM, CTX, CAZ, AU, AM, S, C, SXT, CN	9	5	MDR
43	S57	AU, S, CTX, CAZ, CXM, C, CN, SXT, PEF, CPX, OFX	11	6	MDR
44	S71	CXM, CTX, CAZ, AU, AM, S, OFX, CPX, TE, C, SXT, CN	12	7	MDR
45	HS2	CXM, CTX, CAZ, AU, OFX, PEF, CPX	7	2	NMDR
46	S14	CXM, CTX, CAZ, AU, PEF, CLX, C, SXT	8	4	MDR

47	S20	CXM, CTX, CAZ, AU, OFX, PEF, CPX, C, SXT	9	4	MDR
48	S10	CXM, CTX, CAZ, AU, AM, CPX, TE, C, SXT	9	5	MDR
49	S9	CXM, CTX, CAZ, PEF, CPX, C	6	3	MDR
50	S19	SXT, C, CPX, CAZ, CTX, CXM	6	4	MDR
51	S127	CXM, CTX, CAZ, AU, AM, S, OFX, PEF, CPX	9	3	MDR
52	S26	CN, AM, CPX, AM, CAZ, CTX, CXM	6	3	MDR
53	S29	CPX, CXM	2	2	NMDR
54	S76	SXT, CPX, PEF, OFX, CXM, CTX	6	3	MDR
55	S67	CPX, C, SXT, PEF, OFX, AU, AM, CXM, CTZ, CAZ	10	4	MDR
56	S132	CPX, PEF, AU, CAZ, CXM	5	2	NMDR
57	S58	C, CPX, PEF, OFX, AM, AU, CAZ, CTX, CXM	9	3	MDR

Keys: NART = number of antibiotics resistant to, CART = classification of antibiotics resistant to, LR = level of resistance, MDR = multidrug resistance, CN= gentamicin, SXT = cotrimoxazole, C = chloramphenicol, TE = tetracycline, CPX = ciprofloxacin, PEF = pefloxacin, OFX = ofloxacin, S = streptomycin, AM = amoxicillin, AU = augmentin, CAZ = ceftazidime, CTX = cefotaxime, CXM = cefuroxime.

A high percentage [87.7% (50)] of the isolates were multidrug-resistant (MDR i.e. resistant to more than 3 classes of antibiotics tested). The isolates had various patterns, 36.8% (21) of the isolates were resistant to all the beta-lactams tested (AM, AU, CAZ, CTX, CXM), and were also MDR; of which 29.8% (17) were resistant to ≥ 5 classes of antibiotics Table 7.

A significant percentage [47.6% (10)] of the DEC isolates that were MDR, especially those resistant to more than 5 classes of antibiotics tested, exhibited phenotypical characteristics of ESBL (Table 8) and their resistance pattern is presented in Table 9 below.

Table-8. Distribution of extended spectrum β-lactamase among DEC with multidrug resistance profile.

S/N	ISOLATE	AMC	CAZ	CTX	CXM	ESBL
1	S52	22	25	24	24	+
2	S44	23	23	22	20	+
3	S46	10	6	9	6	-
4	S1	14	6	6	6	-
5	HS3	24	27	22	25	+
6	HS7	17	18	14	14	+
7	H21	17	10	15	12	+
8	S4	20	19	17	17	+
9	S114	17	15	15	18	+
10	S3	10	6	6	6	-
11	S115	24	21	22	23	+
12	S113	23	22	26	23	+
13	HS6	13	8	6	10	-
14	S41	12	6	14	6	-
15	S57	12	6	14	6	-
16	S71	9	6	10	6	-
17	S14	12	6	14	6	-
18	S20	6	6	6	6	-
19	S10	22	25	24	24	+
20	S57	12	6	8	10	-
21	S71	15	6	14	9	-

Key: - = negative, + = positive, AMC = amoxicillin-clavulanate, CAZ = ceftazidime, CXM = cefuroxime, CTX = cefotaxime, S =stool, HS = healthy stool.

DNA extraction was done on the 10 isolates that showed phenotypic ESBL production. Half [50% (5)] of the isolates harbored the CTXM genes that amplified at 593 base pair (Plate 1). 90% of the isolated encoded the TEM gene (Plate 2), none of the isolates had SHV (Plate 3), 40% expressed OXA gene (Plate 4), all (100%) the isolate harbored VEB (Plate 5) while only 50% had the PER gene (Plate 6).

Table-9. Resistant patterns of multidrug-resistant DEC that expressed ESBL phenotypically.

S/N	Isolate	Resistant Pattern	NART	CART	LR
1	S52	CN, TE, C, SXT, CPX, S, OFX, PEF, AM, AU, CXM, CTX, CAZ	13	7	MDR
2	S44	CN, SXT, C, PEF, OFX, S, AM, AU, CAZ, CTX, CXM	11	6	MDR
3	S21	CN, SXT, TE, CPX, OFX, S, AM, AU, CAZ, CTX, CXM	11	6	MDR
4	S1	CN, TE, C, SXT, CPX, S, OFX, PEF, AM, AU, CXM, CTX, CAZ	13	7	MDR
5	HS3	CN, SXT, C, OFX, S, AU, CAZ, CTX, CXM	9	6	MDR
6	HS7	CXM, CTX, CAZ, AU, AM, S, OFX, PEF, TE, C, SXT, CN	12	7	MDR
7	S21	CXM, CTX, CAZ, AM, S, OFX, PEF, CPX, TE, C	6	5	MDR
8	S4	CXM, CTX, CAZ, AU, AM, S, OFX PEF, CPX TE, C, SXT	12	6	MDR
9	S114	CXM, CTX, CAZ, AM, S, OFX, PEF, CPX, TE, C, SXT	11	6	MDR
10	S3	CXM, CTX, CAZ, AM, S, OFX, PEF, CPX, C, SXT, CN	10	6	MDR

Keys: NART = number of antibiotics resistant to, CART = classification of antibiotics resistant to, LR = level of resistance, MDR = multidrug resistance, CN= gentamicin, SXT = cotrimoxazole, C = chloramphenicol, TE = tetracycline, CPX = ciprofloxacin, PEF = pefloxacin, OFX = ofloxacin, S = streptomycin, AM = amoxicillin, AU = augmentin, CAZ = ceftazidime, CTX = cefotaxime, CXM = cefuroxime.

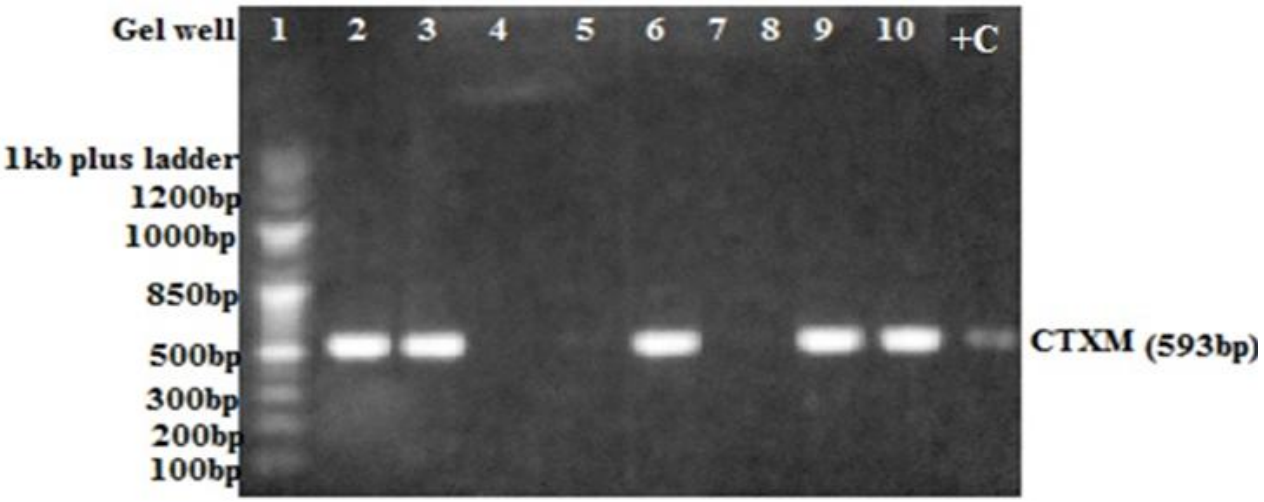

Plate-1. Amplification of CTXM gene from MDR *E. coli* on Gel Electropherogram.

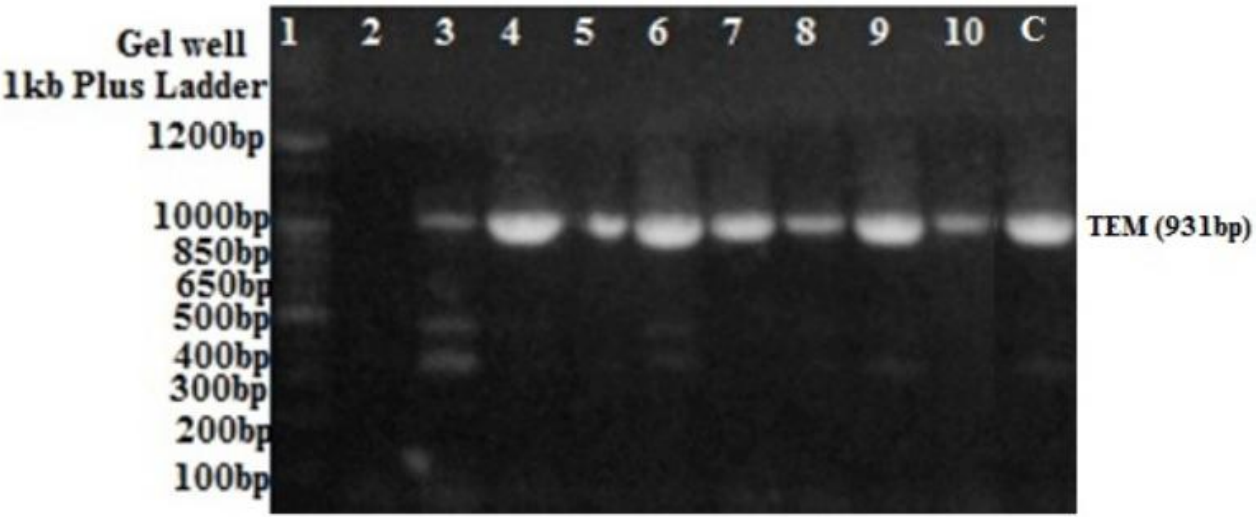

Plate-2. Amplification of TEM gene from MDR *E. coli* on Gel Electropherogram.

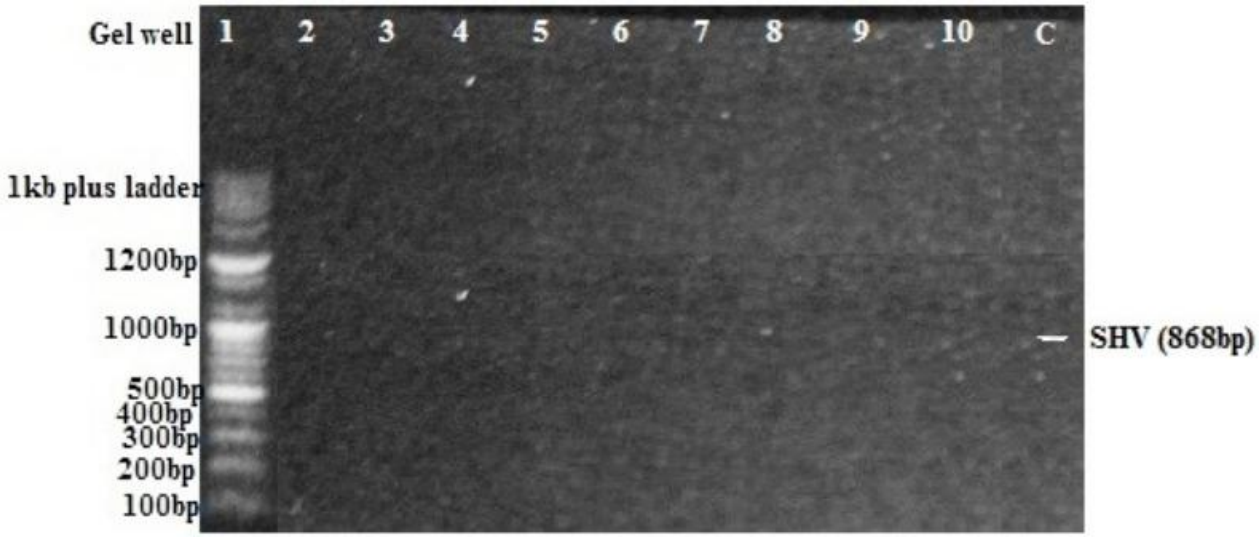

Plate-3. Amplification of SHV gene from MDR *E. coli* on Gel Electropherogram.

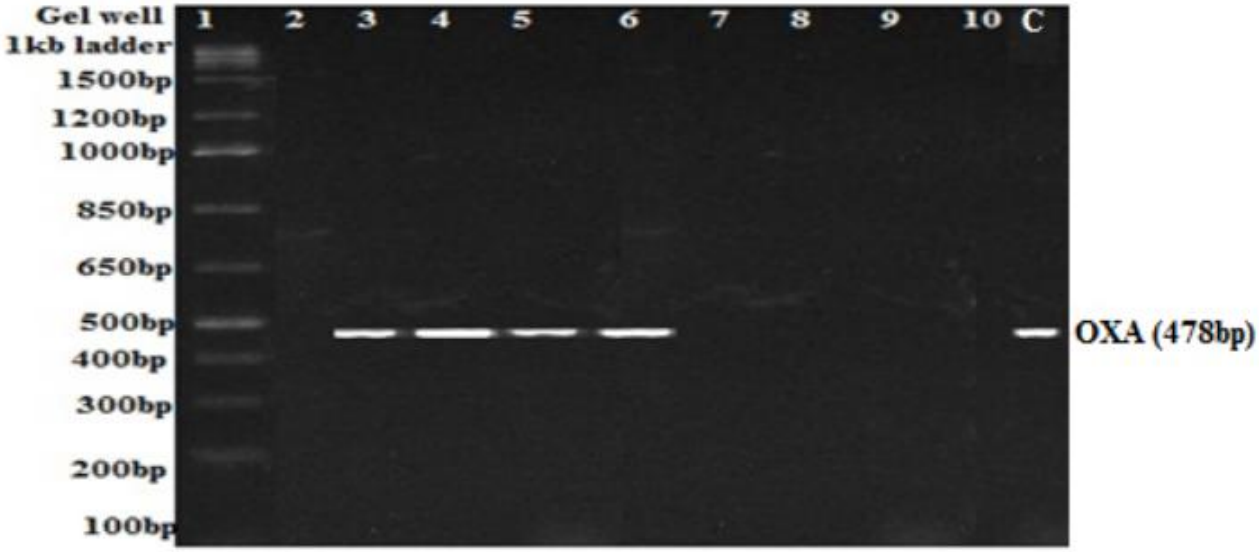

Plate-4. Amplification of OXA gene from MDR *E. coli* on Gel Electropherogram.

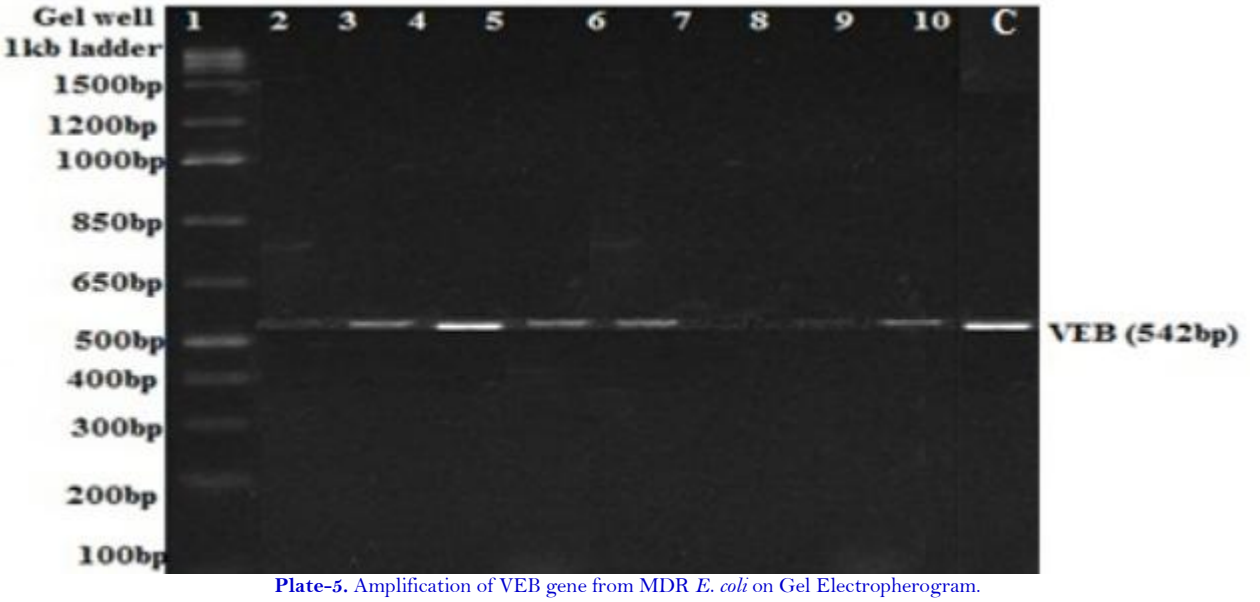

Plate-5. Amplification of VEB gene from MDR *E. coli* on Gel Electropherogram.

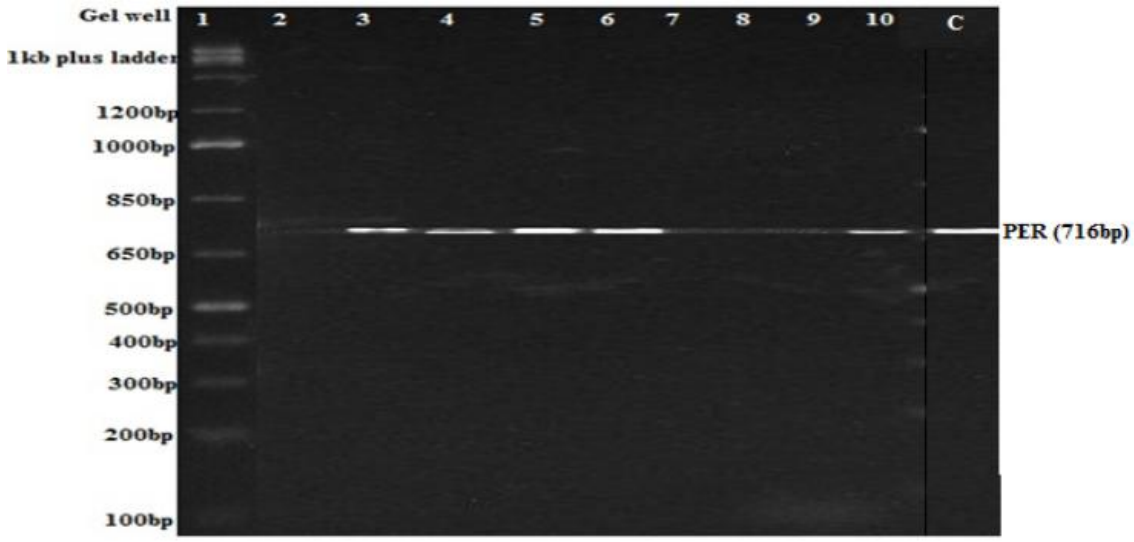

Plate-6. Amplification of PER gene from MDR *E. coli* on Gel Electropherogram.

4. DISCUSSION

According to the 2017 global disease burden estimation, diarrhea accounts for one of the major reasons for hospital visits amongst all ages in developing countries with *E. coli* and Rotavirus documented as the most reported etiological agents. *E.coli* and Rotavirus are reported to be responsible for above 1 million deaths and about 4% of the total global disability-adjusted life-years (DALYS) per year with the highest impact among infants and young children (Khalil et al., 2018; WHO, 2017). A study by Ifeanyi et al. (2015); Onanuga et al. (2014) on children had reported a recovery rate of 18.4% and 12.8% among children with diarrhea in Gwagwalada, Federal Capital Territory, Abuja. Our findings in all age groups observed a total prevalence of 37.7% diarrheagenic *E. coli* from the stools sampled in Minna. These results agree with other findings within Nigeria that *E. coli* is a significant etiological agent of diarrhea (Presterl et al., 2003). However, the prevalence is less than 44.7% reported by Nweze (2010) in South East (Onitsha and Enugu) and 88% reported by Adenipekun et al. (2016) in the South west (Lagos). According to Zhou et al. (2018) who studied the seasonal distribution of DEC, reported that this strain tends to infect young children during summer or autumn. Further analysis of the results showed a significant diarrheagenic *E. coli* isolation rate (53.3%) among healthy individuals in the areas sampled. These asymptomatic carriers according to Onanuga et al. (2014) are likely to be potential spreaders of infectious *E. coli* to susceptible individuals in the study area. However, studies have shown that factors triggering diarrhea in humans abound but the poor quality of food, water, hygiene, and sanitation in developing contributes immensely to high rates commonly reported. Comparative evaluation of age with diarrhea within Minna showed that age groups 21 – 30 years (27.8%) and 31 – 40 years (24.5%) had the highest *E. coli* isolation rate while age groups ≥71 (2.6%) and 0-10 years (3.3%) had the least *E. coli* isolation rate (Table 5). Although the later groups are more susceptible to infection due to low immunity and adaptation, the Centers for Disease Control and Prevention (n.d) had acknowledged that diarrhea is not only attributed to microbial infection rather other factors such as pre-formed microbial toxins, consuming contaminated foods, food allergies, food intolerances (celiac disease or lactose intolerance), some medications, radiation therapy and malabsorption of food (poor absorption). According to Troeger et al. (2018), diarrhea was the eighth leading cause of death among all age groups in 2016 and the study conducted by (Akinjogunla, Eghafona, & Ekoi, 2009); Gupta, Sarker, Rout, Mondal, and Pal (2015) indicates that the prevalence of diarrhea gradually decreased with increasing age. This is contrary to the results observed in this study as diarrheagenic *E. coli* was most common within adults (age 21-40 years). This could be attributed to the report of Barr and Smith (2014) who observed that acute diarrhea associated with bacteria in adults is on the increase globally and it's triggered by increases in travel, co-morbidities, and foodborne illness. Studies conducted by Akinjogunla et al. (2009) in Uyo

(Southern Nigeria) and Nweze (2010) in South-Eastern Nigeria (Onitsha and Enugu) had noted that females (53.33%) had more diarrheagenic *E. coli* than males (46.67%), which supports our findings in Minna. Contrary findings were reported by Olorunshola, Smith, and Coker (2000) in Lagos; Yilgwan and Okolo (2012) in Jos; and Abuzerr et al. (2019) in Gaza who had reported that males are more infected with diarrhea than females. The prevalence of diarrheagenic *E. coli* in different gender is geographically and environmentally influenced. A study by Nweze (2010) showed that gender had no effect on the distribution of diarrheagenic bacteria but individual hygiene plays an important role in the infection rates. Most studies on diarrheagenic *E. coli* have acknowledged antibiotic resistance to commonly prescribed drugs especially the beta-lactams in alarming proportions worldwide due to misuse of antibiotics which do elicit selective force in the bacteria population (Ugwu, Edeani, Ejikeugwu, Okezie, & Ejiofor, 2017). This was also observed in our study as the *E.coli* isolates had significant resistance to most of the beta-lactams tested especially to 3rd generation cephalosporines [Cefotaxime (98.2%), cefuroxime (93%), ceftazidime (84.2%), Augmentin (70.2%), Amoxicillin (59.6%)]. Significant resistance to other classes of antibiotics such as quinolones [pefloxacin (56.1), ciprofloxacin (54.4%), and ofloxacin (45.6)], chloramphenicol (61.4%), cotrimoxazole (61.4%) were also detected. The isolates were observed to show high percentage susceptibility to Tetracycline (77.2%), Streptomycin (56.1%), Ofloxacin (54.4%), and Gentamicin (52.6%). This finding agrees with studies by Ugwu et al. (2017) in Awka Nigeria and Zhang et al. (2018) in China who observed the trend in rising resistance expressed by *E. coli*. Although the WHO guidelines (WHO, 2017) prohibits the use of antibiotics for the treatment of diarrhea especially for undiagnosed cases in Minna Nigeria, antibiotics are often used due to lack of resource personnel, paucity of funds, and impatience in awaiting diagnostic laboratory results (Quan-Cheng, Jian-Guo, Xiang-Hua, & Zhen-Zhen, 2016). These underlining factors have contributed immensely to changes in human microecological factors as observed in nutrition metabolism causing a vast flora imbalance, growth, and development, biological antagonism, and immunity leading to double infections and a decrease in host resistance to infection (Knoop, McDonald, Kulkarni, & Newberry, 2016). According to Kao et al. (2016), the resistant characteristics expressed by the *E.coli* isolates suggest the production of extended-spectrum beta-lactamase enzymes (ESBL), which confers resistance to drugs such as cephalosporins, synthetic oxyimino-β-lactams and penicillin. These attributes have been reported to influence an increase in treatment costs and long hospital stays.

A significant percentage (98.2%) of the isolates had MARI ≥ 0.3. This report is similar to the findings of Igwe, Musa, Olayinka, Ehnimidu, and Onaolapo (2015) who also detected a high MARI among DEC in Zaria, Nigeria. This indicates that the DEC isolates emanated from a source with high-risk contamination where antibiotics are often used. It also shows that large proportions of the bacterial isolates may have been previously exposed to some antibiotics tested in the study (Igwe et al., 2016). A high percentage [87.7% (50) of the isolates had multidrug-resistant (MDR) characteristics i.e. resistant to more than three classes of antibiotics tested. The isolates had various patterns; 36.8% (21) of the isolates showed resistance to all the beta-lactams screened (AM, AU, CAZ, CTX, CXM), and were also MDR; of which 29.8% (17) were resistant to ≥ 5 classes of antibiotics. Studies by Bako et al. (2018) in Burkina Faso and Zhou et al. (2018) in China reported that about 91% and 66.7% of DEC strains were multidrug-resistant (MDR) while Eltai, Al Thani, Al Hadidi, Al Ansari, and Yassine (2020) study in Qatar reported 39.5%. Although the percentage expression of MDR varies across different locations, these studies showcase the possibility of isolating MDR from DEC, which concurs with our finding. This rising resistance profile among the prevalent pathogenic strain of *E. coli*, if allowed to proliferate could pose a significant threat to public health especially among immunocompromised, children, and the elderly. However, the prevalence of DEC is low among the children and elderly in Minna, Nigeria. Further evaluation of results showed that a significant percentage (47.6%) of the DEC isolates that were MDR; especially those that exhibited resistance to more than 5 classes of antibiotics tested, produced phenotypic characteristics of ESBL. Molecular analysis of the isolates indicated that 50%, 90%, 0% of the isolates harbored the CTXM, TEM, SHV genes respectively while 40%, 50%, and 100% expressed OXA, PER, and VEB genes respectively. A significant percentage of the isolates were also seen encoding

more than one form of ESBL gene and also multidrug-resistant. This is similar to the report of Zhou et al. (2018) who also observed different multidrug-resistant and plasmid-encoded genes in DEC. The report showed that 60% of the DEC evaluated possessed carbapenemase genes (2 *blaNDM-1* and 1 *blaKPC-2*) whereas, amongst 30 cephalosporin-resistant DEC, 93.3% possessed extended-spectrum β-lactamase (ESBL) genes, with *blaTEM-1* and *blaCTX-M-55* being the prevalent ESBL genes. Bako et al. (2018) acknowledged the possibility of isolating more than one ESBL gene in DEC isolates. The isolation of DEC with varying resistant profiles in Minna is worrisome as it calls for public health emergency and surveillance. Diarrheagenic *E.coli* with such characteristics are proclaimed to be the major etiological agent responsible for pediatric bacterial diarrhea among children under the age of 5 and the 8th most prominent cause of mortality in developing regions (Soller, Schoen, Bartrand, Ravenscroft, & Ashbolt, 2010). An increasing prevalence of such pathogenic DEC in an environment could lead to fecal contamination of water bodies in resource-limited environments as they are found in waste from diseased humans and animals and may be transported via the sewer lines and agricultural run-offs (Johannessen, Wennberg, Nesheim, & Tryland, 2015).

5. CONCLUSION AND RECOMMENDATIONS

This research work evaluated the existence of ESBL among DEC in Minna and observed a prevalence of 37.7% DEC in the stools sampled. Age group 21 – 30 years had the highest *E. coli* isolation rate (27.8%) while group ≥71 years had the least (2.6%). Females were more affected (64.9%) compared to males (35.1%). The DEC isolates expressed substantial resistance to the majority of the beta-lactams tested and to other classes of antibiotics. High percentages (98.2% and 87.7%) of DEC had MARI ≥ 0.3 and MDR respectively. The isolates had varying patterns of resistance with 47.6% expressing resistance to more than 5 different classes of antibiotics tested and exhibited ESBL characteristics. Molecular evaluation showed that 40%, 50%, and 90%, of the isolates, harbored the OXA, CTXM, and TEM genes respectively while 50% harbored VEB and PER genes. Periodic prevalence surveillance, especially for DEC and its antibiotic resistance profile would provide valuable information in identifying outbreaks, potential reservoirs, and transmission routes. This will go a long way in reducing infant mortality and morbidity associated with diarrhea in the future. More so, promoting various effective intervention and treatment strategies will assist in the prevention of Diarrhea globally.

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