Original Research Article

Molecular diversity and extended spectrum beta-lactamase resistance of diarrheagenic *Escherichia coli* from patients attending selected health care facilities in Nasarawa State, Nigeria

ABSTRACT

Aims: This study investigated the molecular diversity and extended spectrum beta-lactamase resistance of diarrheagenic *E. coli* isolated from patients attending selected healthcare facilities in Nasarawa State, Nigeria.
Place and Duration of Study: Department of Microbiology, Nasarawa State University, P.M.B 1022, Keffi, Nasarawa State, Nigeria; between December 2017 and June, 2019.
Methodology: A total of 207 confirmed *E. coli* isolates from loose stool samples of patients with suspected cases of diarrhea (69 from Federal Medical Centre Keffi [MCK] 69 from General Hospital Akwanga [GHA] and 69 from DalhatuAraf Specialist Hospital Lafia [DASHL]) were included in this study.
Results: Phenotypic detection of ESBL production by β-lactam resistant isolates was done using double disc synergy test. Molecular detection of ESBL genes in phenotypically confirmed ESBL producers was done using Polymerase Chain Reaction. Out of 56 isolates jointly resistant to cefotaxime and/or ceftazidime and ciprofloxacin from DASHL, FMCK and GHA, 53.6% (30/56) were ESBL producers, distributed in relation to the hospitals as follows: $bla_{CTX\text{-}M}$ in DASHL was 6(66.7%), FMCK was 11(100.0%), and GHA was 10(100.0%); bla_{SHV} in DASHL was 8(88.9%), FMCK was 7(63.6%), and GHA was 10(100.0%), and bla_{TEM} in DASHL was 9(100.0%), FMCK was 10(90.9%), and GHA was 10(100.0%). Also, the occurrence of bla_{SHV} was 100.0% in GHA but 88.9% in DASHL.The detection DEC was high in DASHL (88.9%) but low inGHA (58.8%). The occurrence of ETEC was high in GHA (60.0%) while EAEC was also high in FMCK (81.8%) and GHA (70.0%). The isolates were distributed into strain A – J based on RFLP pattern and the occurrence of strain A was high in GHA (70.0%) but low in DASHL (33.3%). **Conclusion:** Most of the isolates were both diarrheagenic and ESBL resistant, and the predominant ESBL and pathotypes genes were $bla_{CTX\text{-}M}$, bla_{TEM} and EAEC. Further studies on molecular detection of sub-types of ESBL and sequencing of diarrheagenic pathotypes genes should be carried out.

1. INTRODUCTION

Diarrhea is defined as the passage of three or more loose or liquid stools per day (or more frequent passage than is normal for the individual); frequent passing of formed stools is not diarrhea, nor is the passing of loose, "pasty" stools by breastfed babies [1].

Common causes of diarrhea in humans include: Rotavirus, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Entamoeba histolytica,* and *Giardia lamblia* [2].The bacterial causes, *Escherichia*

coli (E. coli) has been implicated more frequently [3,4]. Worldwide, reports have shown that *E. coli* causing diarrhea, so-called diarrhoeagenic *Escherichia coli* (DEC), belong to six pathotypes namely: enteroaggregative *Escherichia coli* (EAEC), Enteroinvasive *Escherichia coli* (EIEC) Enterohemorrhagic *Escherichia coli* (EHEC)/Shiga-toxin producing *Escherichia coli* (STEC), enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC) and diffusely adherent *Escherichia coli* (DAEC) [5, 6, 7]. Among the DEC pathotypes, EAEC along with the well-established ETEC and EPEC cause a substantial health burden of infant diarrheal cases and a variety of animal's species [8]. Mostly, DEC outbreaks are often found to be associated with direct contact with infected animals or indirectly through consumption of vegetables, fruits, and water contaminated with infected animal feces [9, 10].This study thus focused on molecular diversity and extended spectrum beta-lactamase resistance of diarrheagenic *E. coli* isolated from patients attending selected healthcare facilities in Nasarawa State, Nigeria.

2. MATERIAL AND METHODS

2.1??

2.2 Sample Collection

A total of 207 (69 from Federal Medical Centre Keffi, 69 from General Hospital Akwanga and 69 from Dalhatu Araf Specialist Hospital Lafia) loose stool samples of patients with suspected cases of diarrhea were randomly collected over a period of three (3) months using sterile container and transported using ice pack to Microbiology Laboratory, Nasarawa State University, Keffi for analysis. The consents of the suspected diarrheic patients were obtained before sample collection.

2.3 Isolation and Identification of *Escherichia coli*

Escherichia coli were isolated from loose stool samples of patients with suspected cases of diarrhea: With the aid of a wire loop, the stool sample was streaked on MacConkey agar (Oxoid Ltd., Basingstoke, UK) plate and incubated at 37°C for 24 h. Pinkish colonies that grew on MacConkey agar were further inoculated on Eosin Methylene Blue agar (Oxoid Ltd., Basingstoke, UK) and incubated at 37°C for 24 h. Greenish metallic sheen colonies that grew on the Eosin Methylene Blue agar plate were selected as presumptive *E. coli* based on method already described [11]. Presumptive *E. coli* were identified by microscopical (Gram stain) and minimum biochemical tests for *E. coli* identification namely "IMViC" (Indole, Methyl red, Voges-Proskauer, Citrate). Indole positive, Methyl red positive, Voges-Proskauer

negative and citrate negative isolates were further confirmed as *E. coli* using a commercial kit B004HI^{TW} (HiMedia Ltd, India) in accordance with the manufacturer's instructions. The bacterium was stored in the refrigerator at 4°C on nutrient agar slants and reactivated by sub-culturing on MacConkey agar and used in the further experiments.

2.4 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the confirmed *E. coli* isolates was carried out as earlier described [8]. Briefly, (3) pure colonies of isolated *E. coli* from loose stool samples of patients with suspected cases of diarrhea was inoculated in to 5 ml sterile 0.85% (w/v) NaCl (BDH Chemicals Ltd., England) and the turbidity of the bacteria suspension was adjusted to the turbidity equivalent to 0.5 McFarland's standard. The McFarland's standard was prepared as follows; 0.5 ml of 1.172% (w/v) $BaCl_2.2H_2O$ (BDH Chemicals Ltd., England) was added into 99.5 ml of 1% (w/v) H_2SO_4 (BDH Chemicals Ltd., England).

A sterile swab stick was soaked in the standardized bacteria suspension and streaked on Mueller- Hinton agar (Oxoid Ltd., Basingstoke, UK) plates and the antibiotic discs (Oxoid Ltd., Basingstoke, UK) were aseptically placed at the center of the plates and allowed to stand for 1 h for pre-diffusion. The plates were placed in an incubator (Model 12-140E, Quincy Lab Inc.) set at 37°C for 24 h. The diameter zone of inhibition in millimeter was measured and the result of the susceptibility was interpreted in accordance with the susceptibility break point earlier described [12].

2.5 Extended Spectrum β-Lactamase (ESBL) Production Test

The confirmatory test for Extended Spectrum β-Lactamase (ESBLs) Production against *E. coli* isolates jointly resistance to cefotaxime, ceftazidime and ciprofloxacin was carried using two-disc method earlier described [13]. Briefly, 10^5 CFU *E. coli* suspensions jointly resistance to cefotaxime, ceftazidime and ciprofloxacin were streaked on sterilized Mueller Hinton agar plates and Amoxicillin-clavulanic acid (30µg) disc was placed in the centre of the plate and cefotaxime (30µg), cefpodoxime (10µg), ceftaxidime (30µg) and ceftriaxone (30µg) disks were placed 15mm (edge-to-edge) from the centre disc. Enhancement of zone of inhibition in the area between the amoxicillin-clavulanic acid disc and any one of the β-lactam disks in comparism with the zone of inhibition on the far side of the drug disc was interpreted as indicative of the presence of an ESBL in the test strain.

2.6 Molecular Detection of ESBL genes

2.6.1 DNA Extraction

The DNAs of *E. coli* isolates that were ESBL-positive by DDST confirmatory test was extracted by a method described previously with minor modifications [14]. Briefly, a sweep of five *E coli* colonies plated on LBA plates was taken, mixed with 200 µl of double-distilled water in 1.5-ml microcentrifuge tubes and boiled for 10minutes in a water bath followed by snap chilling in ice for 5 min. The heat-treated bacterial suspensions were centrifuged at 10000 rpm for 5 min to pellet down the cell debris, and the supernatants were used as DNA templates in the PCR.

2.6.2 Amplification of Primers:

Primers (as in Table 1) for the ESBL genes were amplified by PCR method [9]. Reaction mixtures in final volume of 25 µl was prepared with 10 pmol of each primer, 200 mM of dNTP, 1 unit of Taq polymerase, 2.5 µl of 10Xreaction buffer, 1.5 mM $MgCl_2$ in final concentration, and 100 ng DNA template. Amplification reactions was carried out in a thermocycler (Eppendorf master cycler, MA) under the following conditions: 94^{o}C for 5min, followed by 30cycles of 94^{o}C for 25sec, 52^{o}C for 40sec, 72^{o}C for 50sec, and 72^{o}C for 6min for the final elongation step.

2.6.3 Amplification of Diarrheagenic *Escherichia coli* Genes

The amplification of DEC genes was done by mPCR assay of the DNA extracted from *E. coli* isolates as described [9]. The DNA templates were subjected to multiplex PCR with specific primers for the detection of the following virulence markers: *eaeA* (structural gene for intimin of EHEC and EPEC), *bfpA* (structural gene for the bundle-forming pilus of EPEC), *vt1* and/or *vt2* (Shiga toxins 1 and 2 of EHEC), *eltB* and/or *estA* (enterotoxins of ETEC), *ial* (invasion-associated locus of the invasion plasmid found in EIEC and *Shigella*) and *pCVD* (the nucleotide sequence of the EcoRI-PstI DNA fragment of *pCVD432* of EAEC) as shown in Table 2.

The mPCRs was performed with a 25 µl reaction mixture containing 5 µl of template DNA, 0.2 µl of 18x PCR buffer II, 1.6 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 1.6 µl of 25 mM $MgCl_2$, 0.1 µl of 5 U of AmpliTaq Gold DNA polymerase per µl and a 0.2 µM concentration of each primer except

primer VT1, which was used at a concentration of 0.4 µM. The thermocycling conditions used are as follows: 95°C for 5 min (Initial denaturation), 94°C for 20 sec. (denaturation) 55°C for 30 sec. (Annealing) and 72°C for 30 sec. (initial extension) for 30 cycles, with a final 7 min extension at 72°C [9].

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127 **Table 1**: Primers and target genes with amplicon sizes for Extended Spectrum Beta-lactamase gene in *Escherichia coli*

S/N	Tareget genes	Gene sequence	Amplicon size	References
1	*bla*$_{TEM}$	5´-TCGGGGAAATGTGCGCG-3´ 5´-TGCTTAATCAGTGAGGCACC-3´	972	[15]
2	*bla*$_{SHV}$	5´-GGGTTATTCTTATTTGTCGC-3´ 5´-TTAGCGTTGCCAGTGCTC-3	615	[15]
3	*bla*$_{CTX-M}$	5´-ACGCTGTTGTTAGGAAGTG-3´ 5´-TTGAGGCTGGGTGAAGT-3´	857	[15]

Table 2: Primers and ~~aplicon~~ amplicon size of diarrheagenic *Escherichia coli* pathotypes that was used

Primer	Target gene	Primer sequence	~~Amplimer~~Amplicon size (bp)	Reference
LT	*eltB*	5-TCTCTATGTGCATACGGAGC-3 5-CCATACTGATTGCCGCAAT-3	322	[16]
ST	*estA*	5-GCTAAACCAGTAGAGGTCTTCAAAA-3 5-CCCGGTACAGAGCAGGATTACAACA-3	147	[16]
VT1	*vt1*	5-GAAGAGTCCGTGGGATTACG-3 5-AGCGATGCAGCTATTAATAA-3	130	[16]
VT2	*vt2*	5-ACCGTTTTTCAGATTTTGACACATA-3 5-TACACAGGAGCAGTTTCAGACAGT-3	298	[16]
Eae	*eaeA*	5-CACACGAATAAACTGACTAAAATG-3 5-AAAAACGCTGACCCGCACCTAAAT-3	376	[16]
SHIG	*Ial*	5-CTGGTAGGTATGGTGAGG-3 5-CCAGGCCAACAATTATTTCC-3	320	[16]
BfpA	*bfpA*	5-TTCTTGGTGCTTGCGTGTCTTTT-3 5-TTTTGTTTGTTGTATCTTTGTAA-3	267	[16]
EA	*pCVD*	5-CTGGCGAAAGACTGTATCAT-3 5-CAATGTATAGAAATCCGCTGTT-3	630	[16]

LT= Enterotoxigenic *E. coli* (ETEC); ST=Enterotoxigenic *E. coli* (ETEC);VT=Enterohemorrhagic *E. coli* (EHEC); Eae=Enterohemorrhagic *E. coli* (EHEC); SHIG=Enteroinvasive *E. coli* (EIEC); BfpA=Enteropathogenic *E. coli* (EPEC); EA= Enteroaggregative *E. coli* (EAEC) [16].

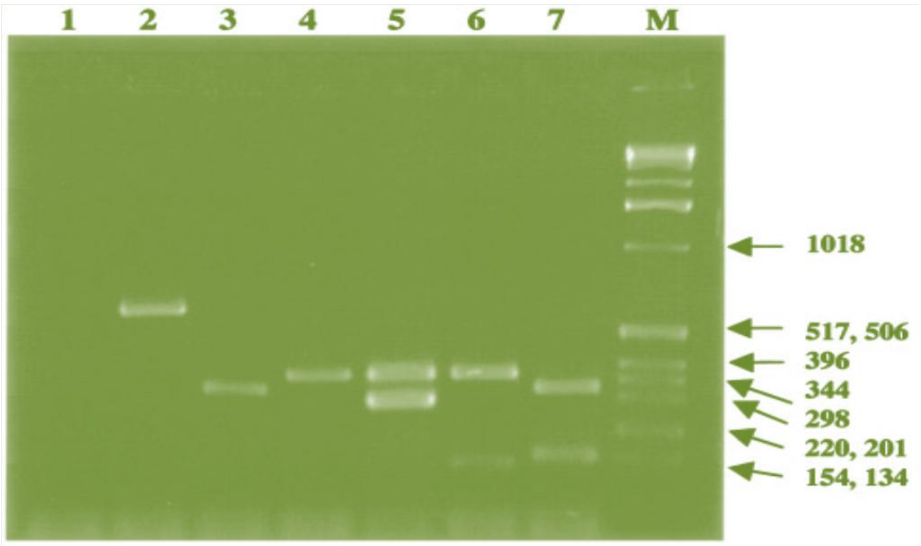

Plate 1: Multiplex PCR amplification of reference strains of diarrheagenic*E. coli* from pure cultures (Lane 1, *E. coli* ATCC 11775; lane2, EAEC 97R; lane 3, EIEC ATCC 43893; lane 4, EPEC ATCC 43887; lane 5, EHEC ATCC 43889; lane 6, EHEC ATCC 43890; lane 7, ETEC ATCC 35401; lane M, marker (1-kb DNA ladder; Gibco/BRL). Numbers on the right are in base pairs).

2.6.4 Amplification of 16S rRNA Gene

The 16S rRNA genes of the ESBL producing isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 min; denaturation, 95ºC for 30 sec; annealing, 52ºC for 30 sec; extension, 72ºC for 30 sec for 35 cycles and final extension, 72ºC for 5 min. The product was resolved on a 1% agarose gel at 120V for 15 min and visualized on a UV transilluminator.

2.6.5 Restriction Endonuclease digestion of amplified 16S rRNA Gene

The endonuclease activity of against The amplified 16S rRNA gene was digested using *BsGr* following the manufacturer's instruction as follows: 2 µl of enzymes solution was added to 36 µl of reaction mixture (10 mMTris-HCl (pH 7.8), 5 mM $MgCl_2$, 20 mMNaCl, 10 mM 2-mercaptoethanol, 10 µg/ml albumin), followed by 2 µl amplified 16S rRNA gene. The mixture was incubated at 37ºC for 1 h; and the restriction

Comment [KA1]: Rephrase

fragment were separated in 1% agarose gel and visualized on a UV transilluminator.

2.6.4 Agarose Gel Electrophoresis

The agarose gel electrophoretic assay for detection of amplified genes for different DEC pathotypes was carried out as described [16]. Briefly, 8µl of PCR products stained with ethidium bromide was loaded into 1.0% (wt/vol) agarose gel wells with a molecular marker run concurrently at 120 V for 30 min. The DNA bands were visualized and photographed under UV light 595nm.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Escherichia coli*

The cultural, morphological and biochemical finger print of *E. coli* isolated from stool of suspected diarrheic patients in Dalhatu Araf Specialist Hospital, Lafia (DASHL), Federal Medical Centre, Keffi (FMCK) and General Hospital, Keffi, Nigeria is as shown in Table 3. Pinkish colony on MCA which grew with greenish metallic sheen on EMB agar was Gram negative rod and had biochemical reactions namely: indole-positive, methyl red-positive, Voges-Proskauer-negative, citrate-negative, ONPG-positive, among others indicated *E. coli*.

3.2 Occurrence of *Escherichia coli*

The occurrence of *Escherichia coli* from stool of patients with suspected cases of diarrhea in the selected health facilities in Nasarawa State, Nigeria is as shown in Figure 1. All (100%) stool samples collected (207) harbored *E. coli* in all the hospitals. The occurrence in relation to age and gender is distributed as shown in Table 3 and 4 respectively.

Comment [KA2]: Table 4& 5

3.3 Molecular Detection of Extended Spectrum Beta-Lactamase

The molecular detection of ESBL production in *E. coli* isolates is as shown in Table 5. Out of 56 isolates jointly resistant to cefotaxime and/or ceftazidime and ciprofloxacin from DASHL, FMCK and GHA, 53.6% (30/56) were ESBL producers, distributed in relation to the hospitals as follows: bla_{CTX-M} in DASHL was 6(66.7%), FMCK was 11(100.0%), and GHA was 10(100.0%); bla_{SHV} in DASHL was 8(88.9%), FMCK was 7(63.6%), and GHA was 10(100.0%), and bla_{TEM} in DASHL was 9(100.0%), FMCK was 10(90.9%), and GHA was 10(100.0%).

Comment [KA3]: Table 6

3.4 Co-existence of the Extended Spectrum Beta-Lactamase Resistance Genes.

The co-existence of the extended spectrum beta-lactamase resistance genes in relation to the hospitals as follows: DASHL $bla_{CTX\text{-}M/SHV/TEM}$ 6(66.7%), FMCK 6(54.5%) and GHA 10(100.0%); $bla_{CTX\text{-}M/SHV}$DASHL 1(11.1%), FMCK 1(9.1%) and GHA 0(0.0%); $bla_{CTX\text{-}M/TEM}$ DASHL 0(0.0%), FMCK 4(36.4%), GHA 0(0.0%); $bla_{SHV/TEM}$ DASHL 2(22.2%), FMCK 0(0.0%) and GHA 0(0.0%) and Bla_{TEM} DASHL 1(11.1%), FMCK 0(0.0%) and GHA 0(0.0%) as shown in Table 6.

Comment [KA4]: Table 7 not 6

3.5 Distribution of Strains of Extended Spectrum Beta-Lactamase Resistant Diarrheagenic *Escherichia coli*.

The distribution of ESBL resistant diarrheagenic *E. coli* into different strains base on their RFLP pattern is as shown in Table 7. The isolates were distributed into strain A – J and the occurrence of strain A was high in GHA (70.0%) but low in DASHL (33.3%). The percentage distribution of strain D, F, H, I, and J were 11.1% in DASHL while the occurance of I was 18.2% in FMCK. In addition the occurrence of strains C, D, and G were 9.1% in FMCK while the occurrence of C, E, and G were 10.0% in GHA.

Comment [KA5]: Table 9

Table 3: Cultural, Morphological and Biochemical characteristics of *Escherichia coli* from stool of patients with suspected cases of diarrhea in Nasarawa State.

Cultural characteristics	Morphological characteristics		Biochemical Characteristics												Inference
	Gram reaction	Morphology	IND	MR	VP	CT	TDA	ONPG	LYS	ORN	UR	NT	H_2S	MAL	
Pinkish colonies on MCA and Greenish metallic sheen on EMB agar	-	Rod	+	+	-	-	-	+	+	+	-	+	-	-	*E. coli*

+ = Positive, - = negative, IND = Indole; MR = Methyl red; Vp = Voges-Proskauer, CT = Citrate, LYS = Lysine, ORN = Ornithine; ONPG = Ortho-Nitrophenyl-β-galactosidase,UR = Urease, NT = Nitrate, H2S = Hydrogen Sulphide, Mal = Malonate, TDA = Phenylalanine deaminas

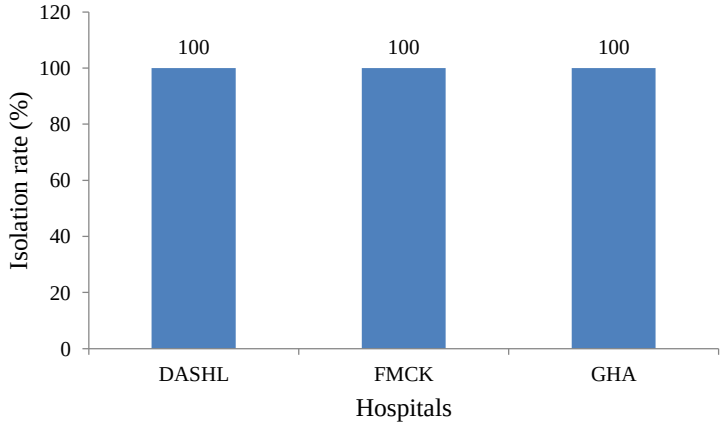

Figure 1: Occurrence of *Escherichia coli* from stool of patients with suspected cases of diarrhea in Nasarawa State in relation to Hospital (DASHL= DalhatuAraf Specialist Hospital Lafia, FMCK= Federal Medical Centre Keffi, GHA= General Hospital Akwanga).

Table 4: Occurrence of *Escherichia coli* in the stool of patients in relation to Age

Age (Years)	No. of Samples			No. (%) *Escherichia coli*		
	DASHL	FMCK	GHA	DASHL	FMCK	GHA
0-5	28	23	29	28(100.0)	23(100.0)	29(100.0)
6-10	17	18	16	17(100.0)	18(100.0)	16(100.0)
11-15	5	6	5	5(100.0)	6(100.0)	5(100.0)
16-20	8	6	1	8(100.0)	6(100.0)	1(100.0)
21-25	4.0	0.0	2.0	4.0(100)	0.0(0.0)	2.0(100)
26-30	6.0	3.0	5.0	6.0(100)	3.0(100)	5.0(100)
31-35	0.0	0.0	6.0	0.0(0.0)	0.0(0.0)	6.0(100)
36-40	0.0	1.0	0.0	0.0(0.0)	1.0(100)	0.0(0.0)
41-45	0.0	5.0	0.0	0.0(0.0)	5.0(100)	0.0(0.0)
>45	1.0	7.0	5.0	1.0(100)	7.0(100)	5.0(100)
Total	**69**	**69**	**69**	**69(100)**	**69(100)**	**69(100)**

DASHL= DalhatuAraf Specialist Hospital, Lafia; FMCK= Federal Medical Centre Keffi; GHA= General Hospital, Akwanga; No.=Number, %= Percentage.

Table 5 Occurrence of *Escherichia coli* in the stool of patients in relation to Gender

Gender	No. of Sample			No. (%) *E. coli*		
	DASHL	FMCK	GHA	DASHL	FMCK	GHA
Male	27	33	29	27(100.0)	33(100.0)	29(100.0)
Female	42	36	40	42(100.0)	36(100.0)	40(100.0)
Total	**69**	**69**	**69**	**69(100.0)**	**69(100.0)**	**69(100.0)**

DASHL= DalhatuAraf Specialist Hospital Lafia; FMCK= Federal Medical Centre, Keffi; GHA= General Hospital, Akwanga; No. = Number; % = Percentage.

Table 6: Molecular detection of Extended Spectrum Beta-Lactamase Resistance Genes in phenotypically confirmed ESBL producing *Escherichia coli* from the stool of the patients

ESBL Resistance Genes	No. (%) Isolates		
	DASHL (n=9)	FMCK (n=11)	GHA (n=10)
bla_{CTX-M}	6(66.7)	11(100.0)	10(100.0)
bla_{SHV}	8(88.9)	7(63.6)	10(100.0)
bla_{TEM}	9(100)	10(90.9)	10(100.0)

DASHL= DalhatuAraf Specialist Hospital, Lafia; FMCK= Federal Medical Centre, Keffi; GHA= General Hospital, Akwanga; No.=Number; %= Percentage.

Table 7: Co-existence of the Extended Spectrum Beta-Lactamase Resistance Genes in the *Escherichia coli* from the stool of the patients

ESBL Resistance Genes	No. (%) Isolates		
	DASHL (n=9)	FMCK (n=11)	GHA (n=10)
$bla_{CTX-M/SHV/TEM}$	6(66.7)	6(54.5)	10(100.0)
$bla_{CTX-M/SHV}$	1(11.1)	1(9.1)	0(0.0)
$bla_{CTX-M//TEM}$	0(0.0)	4(36.4)	0(0.0)
$bla_{SHV/TEM}$	2(22.2)	0(0.0)	0(0.0)
BlaTEM	1(11.1)	0(0.0)	0(0.0)

DASHL= DalhatuAraf Specialist Hospital, Lafia; FMCK= Federal Medical Centre, Keffi; GHA= General Hospital, Akwanga; No.=Number; %= Percentage.

Comment [KA6]: n=10

Table 8: Occurrence of Diarrhegenic *Escherichia coli* genes in Extended Spectrum Beta-Lactamase Resistance Producing *Escherichia coli* from the stool of the patients

Hospitals	ESBL Producers	No. (%) of DEC Pathotypes					Total (%) DEC
		ETEC	EHEC	EPEC	EIEC	EAEC	
DASHL	9	2(22.2)	0(0.0)	0(0.0)	1(11.1)	5(55.6)	**8(88.9)**
FMCK	11	4(36.4)	2(18.2)	0.(0.0)	3(27.3)	9(81.8)	**18(61.1)**
GHA	10	6(60.0)	4(36.4)	0(0.0)	0(0.0)	7(70.0)	**17(58.8)**
Total	**30**	**12(40.0)**	**6(20.0)**	**0(0.0)**	**4(13.3)**	**21(70.0)**	

ESBL= Extended Spectrum Beta-lactamase; DEC= Diarrheagenic*E. coli*; ETEC= Enterotoxigenic*E. coli*; EHEC= Enterohemorrhagic *E. coli*; EPEC= Enteropathogenic *E. coli*; EIEC= Enteroinvasive*E. coli*; EAEC= Enteroaggregative*E. coli*; DASHL= Dalhatu Araf Specialist Hospital, Lafia; FMCK= Federal Medical Centre, Keffi; GHA= General Hospital, Akwanga; No.=Number; %= Percentage.

Table 9: Distribution of different strains of Extended Spectrum Beta-Lactamase diarrheagenic *Escherichia coli* from the stool of the patients

Stains	No. (%) Isolates		
	GHA (n=10)	FMCK (n=11)	DASHL (n=9)
A	7(70.0)	6(54.5)	3(33.3)
B	0(0.0)	0(0.0)	1(11.1)
C	1(10.0)	1(9.1)	0(0.0)
D	0(0.0)	1(9.1)	1(11.1)
E	1(10.0)	0(0.0)	0(0.0)
F	0(0.0)	0(0.0)	1(11.1)
G	1(10.0)	1(9.1)	0(0.0)
H	0(0.0)	0(0.0)	1(11.1)
I	0(0.0)	2(18.2)	1(11.1)
J	0(0.0)	0(0.0)	1(11.1)

ESBL= Extended Spectrum Beta-lactamase; DASHL= Dalhatu Araf Specialist Hospital, Lafia; FMCK= Federal Medical Centre, Keffi; GHA= General Hospital, Akwanga; No.=Number; %= Percentage.

Plate 2: Agarose gel electrophoresis of the 16S rRNA gene of ESBL*E.coli* isolates from DASHL. Lanes D1-D9 represents the 16SrRNA gene bands (1500bp), Lane N represents the negative control, and lane M represents the 1500bp molecular ladder.

Plate 3: Agarose gel electrophoresis of the 16S rRNA gene of ESBL *E. coli* isolates from FMCK. Lane F1, failed amplification, Lanes F2-F11 represents the 16SrRNA gene bands (1500bp), Lane N represents the negative control, lane M represents the 1500bp molecular

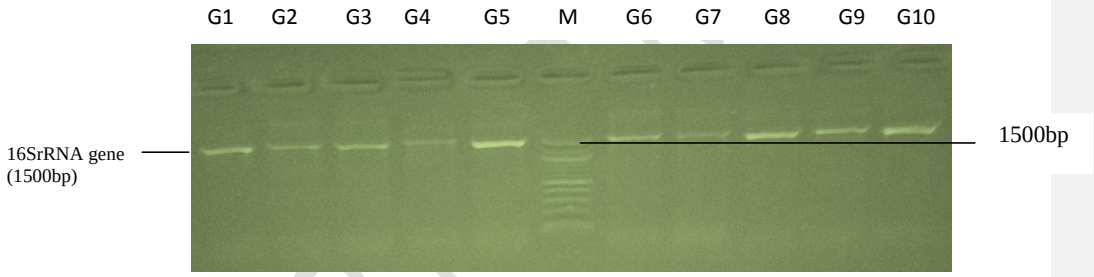

Plate 4: Agarose gel electrophoresis of the 16S rRNA gene of ESBL *E. coli* isolates from GHA. Lanes G1-G10 represents the 16SrRNA gene bands (1500bp), Lane M represents the 1500bp molecular ladder.

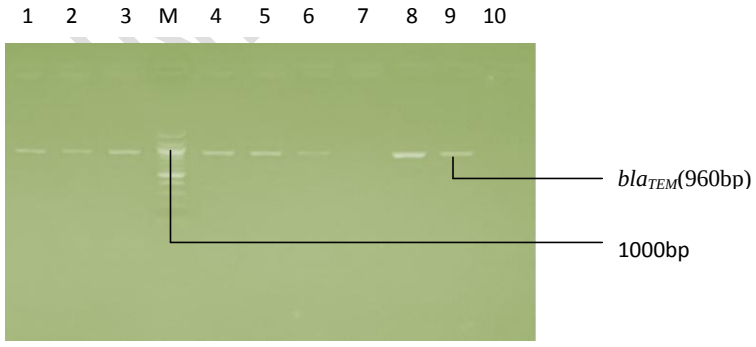

Plate 5: Agarose gel electrophoresis of the amplified bla_{TEM} genes from the *E. coli* isolates from DASHL. Lanes 1, 2, 3, 4, 5, 6, 8, 9 and 10 represent the bla_{TEM} bands, Lane M represents the 1500bp molecular ladder, while other lanes show no bands.

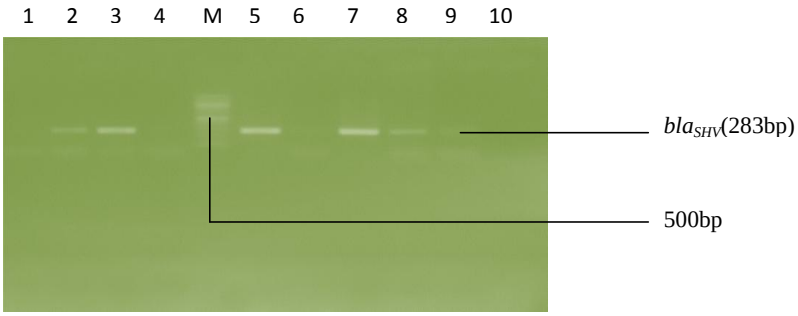

Plate 6: Agarose gel electrophoresis of the amplified *bla*$_{SHV}$gene from the *E. coli* isolates DASHL. Lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent the *bla*$_{SHV}$ bands, Lane M represents the 1500bp molecular ladder, while other lanes show no bands.

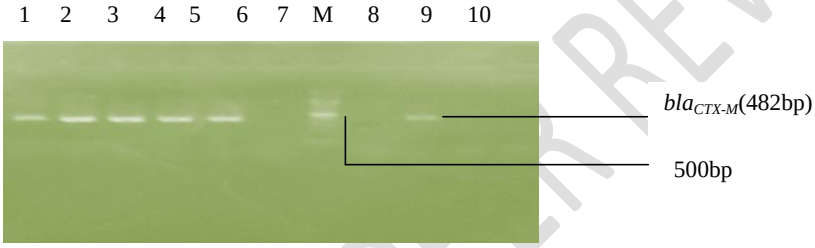

Plate 7: Agarose gel electrophoresis of the amplified *bla*$_{CTX-M}$ gene from the *E. coli* isolates DASHL. Lanes 2, 3, 4, 5, 6 and 9 represent the *bla*$_{CTX-M}$ bands, Lane M represents the 1500bp molecular ladder, while other lanes show no bands.

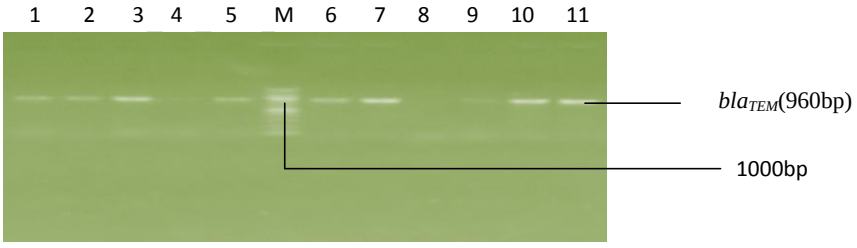

Plate 8: Agarose gel electrophoresis of the amplified *bla*$_{TEM}$ genes from the *E. coli* isolates from FMCK. Lanes 1- Lane and Lane 9- Lane11represent the bla$_{TEM}$ bands, Lane M represent the 1500bp molecular ladder, while Lane 8 showed no bands.

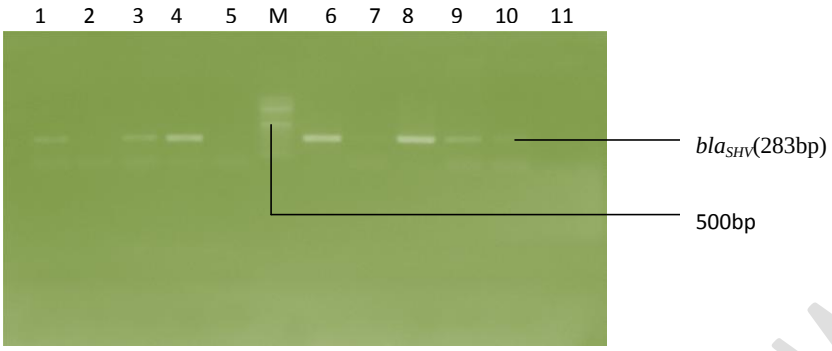

Plate 9: Agarose gel electrophoresis of the amplified *bla_{SHV}* genes from the *E. coli* isolates from FMCK. Lanes 1, 3, 4, 6, 8, 9 and 10 represent the bla$_{SHV}$ bands, Lane M represents the 1500bp molecular ladder, while other lanes show no bands.

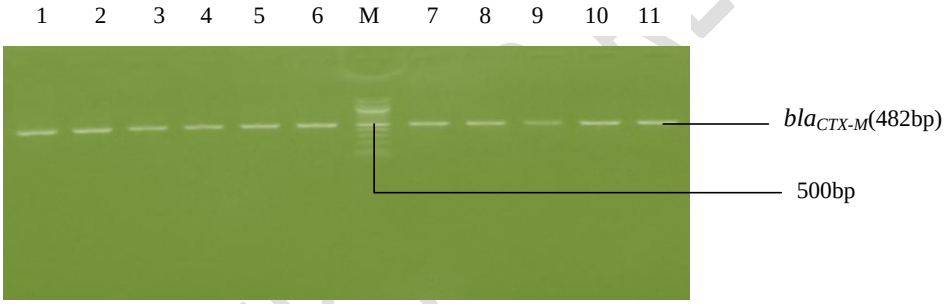

Plate 10: Agarose gel electrophoresis of the amplified *bla_{CTX-M}* gene from the *E. coli* isolates FMCK Lanes 1-Lane 11 represent the *bla_{CTX-M}* bands, Lane M represents the 1500bp molecular ladder.

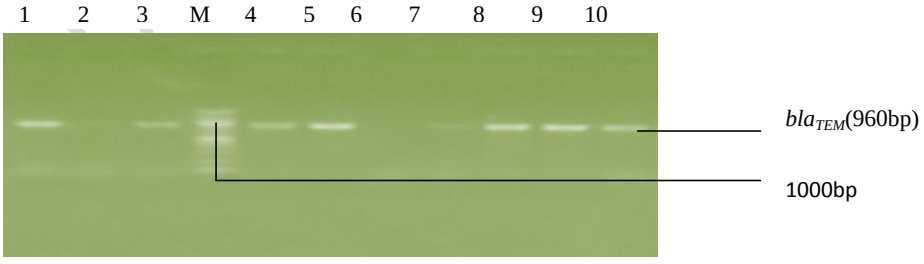

Plate 11: Agarose gel electrophoresis of the amplified *bla_{TEM}* genes from the *E. coli* isolates from GHA. Lanes 1 – Lane 4 and Lane 7 - Lane 10 represent the *bla_{TEM}* bands, Lane M represents the 1500bp molecular ladder, while Lane 6 showed no band.

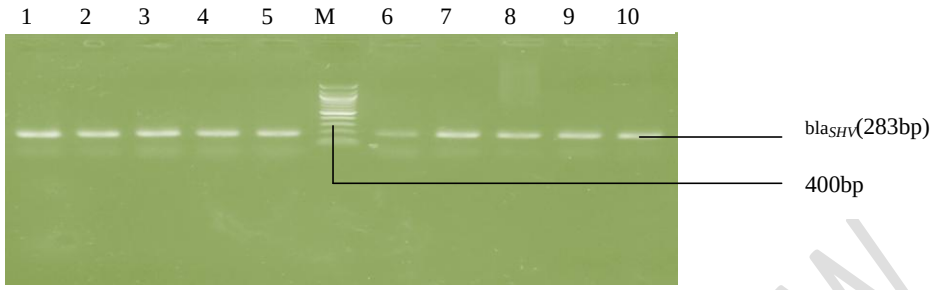

Plate 12: Agarose gel electrophoresis of the amplified *bla*$_{SHV}$ genes from the *E. coli* isolates from GHA. Lanes 1-Lane 10 represent the bla$_{SHV}$ bands, Lane M represents the 1500bp molecular ladder.

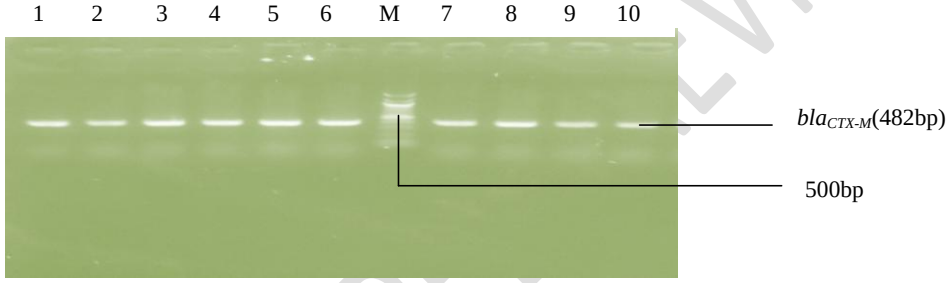

Plate 13: Agarose gel electrophoresis of the amplified *bla*$_{CTX-M}$gene from the *E. coli* isolates GHA. Lanes 1-Lane 10 represent the *bla*$_{CTX-M}$ bands, Lane M represents the 1500bp molecular ladder.

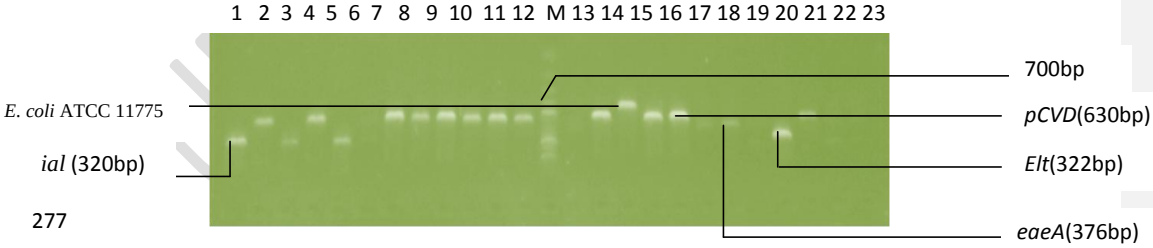

Plate 14: Agarose gel electrophoresis of the diarrheagenic *Escherichia coli*pathotypes from stools of diarrheic patients in Nasarawa State, Nigeria. Amplification; L1, L4 & L6=*ial* (EIEC); L2, L8-L12, L14, L16 & L17=*pCVD* (EAEC); L15= *E. coli* (ATCC 11775); L18-L19= *eaeA*(EHEC); L21*Elt* (ETEC); L23= negative; M= 1500bp; while L7, L13 & L22 showed no band.

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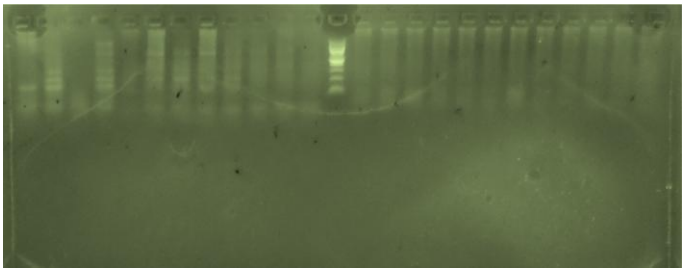
1 2 3 4 5 6 7 8 9 10 11 12 M 13 14 15 16 17 18 19 20 21 22 23



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284 **Plate 15**: RFLP Agarose gel electrophoresis of the 16S rRNA gene of the *Escherichia coli* isolates from DASHL, FMCK and GHA showing different bands pattern after digestion

285 with BsGr.

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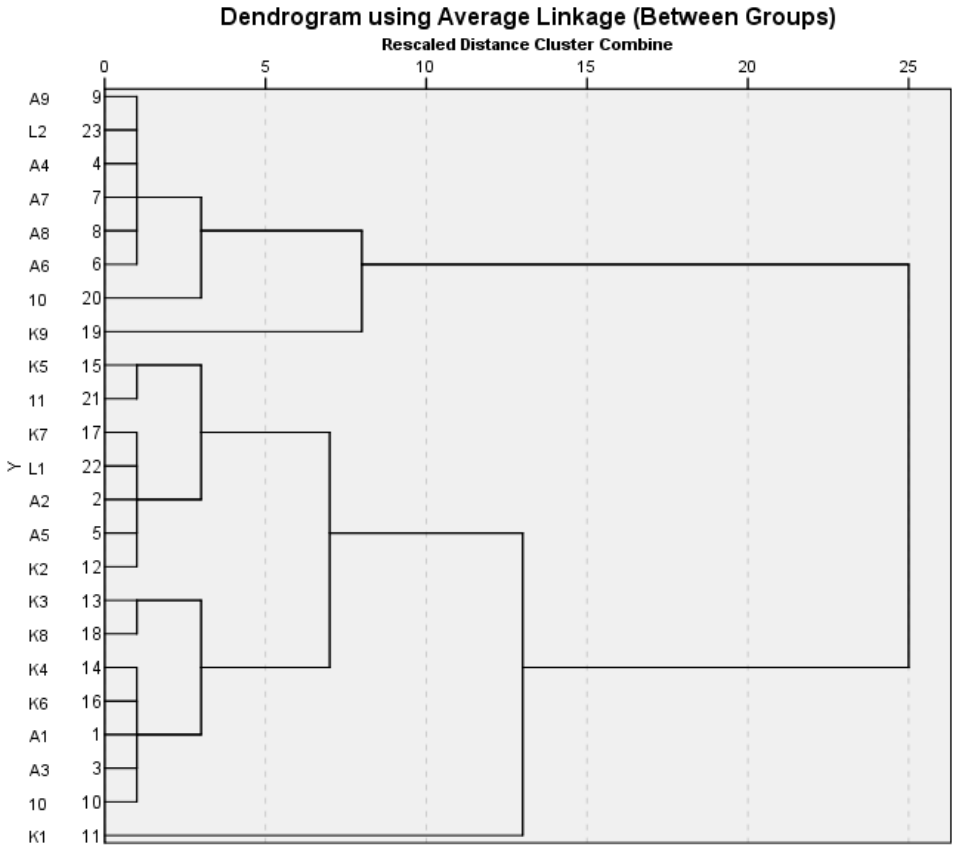

Figure 2: Dendrogram of 16srRNA of Extended Spectrum Beta-lactamase producing *Escherichia coli* after digestion with endonuclease *BsGr*.

The number of infections due to ESBL *E. coli* is increasing, especially in African countries [17]. Diarrheagenic *Escherichia coli* (DEC) are important intestinal pathogens causing a wide variety of gastrointestinal diseases, particularly among children in developing countries [18]. Studies on molecular diversity and extended spectrum beta-lactamase resistance of diarrheagenic *Escherichia coli* isolated from diarrheic patients in Nasarawa State, Nigeria was carried out. The isolation of *E. coli* in all stool samples(100%) in the study locations is in agreement with studies reported [4, 7, 18]; and confirms the fact that *E. coli* is a common bacteria isolated in stool of human.

The occurrence of *Escherichia coli* from stool of patients with suspected cases of diarrhea in relation to age; age group 0-5 and 6-10 years have the highest number of samples collected while age group 35 – >45 have the least number collected. However, it was observed that between age groups the presence of the bacterial isolates with age group 0-5 and 6-10 years having the highest occurrence of bacterial isolates and the least is age group 35 – >45. This follows the same trend with a study done in Abuja [4, 19], which shows that diarrhea is statistically associated with age and majority of the cases occurring in children between 7 months and 2 years of age. The reason for high incidence of bacteria isolates in age group 0-5 and 6-10 years could be due to the fact that children within this age group on their own cannot differentiate between what to eat and what not to eat; they have not learnt the rudiment of adherence to aseptic or hygienic practice; they can barely express themselves. Most diarrhea occur during the first 2 years of life due to combined effects of declining levels of maternally acquired antibodies, the lack of active immunity in the infant, the introduction of food that may be contaminated with faecal bacteria and direct contact with human or animals faeces when the infant start to grow [4, 19]. Most enteric pathogens stimulate at least partial immunity against repeated infection or illness, which helps to explain the declining incidence of disease in older children and adults [20].

The occurrence of ESBL producers in *E. coli* isolates jointly resistant to ceftazidine and cefotaxime observed in this study was higher than 26.3% reported [21], 16.5% reported in Egypt [22]. This study showed that bla_{TEM},bla_{SHV} and $bla_{CTX\text{-}M}$ ESBL gene were expressed in GHA followed by FMCK and DASHL. This finding does not in agree with the study earlier described [23]. The occurrence of $bla_{CTX\text{-}M}$andbla_{TEM} genes was higher in all study location than bla_{SHV} and this finding seems to agree with the study reported [22, 24]. The occurrence of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ ESBL gene observed in this study is higher than that reported [25]. Observation from this study indicated that not all the *E. coli* isolate jointly resistance to both cefotaxime and ceftazidine were ESBL producers and this finding is also in agreement with the study earlier reported [26]. However, the mechanism of resistance to *E. coli* isolates that were jointly resistance to both cefotaxime and ceftazidine may not be due to production of ESBL but may be due to other mechanisms of metabolic resistance.

The Restriction Fragment Length Polymorphism (RFLP) of diarrheagenic *E. coli* of amplified 16S rRNA gene digested with *BsGr* enzymes were distributed into strain A – J and the occurrence of strain A was

high in GHA (70.0%) but low in DASHL (33.3%). The percentage distribution of strain D, F, H, I, and J were 11.1% in DASHL while the occurrence of I was 18.2% in FMCK. In addition, the occurrence of strains C, D, and G were 9.1% in FMCK while the occurrence of C, E, and G were 10.0% in GHA.

The RFLP amplified *16SrRNA* gene digested with *BsGr* is the first study ever conducts in Nasarawa State, Nigeria. However other similar studies on diarrheagenic *E. coli* have been reported elsewhere.

The high frequency of detection of EAEC 81.8% in FMCK, 70.0% in GHA and 55.6% in DASHL observed in this study was not surprising. It is in agreement with 7.2% [9] and 22.0% [7], earlier reported in Kenya and Keffi (in Nigeria). EAEC was previously reported to be endemic in Southern Nigeria as well as in sub-Saharan Africa [27]. So, our observation on the occurrence of EAEC 9(81.8) in FMCK, 7(70.0) in GHA and 5(55.6) in DASHL concurred with what was reported in Southwestern Nigeria and elsewhere especially in the sub-Saharan Africa 18(7.2%) [27].

The frequency of detection of EAEC in this study is higher than that reported [28]; but the detection of ETEC 4(36.4) in FMCK, 6(60.0) in GHA and 2(22.2) in DASHL and EIEC3 (27.3) in FMCK and 1(11.1) in DASHL followed by EHEC 4(36.4) in GHA and 2(18.2) in FMCK were low (1.0 and 1.9%) reported [9, 28] respectively. The very low frequency of detection of diarrheagenic *E. coli* obtained in this study is in close agreement with the study reported [29] with prevalence of *E. coli* O157: H7 in children with diarrhea as 5.4% in Zaria, Nigeria. Also, [30, 31], reported a prevalence of 5% EHEC O157:H7 in humans, in Lagos, Nigeria. But it is in contrast with the study conducted [32], who reported 19.6% prevalence of diarrheagenic *E. coli* in a study conducted in Southeastern Nigeria. An incidence higher than 40% has been reported in Bangladesh by [33]. It was observed that EPEC were not detected in any of the study location, reason may be so because isolation rate of different pathotypes of diarrheagenic *E. coli* have been reported to be vary in different geographical areas although other studies in other parts of the country reported low frequency of detection of EPEC [28, 33], which is in total disagreement with studies carried out in Southeast Nigeria, which reported that EPEC was the most isolated of all DEC pathotypes followed by EAEC, ETEC, EIEC and EHEC in that order [32].

Outbreaks and sporadic cases of EHEC have been reported in developed countries of North America, Japan, Europe and even Australia [34]. However there have been few reports of sporadic EHEC in African countries. Three large EHEC outbreaks were previously reported in Swaziland, Central African

Republic and the Cameroon [34, 35]; but some authors criticized the methodology used in those studies as being nonspecific or insensitive [27]. Despite this, our findings tend to align with the earlier observation that EPEC and EHEC may be rare after all [32, 35].The patients employed in this study may be infected by other pathogens other than diarrheagenic *E. coli* since there are different pathogens that can cause diarrhea in children and adults.

4. CONCLUSION

Diarrheagenic *Escherichia coli* was found in all the study locations; and mostly among children within the Age group 0-5 and 6-10 years and were antibiotic resistance as well as ESBL resistant. The predominant ESBL and pathotypes genes were $bla_{CTX\text{-}M}$, bla_{TEM} and EAEC.

ETHICAL APPROVAL

"All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki."

REFERENCES

1. World Health Organization (2017).
2. Nweze EI. Virulence Properties of Diarrhoeagenic *E. coli* and Etiology of Diarrhoea in Infants, Young Children and Other Age Groups in Southeast, Nigeria. American-Eurasian Journal of Scientific Research, 2009; 4(3):173-179.
3. Ali MMM, Ahmed SF, Klena JD, Mohamed ZK, Moussa TAA, Ghenghesh KS. Enteroaggregative *Escherichia coli* in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. Journal of Infection Developing Countries, 2014; 8(5):589-596.
4. Onanuga A, Igbeneghu O, Lamikanra A. A study of the prevalence of diarrhoeagenic *Escherichia coli* in children from Gwagwalada, Federal Capital Territory, Nigeria. Pam African Medical Journal, 2014; 17:146.
5. Chen HD, Frankel G. Enteropathogenic *Escherichia coli*: unraveling pathogenesis. *FEMS* Microbiological Review; 2014; 29: 83-98.
6. Sani A, Onaolapo JA, Ibrahim YKE, Idris HW, Igwe JC, Nworie A. Prevalence of *Escherichia coli* pathotypes among children with diarrhoea in Zaria, Nigeria; British Journal of Medicine and Medical Research, 2015; 7:17-24.
7. Abimiku RH, Ngwai YB, Nkene IH, Tatfeng YM. Molecular detection of diarrheagenic pathotypes of *Escherichia coli* from diarrheic patients in Keffi, Nigeria. Microbioz Journals, Journal of Microbiology and Biomedical Research, 2016; 2 (3),1-6.
8. Puño-Sarmiento J, Medeiros L,Chiconi C, Martins F, Pelayo J, Rocha S. Detection of diarrheagenic *Escherichia coli* strains isolated from dogs and cats in Brazil. Vetrenary Microbiology; 2013; 166: 676–80.

9. Nguyen TV, Le VP, Le, HC, Gia KN, Weintraub A. Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. Journal of Clinical Microbiology, 2005; 43(2):755-760.

10. Bautista DH, Gómez CA, Rangel VE, Vázquez BE, Castro RJ. Frequency of indicator bacteria, *Salmonella* and diarrhoeagenic *Escherichia coli* pathotypes on ready-to-eat cooked vegetable salads from Mexican restaurants. *Letters of Applied Microbiology*, 2013; 56: 414–20.
11. Cheesbrough M. District Laboratory practice in Tropical Countries, Cambridge University United Kingdom, Part 2, 2006; 63–70.,
12. Clinical and Laboratory Standards Institute (CLSI). 2015.
13. Jarlier V, Nicolas MH, Fournier G, Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Review of Infectious Diseases, 1998; 10: 867-8.

14. Croxen MA, Law RJ, Scholz R. Recent advances in understanding enteric pathogenic *Escherichia coli.* Clinical Microbiology Review; 2013; 26:822–880.

15. Feizabadi MM, Delfani S, Raji N, Majnooni A, Aligholi M, Shahcheraghi F, *et al.* Distribution of bla TEM, bla SHV, bla CTX-M genes among clinical isolates of Klebsiella pneumoniae at Labbafinejad Hospital, Tehran, Iran. Microbial Drug Resistance, 2010; 16(1):49-53.
16. Toma CY, Lu N, Higa N, Nakasone I, Chinen A, Baschkier M, Rivas M. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. J. Clin. Microbiol. 2003; 41:2669–2671.
17. Akbariqomi M, Ghafourian S, Taherikalani M, Mohammadi S, Pakzad I, Sadeghifard N. Antibiotic Susceptibility Patterns of Extended Spectrum Beta-Lactamase and Non Extended Spectrum Beta-LactamasePseudomonas aeruginosa Clinical Isolates. Recent Patents on Anti-infective Drug Discovery, 2015; 10(2), 128-133.
18. Ali MMM, Ahmed SF, Klena JD, Mohamed ZK, Moussa TAA, Ghenghesh KS. Enteroaggregative *Escherichia coli* in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. Journal of Infection Developing Countries, 2014; 8(5):589-596.

19. Ifeanyi CI, Isu RN, Akpa AC, Ikeneche NF. Enteric bacteria pathogens associated with diarrhoea of children in the federal capital territory Abuja, Nigeria. New York Science Journal; 2010; 3(1).

20. Abdullahi M, Olonitola SO, Inabo IH. Isolation of bacteria associated with diarrhoea among children attending some hospitals in Kano Metropolis, Kano State, Nigeria. Bayero Journal of Pure and Applied Sciences. 2010; 3(1):10–15.
21. Fody AM, Boubou L, Moussa A, Bawa HI, Konate A, Yaou C, Zongo C, Salaou C, Daouda A, Sidikou R, Traoure AB, Barro N. Phenotypic Detection of ESBL in multidrug resistant *E. coli* from clinical isolates in Niamey, Niger. Africa .Final Microbiology Research, 2017; 713-717.

22. Ahmed OL, Ahmed SA, Ahmed IZ. Detection of blaSHV and blaCTX-M genes in ESBL producing *Klebsiella pneumonia* isolated from Egyptian patients with suspected nosocomial infections. *Egyptian Journal of Medical Human Gene*, 2013; 14:283-297.
23. Sedighi I, Mohammad RA, Ali R, Zahra K, Mohammad YA. Dissemination of Extended-Spectrum β-Lactamases and Quinolone Resistance Genes among Clinical Isolates of Uropathogenic *Escherichia coli* in Children. Jundishapur Journal of Microbiology, 2015; 8(7): 1-6.
24. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrobial Agent Chemotherapy; 2010; 54: 965-967.
25. Mohadjer S, Bidanjiri A, Hafezi R, Hamidi AH. The first report on the isolation of enterotoxigenic *Escherichia coli* as a cause of infantile diarrhea in Iran; Iranian Journal of Public Health, 1982; 11: 77-88.
26. Nkene IH, Ngwai YB, Omede MU, Samuel J, Envuladu EY, Abimiku RH. Extended spectrum beta-lactamase production in Escherichia coli from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria. International Journal of Research Studies in Biosciences, 2015; 3(12):19-25.

27. Okeke IN. Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status, uncertainties and necessities. Journal of Infectious in Developing Countries; 2009; 3: 817-842.
28. Sani A, Onaolapo JA, Ibrahim YKE, Idris HW, Igwe JC, Nworie A. Prevalence of *Escherichia coli* pathotypes among children with diarrhoea in Zaria, Nigeria; British Journal of Medicine and Medical Research, 2015; 7:17-24.
29. Scaletsky ICA, Aranda KRS, Souza TB, Silva NP. Adherence factors in atypical enteropathogenic *Escherichia coli* strains expressing the localised adherence-like pattern in Hep-2 cells. *J Clinical Microbiology*; 2010; 48: 302-306.
30. Ogunsanya TI, Rotimi VO, Adenuga AA. Study of the aetiological agents of childhood diarrhoea in Lagos, Nigeria. J Med Microb*;* 2009.40:10–14.
31. Olorunshola ID, Smith SI, Coker AO. Prevalence of EHEC O157:H7 in patients with diarrhoea in Lagos, Nigeria. Acta Pathologica Microbiological Immunologica Scandi navica*,* 2000; 108:761-763.
32. Nweze EI. Virulence Properties of Diarrhoeagenic *E. coli* and Etiology of Diarrhoea in Infants, Young Children and Other Age Groups in Southeast, Nigeria. American-Eurasian Journal of Scientific Research, 2009; 4(3):173-179.
33. Albert MJ, Faruque AS, Faruque SM, Sack RB,Mahalanabis, D. Case-control study of enteropathogens associated with childhood diarrhoea in Dhaka, Bangladesh. J Clin. Microbio1999; 37:3458-3464*.*
34. Effler E, Isaa¨cson ML, Arntzen RH, Canter TP, Barrett L, Lee C, Mambo W, Levine AZ, Griffin PM. Factors contributing to the emergence of *Escherichia coli* O157 in Africa *Emerging Infect Dis* 2001; 7: 812-819.
35. Germanii YB, Soro MV, MorelOJM. Enterohaemorrhagic *Escherichia coli* in the Central African Republic *Lancet* 1997; 349: 1670