1 *Original Research Article*

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

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10 **ABSTRACT**

11 **Aims:** This study investigated the molecular diversity and extended spectrum beta-lactamase resistance
12 of diarrheagenic *E. coli* isolated from patients attending selected healthcare facilities in Nasarawa State,
13 Nigeria.
14 **Place and Duration of Study:** Department of Microbiology, Nasarawa State University, P.M.B 1022,
15 Keffi, Nasarawa State, Nigeria; between December 2017 and June, 2019.
16 **Methodology:** A total of 207 confirmed *E. coli* isolates (using standard microbiological methods) from
17 loose stool samples of patients with suspected cases of diarrhea (69 from Federal Medical Centre Keffi
18 [MCK] 69 from General Hospital Akwanga [GHA] and 69 from Dalhatu Araf Specialist Hospital Lafia
19 [DASHL]) were included in this study. **Results:** Phenotypic detection of ESBL production by β-lactam
20 resistant isolates was done using double disc synergy test. Molecular detection of ESBL genes in
21 phenotypically confirmed ESBL producers was done using Polymerase Chain Reaction. Out of 56
22 isolates jointly resistant to cefotaxime and/or ceftazidime and ciprofloxacin from DASHL, FMCK and GHA,
23 53.6% (30/56) were ESBL producers, distributed in relation to the hospitals as follows: $bla_{CTX\text{-}M}$ in DASHL
24 was 6(66.7%), FMCK was 11(100.0%), and GHA was 10(100.0%); bla_{SHV} in DASHL was 8(88.9%),
25 FMCK was 7(63.6%), and GHA was 10(100.0%), and bla_{TEM} in DASHL was 9(100.0%), FMCK was
26 10(90.9%), and GHA was 10(100.0%). Also, the occurrence of bla_{SHV} was 100.0% in GHA but 88.9% in
27 DASHL. The detection DEC was high in DASHL (88.9%) but low inGHA (58.8%). The occurrence of
28 ETEC was high in GHA (60.0%) while EAEC was also high in FMCK (81.8%) and GHA (70.0%). The
29 isolates were distributed into strain A – J based on RFLP pattern and the occurrence of strain A was high
30 in GHA (70.0%) but low in DASHL (33.3%). **Conclusion:** Most of the isolates were both diarrheagenic
31 and ESBL resistant, and the predominant ESBL and pathotypes genes were $bla_{CTX\text{-}M}$, bla_{TEM} and EAEC.
32 Further studies on molecular detection of sub-types of ESBL and sequencing of diarrheagenic pathotypes
33 genes should be carried out.

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36 ## 1. INTRODUCTION

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

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

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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 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.

2.2 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

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

75 **2.3 Antimicrobial Susceptibility Testing**

76 Antimicrobial susceptibility testing of the confirmed *E. coli* isolates was carried out as earlier described [8].
77 Briefly, (3) pure colonies of isolated *E. coli* from loose stool samples of patients with suspected cases of
78 diarrhea was inoculated in to 5 ml sterile 0.85% (w/v) NaCl (BDH Chemicals Ltd., England) and the
79 turbidity of the bacteria suspension was adjusted to the turbidity equivalent to 0.5 McFarland's standard.
80 The McFarland's standard was prepared as follows; 0.5 ml of 1.172% (w/v) $BaCl_2.2H_2O$ (BDH Chemicals
81 Ltd., England) was added into 99.5 ml of 1% (w/v) H_2SO_4 (BDH Chemicals Ltd., England).
82 A sterile swab stick was soaked in the standardized bacteria suspension and streaked on Mueller- Hinton
83 agar (Oxoid Ltd., Basingstoke, UK) plates and the antibiotic discs (Oxoid Ltd., Basingstoke, UK) were
84 aseptically placed at the center of the plates and allowed to stand for 1 h for pre-diffusion. The plates
85 were placed in an incubator (Model 12-140E, Quincy Lab Inc.) set at 37°C for 24 h. The diameter zone of
86 inhibition in millimeter was measured and the result of the susceptibility was interpreted in accordance
87 with the susceptibility break point earlier described [12].

88 **2.4 Extended Spectrum β-Lactamase (ESBL) Production Test**

89 The confirmatory test for Extended Spectrum β-Lactamase (ESBLs) Production against *E. coli* isolates
90 jointly resistance to cefotaxime, ceftazidime and ciprofloxacin was carried using two-disc method earlier
91 described [13]. Briefly, 10^5 CFU *E. coli* suspensions jointly resistance to cefotaxime, ceftazidime and
92 ciprofloxacin were streaked on sterilized Mueller Hinton agar plates and Amoxicillin-clavulanic acid
93 (30µg) disc was placed in the centre of the plate and cefotaxime (30µg), cefpodoxime (10µg), ceftaxidime
94 (30µg) and ceftriaxone (30µg) disks were placed 15mm (edge-to-edge) from the centre disc.
95 Enhancement of zone of inhibition in the area between the amoxicillin-clavulanic acid disc and any one of
96 the β-lactam disks in comparison with the zone of inhibition on the far side of the drug disc was
97 interpreted as indicative of the presence of an ESBL in the test strain.

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98 ## 2.5 Molecular Detection of ESBL genes

99 ### 2.5.1 DNA Extraction

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

106 ### 2.5.2 Amplification of Primers:

107 Primers (as in Table 1)for the ESBL genes were amplified by PCR method[9]. Reaction mixtures in final
108 volume of 25 µl was prepared with 10 pmol of each primer,200 mM of dNTP, 1 unit of Taq polymerase,
109 2.5 µl of 10Xreaction buffer, 1.5 mM $MgCl_2$in final concentration, and100 ng DNA template. Amplification
110 reactions was carried out in a thermocycler (Eppendorf master cycler, MA) under the following conditions:
111 94°C for 5min, followed by 30cycles of 94°C for 25sec, 52°C for 40sec, 72°C for 50sec, and72^{o}C for 6min
112 for the final elongation step.

113 ### 2.5.3 Amplification of Diarrheagenic *Escherichia coli* Genes

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

121 The mPCRs was performed with a 25 µl reaction mixture containing 5 µl of template DNA, 0.2 µl of 18x
122 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
123 µl of 5 U of AmpliTaq Gold DNA polymerase per µl and a 0.2 µM concentration of each primer except

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124 primer VT1, which was used at a concentration of 0.4 μM.The thermocycling conditions used are as

125 follows: 95°C for 5 min (Initial denaturation), 94°C for 20 sec. (denaturation) 55°C for 30 sec. (Annealing)

126 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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128 **Table 1**: Primers and target genes with amplicon sizes for Extended Spectrum Beta-lactamase gene in *Escherichia coli*

S/N	Target 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	$bla_{CTX\text{-}M}$	5´-ACGCTGTTGTTAGGAAGTG-3´ 5´-TTGAGGCTGGGTGAAGT-3´	857	[15]

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

Primer	Target gene	Primer sequence	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	*lal*	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]

130 LT= Enterotoxigenic *E. coli* (ETEC); ST=Enterotoxigenic *E. coli* (ETEC);VT=Enterohemorrhagic *E. coli* (EHEC); Eae=Enterohemorrhagic *E. coli* (EHEC); SHIG=Enteroinvasive

131 *E. coli* (EIEC); BfpA=Enteropathogenic *E. coli* (EPEC); EA= Enteroaggregative *E. coli* (EAEC) [16].

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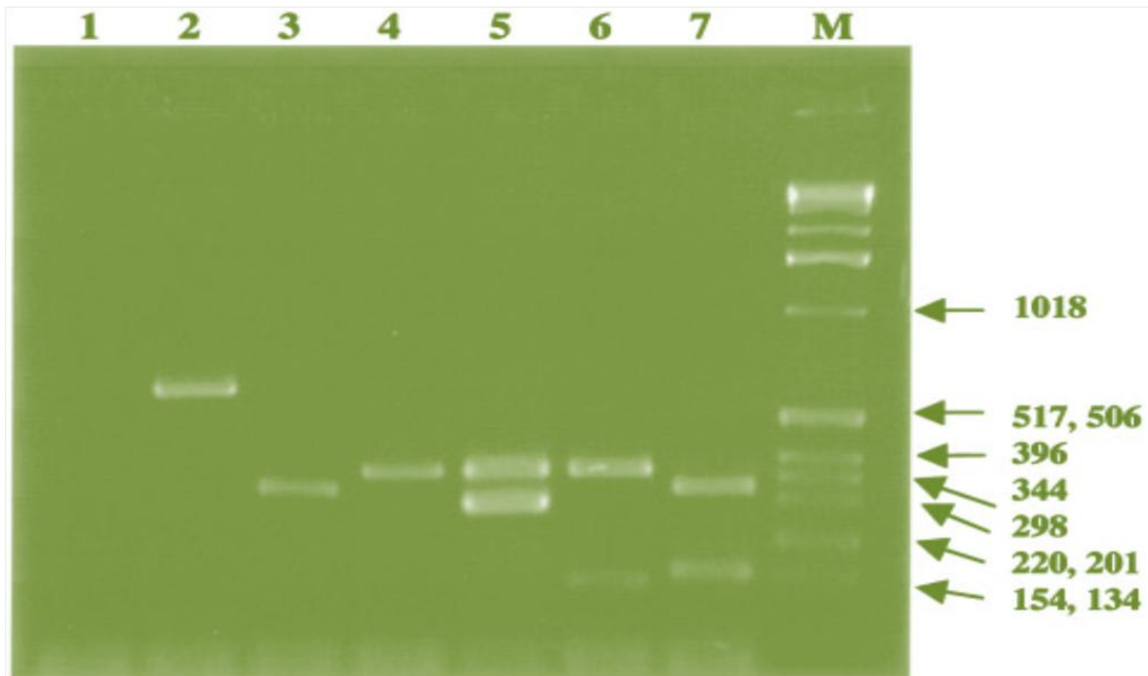

132 **Plate 1:** Multiplex PCR amplification of reference strains of diarrheagenic *E. coli* from pure cultures (Lane 1, *E. coli* ATCC 11775;
133 lane2, EAEC 97R; lane 3, EIEC ATCC 43893; lane 4, EPEC ATCC 43887; lane 5, EHEC ATCC 43889; lane 6, EHEC ATCC 43890;
134 lane 7, ETEC ATCC 35401; lane M, marker (1-kb DNA ladder; Gibco/BRL). Numbers on the right are in base pairs).
135

136 **2.5.4 Amplification of 16S rRNA Gene**

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

145 **2.5.5 Restriction Endonuclease digestion of amplified 16S rRNA Gene**

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

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

151 **2.5.6 Agarose Gel Electrophoresis**

152 The agarose gel electrophoretic assay for detection of amplified genes for different DEC pathotypes was
153 carried out as described [16]. Briefly, 8µl of PCR products stained with ethidium bromide was loaded into
154 1.0% (wt/vol) agarose gel wells with a molecular marker run concurrently at 120 V for 30 min. The DNA
155 bands were visualized and photographed under UV light 595nm.

156 **3. RESULTS AND DISCUSSION**

157 **3.1 Isolation and Identification of *Escherichia coli***

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

164 **3.2 Occurrence of *Escherichia coli***

165 The occurrence of *Escherichia coli* from stool of patients with suspected cases of diarrhea in the selected
166 health facilities in Nasarawa State, Nigeria is as shown in Figure 1. All (100%) stool samples collected
167 (207) harbored *E. coli* in all the hospitals. The occurrence in relation to age and gender is distributed as
168 shown in Table 4 and 5 respectively.

169 **3.3 Molecular Detection of Extended Spectrum Beta-Lactamase**

170 The molecular detection of ESBL production in *E. coli* isolates is as shown in Table 6. Out of 56 isolates
171 jointly resistant to cefotaxime and/or ceftazidime and ciprofloxacin from DASHL, FMCK and GHA, 53.6%
172 (30/56) were ESBL producers, distributed in relation to the hospitals as follows: $bla_{CTX\text{-}M}$ in DASHL was
173 6(66.7%), FMCK was 11(100.0%), and GHA was 10(100.0%); bla_{SHV} in DASHL was 8(88.9%), FMCK
174 was 7(63.6%), and GHA was 10(100.0%), and bla_{TEM} in DASHL was 9(100.0%), FMCK was 10(90.9%),
175 and GHA was 10(100.0%).

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176 ### 3.4 Co-existence of the Extended Spectrum Beta-Lactamase Resistance Genes.

177 The co-existence of the extended spectrum beta-lactamase resistance genes in relation to the hospitals
178 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
179 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
180 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%),
181 FMCK 0(0.0%) and GHA 0(0.0%) as shown in Table 7. Occurrence of Diarrhegenic *Escherichia coli*
182 genes in Extended Spectrum Beta-Lactamase Resistance *Escherichia coli* from the stool of the patients is
183 as shown in Table 8.

184 ### 3.5 Distribution of Strains of Extended Spectrum Beta-Lactamase Resistant
185 ### Diarrheagenic *Escherichia coli*.

186 The distribution of ESBL resistant diarrheagenic *E. coli* into different strains base on their RFLP pattern is
187 as shown in Table 9. The isolates were distributed into strain A – J and the occurrence of strain A was
188 high in GHA (70.0%) but low in DASHL (33.3%). The percentage distribution of strain D, F, H, I, and J
189 were 11.1% in DASHL while the occurrence of I was 18.2% in FMCK. In addition the occurrence of
190 strains C, D, and G were 9.1% in FMCK while the occurrence of C, E, and G were 10.0% in GHA.
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192 **Table 3:** Cultural, Morphological and Biochemical characteristics of *Escherichia coli* from stool of patients with suspected cases of

193 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*

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

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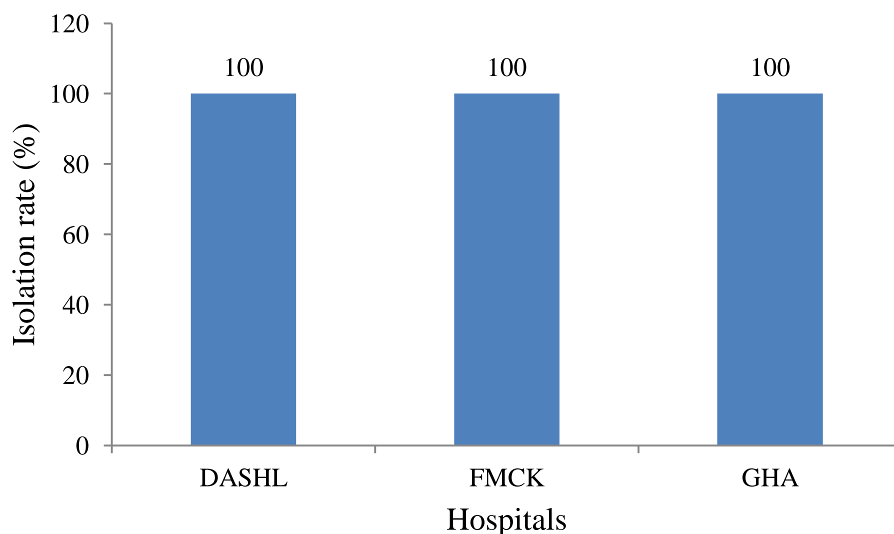

Figure 1: Occurrence of *Escherichia coli* from stool of patients with suspected cases of diarrhea in Nasarawa State in relation to Hospital (DASHL= Dalhatu Araf Specialist Hospital Lafia, FMCK= Federal Medical Centre Keffi, GHA= General Hospital Akwanga).

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

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206 **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)**

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

209

210 **Table 6:** Molecular detection of Extended Spectrum Beta-Lactamase Resistance Genes in phenotypically
211 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)

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

214

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

ESBL Resistance Genes	No. (%) Isolates		
	DASHL (n=10)	**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)

217 DASHL= Dalhatu Araf Specialist Hospital, Lafia; FMCK= Federal Medical Centre, Keffi; GHA= General Hospital, Akwanga;
218 No.=Number; %= Percentage.
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223 **Table 8:** Occurrence of Diarrhegenic *Escherichia coli* genes in Extended Spectrum Beta-Lactamase
224 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)**	

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

229

230 **Table 9:** Distribution of different strains of Extended Spectrum Beta-Lactamase diarrheagenic*Escherichia*
231 *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)

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

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236 **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.

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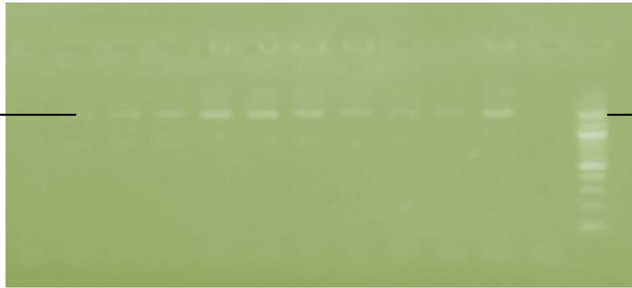

238

239 **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

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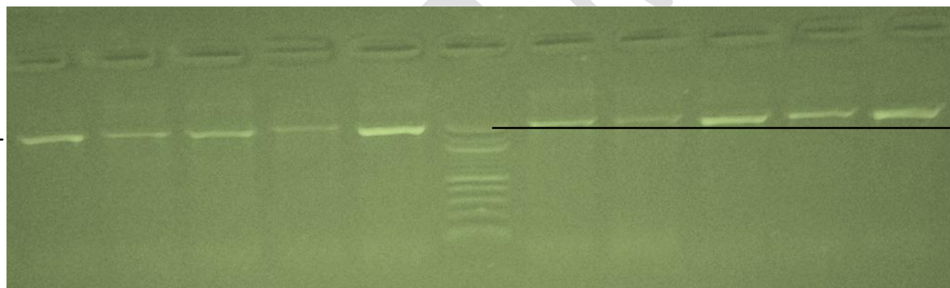

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243 **Plate 4**: Agarose gel electrophoresis of the 16S rRNA gene of ESBL *E. coli* isolates from GHA. Lanes G1-G10 represents the 16SrRNA gene bands (1500bp), Lane M represents the 1500bp molecular ladder.

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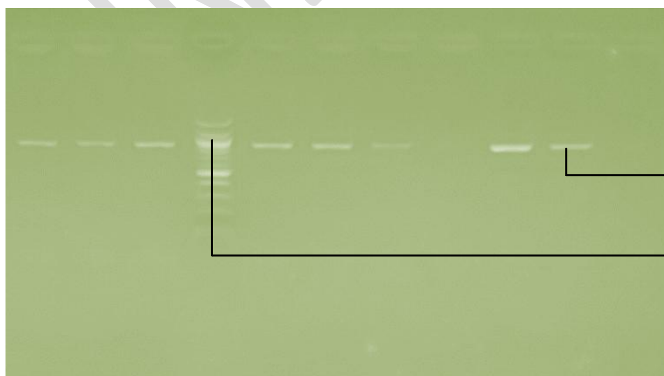

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Plate 5: Agarose gel electrophoresis of the amplified bla_{TEM} genes from the *E. coli* isolates from DASHL. Lanes 1, 2, 3, 4, 5, 6, 8, 9 and 10 represent the bla_{TEM} bands, Lane M represents the 1500bp molecular ladder, while other lanes show no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

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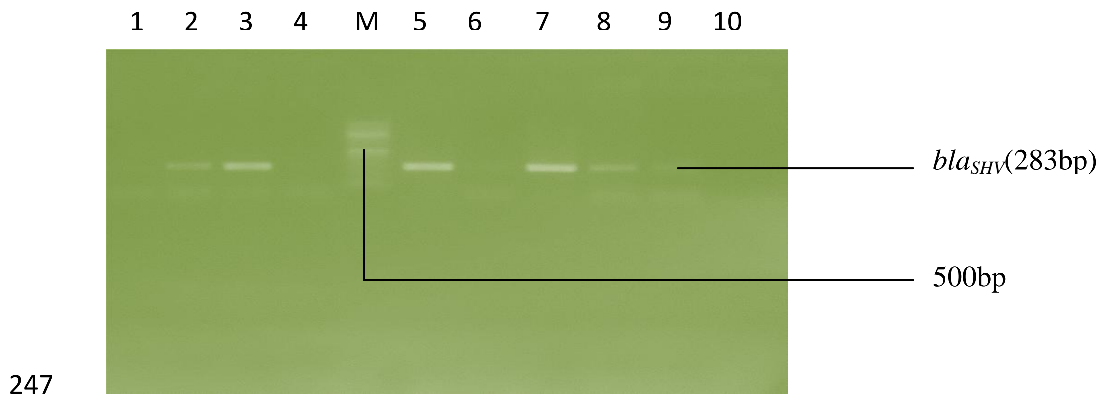

248 **Plate 6**: Agarose gel electrophoresis of the amplified *bla*$_{SHV}$gene from the *E. coli* isolates DASHL. Lanes 2, 3, 4, 5, 6, 7, 8 and 9 represent the *bla*$_{SHV}$ bands, Lane M represents the 1500bp molecular ladder, while other lanes show no bands.

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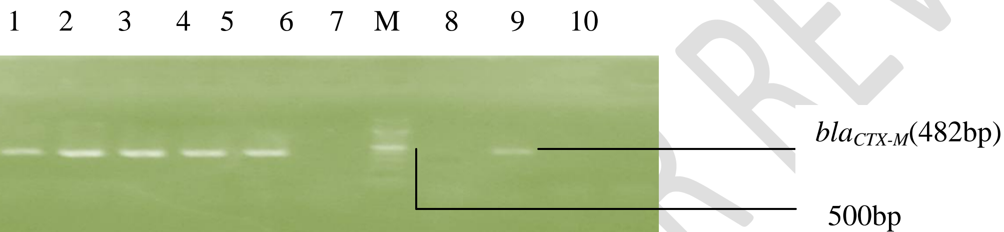

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253 **Plate 7:** Agarose gel electrophoresis of the amplified *bla*$_{CTX-M}$ gene from the *E. coli* isolates DASHL. Lanes 2, 3, 4, 5, 6 and 9 represent the *bla*$_{CTX-M}$ bands, Lane M represents

254 the 1500bp molecular ladder, while other lanes show no bands.

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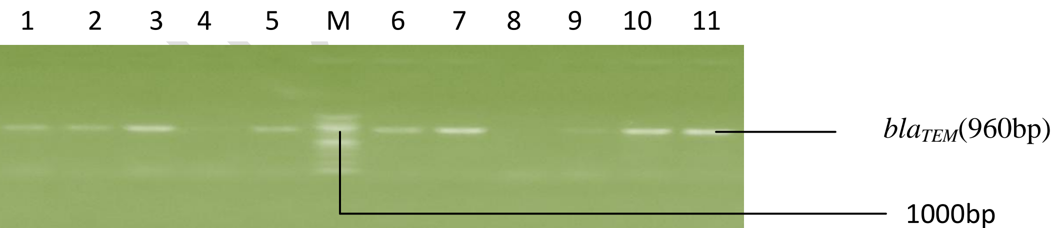

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258 **Plate 8:** Agarose gel electrophoresis of the amplified *bla*$_{TEM}$ genes from the *E. coli* isolates from FMCK. Lanes 1- Lane and Lane 9- Lane11represent the bla$_{TEM}$ bands, Lane M represent the 1500bp molecular ladder, while Lane 8 showed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

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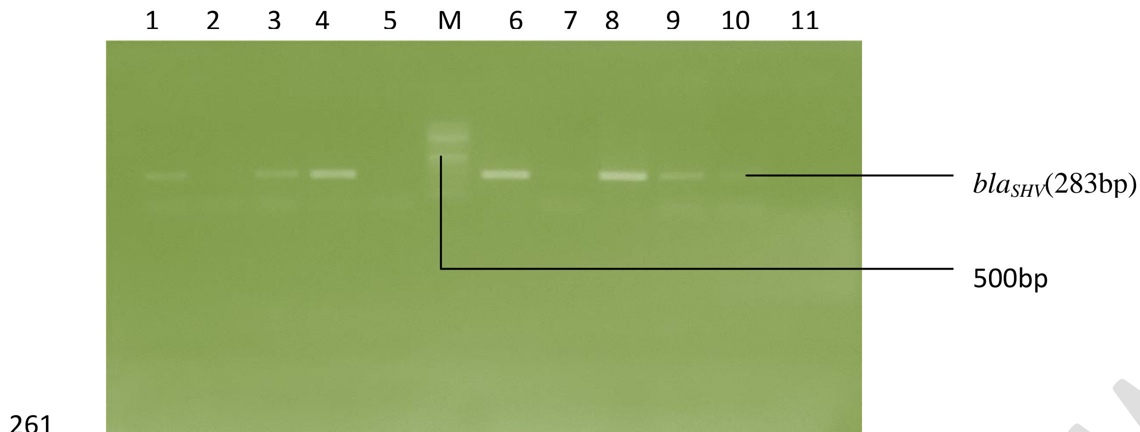

262 **Plate 9:** Agarose gel electrophoresis of the amplified *bla*$_{SHV}$ genes from the *E. coli* isolates from FMCK. Lanes 1, 3, 4, 6, 8, 9 and 10 represent the bla$_{SHV}$ bands, Lane M represents the 1500bp molecular ladder, while other lanes show no bands.

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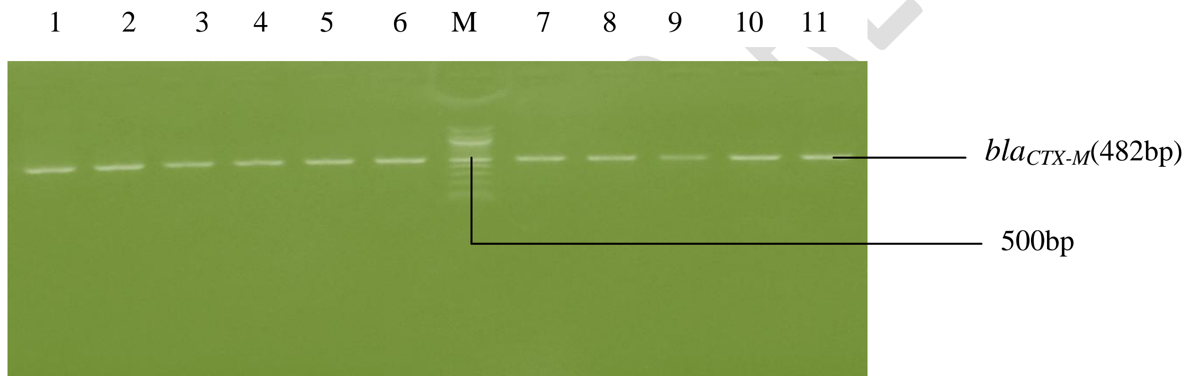

266 **Plate 10:** Agarose gel electrophoresis of the amplified *bla*$_{CTX-M}$ gene from the *E. coli* isolates FMCK Lanes 1-Lane 11 represent the *bla*$_{CTX-M}$ bands, Lane M represents the 1500bp molecular ladder.

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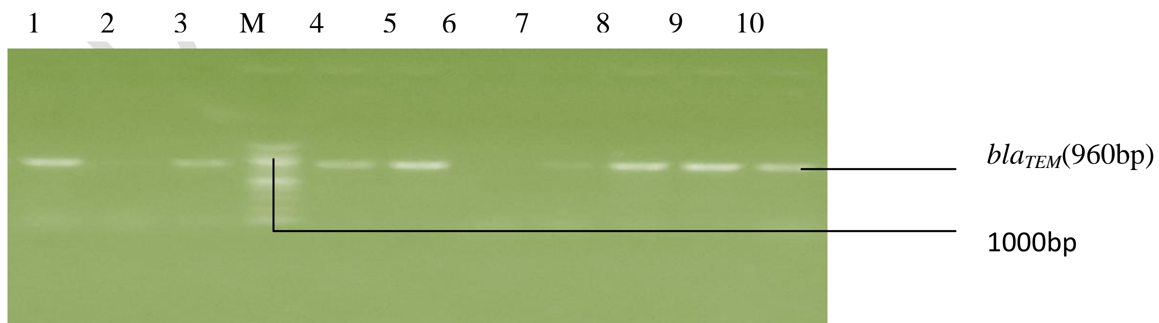

Plate 11: Agarose gel electrophoresis of the amplified *bla*$_{TEM}$ genes from the *E. coli* isolates from GHA. Lanes 1 – Lane 4 and Lane 7 - Lane 10 represent the *bla*$_{TEM}$ bands, Lane M represents the 1500bp molecular ladder, while Lane 6 showed no band.

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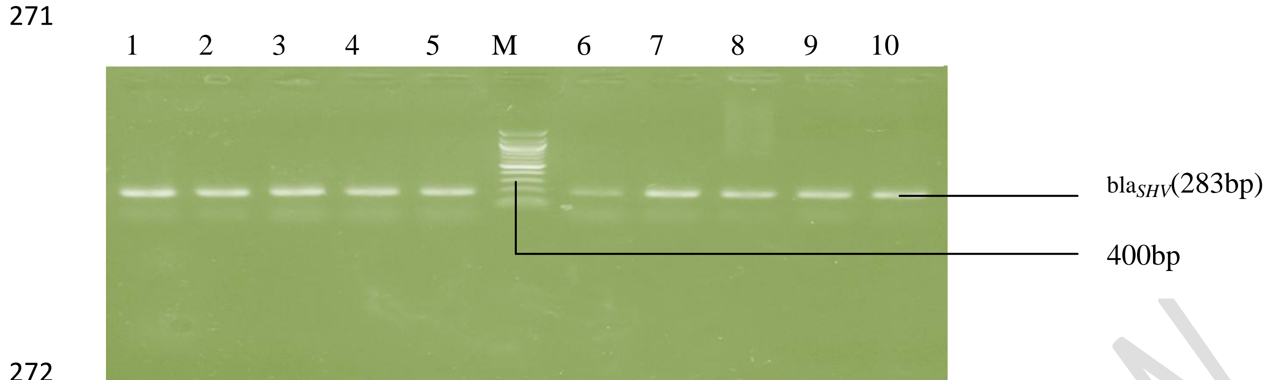

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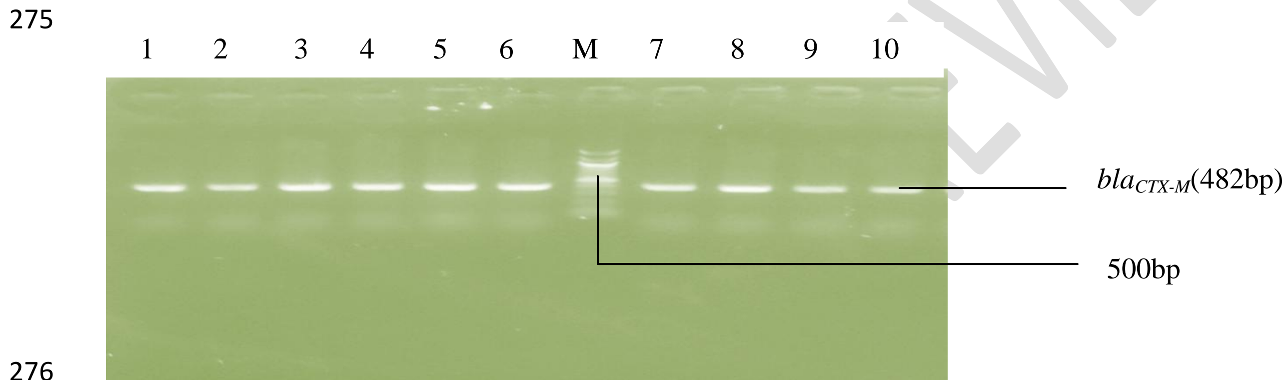
273 **Plate 12:** Agarose gel electrophoresis of the amplified *bla$_{SHV}$* genes from the *E. coli* isolates from GHA. Lanes 1-Lane 10 represent the bla$_{SHV}$ bands, Lane M represents the

274 1500bp molecular ladder.

275



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277 **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

278 1500bp molecular ladder.

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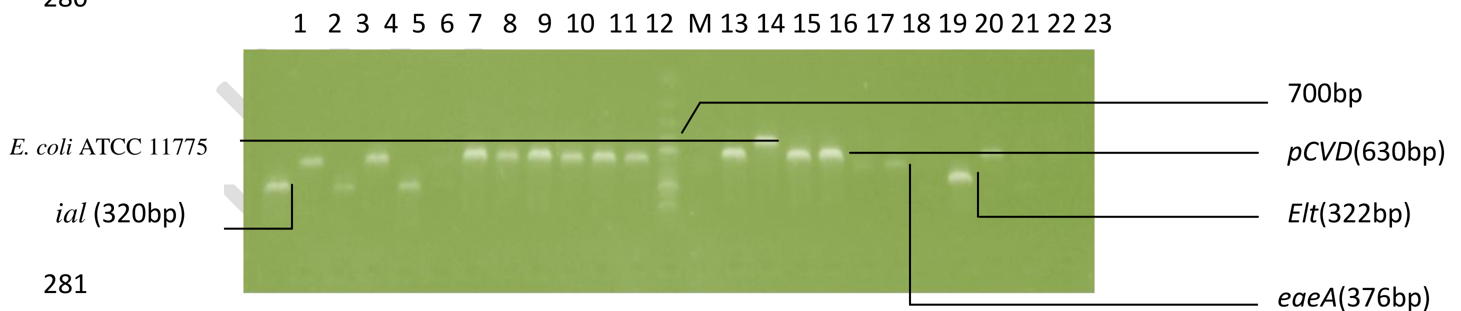

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282 **Plate 14**: Agarose gel electrophoresis of the diarrheagenic *Escherichia coli*

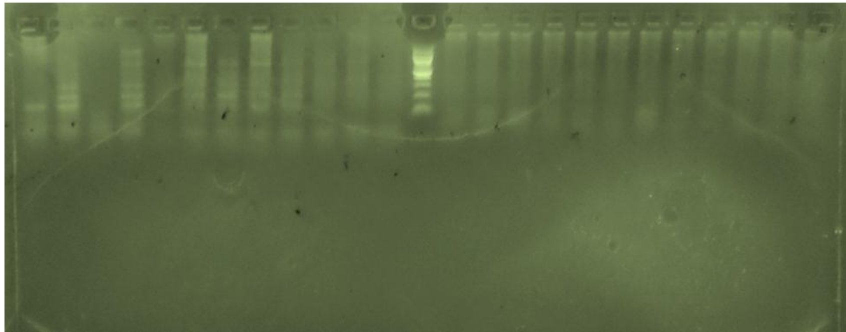
283 pathotypes from stools of diarrheic patients in Nasarawa State, Nigeria. Amplification; L1, L4 & L6=*ial* (EIEC); L2, L8-L12, L14, L16 & L17=*pCVD* (EAEC); L15= *E. coli* (ATCC 11775); L18-L19= *eaeA*(EHEC); L21*Elt*

284 (ETEC); L23= negative; M= 1500bp; while L7, L13 & L22 showed no band.

285

286

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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288 **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
289 with *BsGr*.

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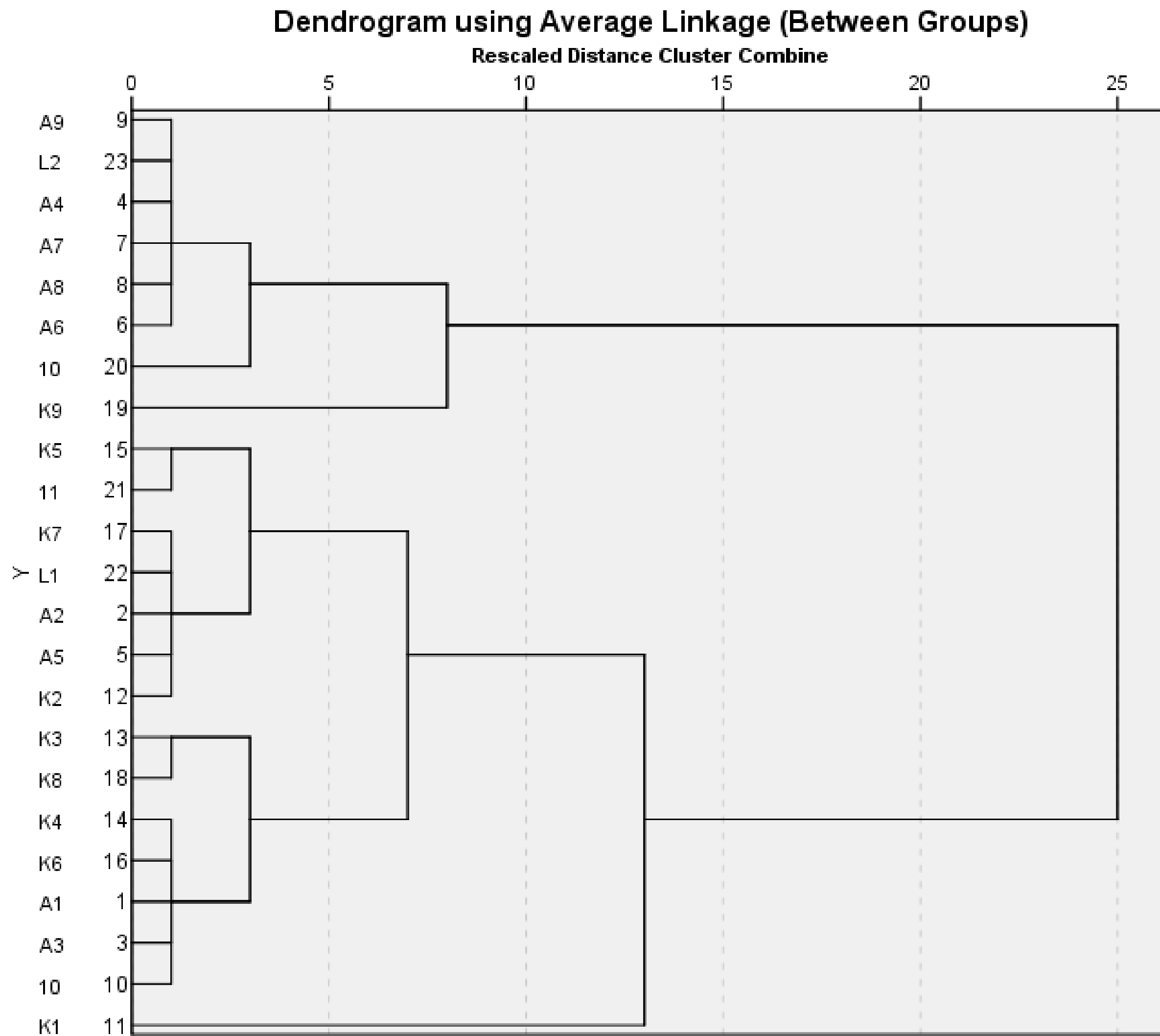

Figure 2: Dendrogram of 16srRNA of Extended Spectrum Beta-lactamase producing *Escherichia coli* after digestion with endonuclease *BsGr*.

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.

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303 The occurrence of *Escherichia coli* from stool of patients with suspected cases of diarrhea in relation to
304 age; age group 0-5 and 6-10 years have the highest number of samples collected while age group 35 –
305 >45 have the least number collected. However, it was observed that between age groups the presence of
306 the bacterial isolates with age group 0-5 and 6-10 years having the highest occurrence of bacterial
307 isolates and the least is age group 35 – >45. This follows the same trend with a study done in Abuja [4,
308 19],which shows that diarrhea is statistically associated with age and majority of the cases occurring in
309 children between 7 months and 2 years of age. The reason for high incidence of bacteria isolates in age
310 group 0-5 and 6-10 years could be due to the fact that children within this age group on their own cannot
311 differentiate between what to eat and what not to eat; they have not learnt the rudiment of adherence to
312 aseptic or hygienic practice; they can barely express themselves. Most diarrhea occur during the first 2
313 years of life due to combined effects of declining levels of maternally acquired antibodies, the lack of
314 active immunity in the infant, the introduction of food that may be contaminated with faecal bacteria and
315 direct contact with human or animals faeces when the infant start to grow [4, 19]. Most enteric pathogens
316 stimulate at least partial immunity against repeated infection or illness, which helps to explain the
317 declining incidence of disease in older children and adults [20].

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

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

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331 high in GHA (70.0%) but low in DASHL (33.3%). The percentage distribution of strain D, F, H, I, and J
332 were 11.1% in DASHL while the occurrence of I was 18.2% in FMCK. In addition, the occurrence of
333 strains C, D, and G were 9.1% in FMCK while the occurrence of C, E, and G were 10.0% in GHA.
334 The RFLP amplified *16SrRNA* gene digested with *BsGr* is the first study ever conducts in Nasarawa
335 State, Nigeria. However other similar studies on diarrheagenic *E. coli* have been reported elsewhere.
336 The high frequency of detection of EAEC 81.8% in FMCK, 70.0% in GHA and 55.6% in DASHL observed
337 in this study was not surprising. It is in agreement with 7.2% [9] and 22.0% [7], earlier reported in Kenya
338 and Keffi (in Nigeria). EAEC was previously reported to be endemic in Southern Nigeria as well as in sub-
339 Saharan Africa [27]. So, our observation on the occurrence of EAEC 9(81.8) in FMCK, 7(70.0) in GHA
340 and 5(55.6) in DASHL concurred with what was reported in Southwestern Nigeria and elsewhere
341 especially in the sub-Saharan Africa 18(7.2%) [27].
342 The frequency of detection of EAEC in this study is higher than that reported [28]; but the detection of
343 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
344 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]
345 respectively. The very low frequency of detection of diarrheagenic *E. coli* obtained in this study is in close
346 agreement with the study reported [29] with prevalence of *E. coli* O157: H7 in children with diarrhea as
347 5.4% in Zaria, Nigeria. Also, [30, 31], reported a prevalence of 5% EHEC O157:H7 in humans, in Lagos,
348 Nigeria. But it is in contrast with the study conducted [32], who reported 19.6% prevalence of
349 diarrheagenic *E. coli* in a study conducted in Southeastern Nigeria. An incidence higher than 40% has
350 been reported in Bangladesh by [33]. It was observed that EPEC were not detected in any of the study
351 location, reason may be so because isolation rate of different pathotypes of diarrheagenic *E. coli* have
352 been reported to be vary in different geographical areas although other studies in other parts of the
353 country reported low frequency of detection of EPEC [28, 33], which is in total disagreement with studies
354 carried out in Southeast Nigeria, which reported that EPEC was the most isolated of all DEC pathotypes
355 followed by EAEC, ETEC, EIEC and EHEC in that order [32].
356 Outbreaks and sporadic cases of EHEC have been reported in developed countries of North America,
357 Japan, Europe and even Australia [34]. However there have been few reports of sporadic EHEC in
358 African countries. Three large EHEC outbreaks were previously reported in Swaziland, Central African

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

364 4. CONCLUSION

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

368 ETHICAL APPROVAL

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

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