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Original Article

Profiling Virulence and Antimicrobial Resistance Markers of Enterovirulent *Escherichia Coli* from Fecal Isolates of Adult Patients with Enteric Infections in West Cameroon

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ABSTRACT

Objectives: This study aimed to identify virulent and antimicrobial resistant genes in fecal *E. coli* in Mbouda, Cameroon.
Methods: A total of 599 fecal samples were collected from patients with enteric infections who were ≥ 20 years old. *E. coli* was isolated on the MacConkey agar and virulent genes were detected by multiplex/simplex PCR. Isolates in which ≥ 1 virulent gene was detected were subjected to antibiotic susceptibility testing. The resulting resistant isolates were subjected to PCR, followed by sequencing for resistant genes detection.
Results: There were 119 enterovirulent *E. coli* identified, amongst which 47.05% were atypical enteropathogenic *E. coli* (EPEC), 36.97% enterotoxigenic *E. coli*, 10.08% Shiga toxin producing *E. coli* (STEC) and 5.88% were enteroinvasive *E. coli* (EIEC). The occurrence of the *eae* gene (47.06%) was higher compared with *CVD432* (33.61%), *aaic* (13.45%), *stx2* (10.08%) and *stx1* (0.84%). High resistance rates were noted for ampicillin (94.64% EPEC, 91.67% STEC, 59.09% EAEC, and 57.14% EIEC) and sulfamethoxazole-trimethoprim (100% EPEC and 83.33% STEC, 81.82% EAEC and 71.43% EIEC). *sul2* (71.43%), *tetB* (64.71%), *tetA* (59.94%) and *blaTEM* (52.10%) were detected. A double mutation (S83L; D87N) was seen in *gyrA* and a single mutation (S80I) was observed in *parC*.
Conclusion: These findings suggested that measures should be taken to reduce the harm of *E. coli* to public health.

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Introduction

Escherichia coli is part of the normal enteric bacterial flora of humans and animals [1]. Nonetheless, some *E. coli* strains have developed pathogenic mechanisms that cause infections in humans and animals [2]. *E. coli* strains can be a source of enteric diarrheagenic infections in human beings [3]. The pathogenicity of *E. coli* is related to the association between many variables, and it is affected by ecological conditions such as microbial species, wellbeing status of the host, association with other microorganisms and antibiotic treatment received [4]. Accordingly, any *E. coli* conveying pathogenicity or antimicrobial resistance (AMR) is possibly pathogenic and harmful to its host [3].

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Diarrheagenic *E. coli* (DEC) can be categorized as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) or Shiga toxin producing *E. coli* (STEC) [5]. The categorization of *E. coli* as EPEC is based on the presence of either the *eae* gene (exterior membrane protein adhesion) and/or the *bfpA* gene (plasmid-encoded bundle-forming pilus) [6]. Isolated *E. coli* carrying the *eae* gene is considered as EPEC and the *bfpA* gene further divides EPEC (*eae*+) into typical and atypical [6]. *E. coli* is classified as ETEC based on the presence of *est* and *elt* genes which are heat-stable and heat-labile enterotoxin genes, respectively [7]. The plasmid-encoded genes *CVD432* (a dispersin transporter) and *aaiC* (AggR-activated Island C) are used to classify EAEC *E. coli* isolates [8]. An enteric *E. coli* infection is marked by an immediate evacuation of liquid, non-bloody diarrhea of considerable volume, accompanied by little or no fever [9]. Other common symptoms are abdominal pain, malaise, nausea, and vomiting [3]. Although diarrhoeal disease is usually less harmful to adults than to children, it can reduce the productivity of its workforce which in turn affects the economy of a country. Diarrheal diseases are one of the principal causes of morbidity and mortality all over the world, especially in developing countries [10]. As reported by the Centre for Disease Control and Prevention (CDC) in 2018, diarrheal diseases are the fifth cause of deaths after HIV infection, pneumonia, lung abscess and acute bronchitis in Cameroon [11].

Bacterial diseases are treated with antimicrobial drugs [12]. Overuse of anti-infectious agents is related to the selection of AMR bacterial populations [13]. Several virulent genes and resistant genes, have been identified by PCR that code for the production of efflux pumps proteins, which decrease the permeability of the bacterial membrane, or modifying the substrate targets of antimicrobials have been identified by PCR thereby destroying antibiotic efficacy [14]. The transmission of, and pathways that may lead to, antibiotic resistance and virulence, can be divided into adaptive resistance, innate resistance, and acquired resistance. Physiological changes that lead to elevated mutation rates, i.e. mutation in metabolic genes and regulatory processes, and classic antibiotic inactivation and resistance mechanisms, could be affected by environmental factors [15]. This type of resistance and increased virulence can potentially be shared among bacteria leading to acquired resistance [15]. The rise and spread of bacterial resistance to antibiotics has been recognized as a worldwide burden, particularly in developing countries [16]. Bacterial resistance to antibiotics in the developing countries has been credited to the indiscriminate use of anti-infectious agents in animals bred for food consumption, and overuse, and imperfect prescription of antibiotics (which may influence the recovery of isolates from patients) [17].

The virulence and resistance profiles of enterovirulent *E. coli* strains in adults with enteric infections residing in Cameroon were determined in this study.

Material and Methods

1. Clinical sample and study population

Participants in this study were adult patients with enteric diseases, being treated in Mbouda Adlucem hospital and district hospital. These 2 health facilities are reference hospitals in the Bamboutos Division, West Region of Cameroon. Using sterile containers, a total of 599 stool specimens were sampled between May 2016 and May 2018, from patients aged ≤ 20 years. Samples were processed immediately after collection. Sociodemographic information of the study participants was collected using a structured questionnaire. Diarrheal diseases in adults are a common problem diagnosed by physicians in Mbouda. Although a diarrheal disease is usually less harmful to adults than to children, it can affect the economy of a country by reducing the output of its workforce.

2. Isolation and identification of bacteria from stool samples

Each stool sample was diluted 1:4 in sterile distilled water and inoculated onto Mac-Conkey (Becton Dickinson-Difco, Franklin Lakes, NJ, USA) agar plates. The plates were incubated in aerobiosis for 24 hours at 37°C. From morphological examination, colonies that appeared pink, flat, dry and lactose fermenting, with a bordering darker pink area of scurried bile salts were preliminarily reported as *E. coli* and confirmed using motility and biochemical tests such as urease production, indole production, catalase test, citrate utilization, carbohydrate fermentation test and methyl red test [18]. From each sample, 3 distinct colonies were collected for molecular determination of *E. coli* pathotypes (EAEC, EPEC, ETEC, EIEC and STEC) using multiplex and simplex PCR [19].

3. DNA extraction

To extract DNA, 3 distinct bacterial colonies were suspended in 200 µL of sterile phosphate buffered saline (1%). The suspension was heated to 100°C for 10 minutes and centrifuged at 10,000 g for 5 minutes. The supernatant fluid containing the DNA was stored at -20°C for supplementary molecular assays.

4. Screening of virulent genes by multiplex and simplex PCR

The detection of 3 types of DEC (EAEC, EPEC and ETEC) was performed using multiplex PCR testing with specific primers (Table 1) for the identification of the following virulence markers: EPEC intimin (*eae* gene) exterior membrane protein

adhesion, EPEC plasmid-encoded bundle-forming pilus (*bfpA* gene), est and elt for the heat-stable and heat-labile enterotoxin genes of ETEC [7], the EAEC plasmid-encoded gene [*aatA* or CVD432 (a dispersin transporter)] and the EAEC chromosomal encoded *aaiC* locus (AggR-Activated Island C). The reaction mixture comprised of 7.4 μL of distilled water, 2.5 μL of 10x PCR buffer with 2 mM of $MgCl_2$ (New England Biolab, UK), 0.4 μL of each LT, ST, *bfpA*, *CVD432*, *aaic* primer and 0.44 μL of each *eae* primer, 2 μL of 10 mM of dNTPs [1.25mM (New England Biolab, UK)], 0.25 μL of 5.0 U Taq DNA (New England Biolab, UK) and 3.0 μL template DNA. The PCR conditions included initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 57°C for 20 seconds and extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The PCR products were stained after electrophoresis on a 2% agarose gel using an ethidium bromide solution, for 25 minutes, and visualized on a transilluminator. The *E. coli* ATCC 29552 (EAEC), ATCC 35401 (ETEC), ATCC 43893 (EIEC), and ATCC 43895 (EPEC, EHEC, and STEC) were used as positive control strains.

All these genes are virulence determinants for their respective pathogens. Isolates positive for 1 or both of the *eae* and *bfpA* genes were assigned as EPEC. Those positive for either 1 or both EAEC factors (*CVD432*, *aaiC* genes) were designated as EAEC. All *eae*-positive and negative *E. coli* isolates from multiplex PCR were further analyzed by simplex PCR for the *ial* gene (invasive associated protein for EIEC detection), *stx1* (shiga toxins 1) and *stx2* (shiga toxins 2) virulent genes [6-8]. Isolates positive for the *ial* gene were assigned as EIEC, and those positive for 1 or both of the *stx1*, *stx2* genes were considered as STEC.

5. Simplex PCR for ial gene detection

The simplex PCR reaction mixture for EIEC detection contained 15.5 μL of distilled water, 2.5 μL of 10x PCR buffer with 2 mM of $MgCl_2$ (New England Biolab, UK), 1.0 μL of each *ial* primer (Table 1), 2.0 μL of 10 mM of dNTPs [1.25mM (New England Biolab, UK)], 0.20 μL of 5.0 U Taq DNA (New England Biolab, UK) and 3.0 μL template DNA. The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation, annealing and extension at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, respectively, and a final extension at 72°C for 7 minutes. Positive control strains were included in each batch of tests. The PCR products were stained after electrophoresis on a 2% agarose gel using an ethidium bromide solution, for 25 minutes, and visualized on a transilluminator.

Table 1. Specific PCR primers used in this study for determination of the virulent genes.

Target gene	Primers	Primer sequences	Amplicon sizes	References
		Primers for virulent genes (5'—3')		
elt/*ETEC*	LT-F LT-R	CACACGGAGCTCCTCAGTC CCCCCAGCCTAGCTTAGTTT	508 bp	[19]
est/ETEC	ST-F ST-R	GCTAAACCAGTAGAGGTCTTCAAAA CCCGGTACAGAGCAGGATTACAACA	147 bp	[19]
bfpA/EPEC	BFPA-F BFPA-R	GGAAGTCAAATTCATGGGGG GGAATCAGACGCAGACTGGT	367 bp	[19]
eae/EPEC	EAE-F EAE-R	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881 bp	[19]
aatA/EAEC	CVD432-F CVD432-R	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630 bp	[19]
aaic/EAEC	AAIC-F AAIC-R	ATTGTCCTCAGGCATTTCAC ACGACACCCCTGATAAACAA	215 bp	[19]
stx1/STEC	EVT1 EVT2	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	349 bp	[20]
stx2/STEC	EVS1 EVC2	TACAGTCGTCACTCACTGGT CTGCTGTCACAGTGACAAA	110 bp	[20]
ial/EIEC	ial-F ial-R	CTGGATGGTATGGTGAGG GGAGGCCAATTATTTCC	700 bp	[21]

EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*, ETEC = enterotoxigenic *E. coli*.

6. Simplex PCR for *stx1* (shiga toxins 1) and *stx2* (shiga toxins 2) genes detection

The simplex reaction mixture comprised of 13.25 µL distilled water, 2.5 µL of 10x PCR buffer with 2 mM of $MgCl_2$ (New England Biolab, UK), 2.0 µL of each *shiga toxin* primer (Table 1 [19-21]), 2 µL of 10 mM of dNTPs [1.25mM (New England Biolab, UK)], 0.25 µL of 5.0 U Taq DNA (New England Biolab, UK) and 3.0 µL template DNA. The PCR conditions involved an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 90 seconds, annealing at 55°C for 90 seconds, an extension at 72°C for 90 seconds, and a final extension at 72°C for 7 minutes.

7. Antimicrobial susceptibility testing

All isolates possessing at least 1 virulence gene as determined by multiplex and simplex PCR were subjected to antibiotic susceptibility testing using the Kirby Bauer disk diffusion method [22] for the following antimicrobials: ampicillin (AMP, 10µg), azithromycin (AZM, 15µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30µg), ceftriaxone (CRO, 30µg), chloramphenicol (CHL, 30µg), ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), meropenem (MEM, 10µg), nalidixic acid (NA, 30 µg), norfloxacin (NOR, 10 µg), ofloxacin (OFX, 5 µg), tetracycline (TET, 5µg), and trimethoprim-sulphamethoxazole (SXT, 23.75/1.25 µg) [Becton Dickinson and Company, Sparks, MD 21152 USA]. *E. coli ATCC 25922* was used as a quality control strain. The expression of the results as sensitive, intermediate and resistant was performed according to Clinical and Laboratory Standards Institute guidelines [23]. Multi drug resistance (MDR) was considered as bacterial resistance to 3 antibiotics belonging to at least 3 classes or families [24].

8. Screening of antibiotic resistant genes

Simplex PCR was utilized for the determination of resistant genes: β-lactamases *blaTEM*, *blaOxa* and *blaPSE-1*, chloramphenicol acetyltransferase (*cat1* and *cat2*), non-enzymatic or plasmid chloramphenicol resistance (*cmlA*) and chloramphenicol efflux pump *floR* (plasmid or chromosome-encoded chloramphenicol exporter), tetracycline efflux pumps *tetA*, *tetB* and *tetG*, and dihydropteroate synthases *sul1*, *sul2* and *sul3* and dihydofolate reductase *dfrA1*, *dfrA7* and *dfrA12* [25]. For these antibiotic resistant genes, the PCR reaction mixture contained 8.5 µL of distilled water, 1.5 µL of 10x PCR buffer with 2 mM of $MgCl_2$ (New England Biolab, UK), 0.15 µL of each primer, 1.5 µL dNTPs [1.25mM (New England Biolab, UK)], 0.20 µL of 5.0 U Taq DNA (New England Biolab, UK) and 3.0 µL template DNA. Table 2 shows the specific primer sequences and PCR conditions [26-36].

9. Screening of mutations in the DNA gyrase (*gyrA*, *gyrB*) and topoisomerase (*parC*) genes

The mutations in chromosomal DNA gyrase (*gyrA*, *gyrB*) and DNA topoisomerase (*parC*) carrying the quinolone resistance-determining regions were investigated using simplex PCR followed by sequencing [37,38]. For each of the *gyrA*, *gyrB* and *parC* genes, the PCR reaction mixture contained 8.5 µL of distilled water, 1.5 µL of 10x PCR buffer with 2 mM of $MgCl_2$ (New England Biolab, UK), 0.15 µL of each primer, 1.5 µL dNTPs [2.5mM (New England Biolab, UK)], 0.20 µL of 5.0 U Taq DNA (New England Biolab, UK) and 3.0 µL template DNA. The PCR conditions and amplicon sizes are shown in Table 2. Amplified DNA products were resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide. PCR products were purified for sequencing by BigDye XTerminator purification method (Foster City, USA). The forward and reverse strands were sequenced in Applied Biosystems Genetic Analyzers 3130/xl (Applied Biosystems) with the same PCR primer sets. Raw sequences of each *gyrA*, *gyrB* and *parC* genes were reviewed by visual inspection using Sequence Scanner software v2.0 (64-bit, [Applied Biosystems genetic analyzer instruments]), studied with the BLAST and FASTA programs of the National Centre of Biotechnology Information (www.ncbi.nlm.nhi.gov) and compared with the *gyrA*, *gyrB* and *parC* reference sequences of *E. coli* str. K-12 substrain MG1655 in the GenBank database.

10. Ethical considerations

Ethical endorsement was acquired from the Cameroon National Ethics Committee, Ministry of Public Health (2018/06/1054/CE/CNERSH). Before data collection, authorizations were obtained from each director of Mbouda, Adlucem and district hospitals. Information sheets presenting the objective and procedures of the study were explained to each participant. Answers were also given to their questions concerning the study. Written informed consent was obtained from each participant before his/her discretionary participation.

11. Statistical analysis

Epi Info version 7.2.2.6 (CDC, Atlanta, USA) was used for the statistical analysis. Frequencies and percentages were computed, and the study variables were compared using 2-tailed Chi-square test and correlation analysis. Results were considered significant if $p < 0.05$.

Table 2. Specific PCR primers used in this study for the determination of antibiotics resistance genes.

Antibiotics	Target gene	Primers	Primer sequences	Amplicon sizes	Annealing temperature (times)	References
			Primers for resistance genes (5'—3')			
AMP	*blaTEM*	OT-1 OT-2	TTGGGTGCACGAGTGGGT TAATTGTTGCCGGGAAGC	503 bp	58°C (1 min)	[26]
	blaOxa	OO-1 OO-2	ACCAGATTCAACTTTCAA TCTTGGCTTTTATGCTTG	598 bp	57°C (1 min)	[26]
	BlaPSE-1	PSE-1-F PSE-1-R	TTT GGT TCC GCG CTA TCT G TAC TCC GAG CAC CAA ATC CG	150	51°C (1 min)	[27]
CHL	*cat1*	CAT1-F CAT1-R	TCCCAATGGCATCGTAAAGAAC TCGTGGTATTCACTCCAGAGCG	293 bp	53°C (30 s)	[28]
	cat2	CAT2-F CAT2-R	AACGGCATGATGAACCTGAA ATCCCAATGGCATCGTAAAG	547 bp	55°C (30 s)	[29]
	floR	floR-F floR-R	ACTCGGCATGGACATGTACT ACGGACTGCGGAATCCATAG	1213 bp	55°C (40 s)	[29]
	cmIA	cmIA-F cmIA-R	TGTCATTTACGGCATACTCG ATCAGGCATCCCATTCCCAT		55°C (30 s)	[26]
TET	*tetA*	tetA-F tetA-R	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	956 bp	58.5°C (1 min)	[30]
	tetB	tetB-F tetB-R	CTCAGTATTCCAAGCCTTTG ACTCCCCTGAGCTTGAGGGG	415 bp	55°C (45 s)	[30]
	tetG	tetG-F tetG-R	CAG CTT TCG GAT TCT TAC GG GAT TGG TGA GGC TCG TTA GC	884 bp	60°C (45 s)	[31]
SXT	*dfrXII (dfrA12, dfrA13)*	dfrXII-F dfrXII-R	GGT GSG CAG AAG ATT TTT CGC TGG GAA GAA GGC GTC ACC CTC	319 bp	60°C (45 s)	[32]
	dfrA7, dfrA17	dfrVII-F dfrVII-R	TTG AAA ATT TCA TTG ATT G TTA GCC TTT TTT CCA AAT CT	474 bp	55°C (1 min)	[32]
	dfrIa (dfrA1, dfrA5, dfrA15)	dfrIa-F dfrIa-R	GTG AAA CTA TCA CTA ATG G TTA ACC CTT TTG CCA GAT TT	474 bp	55°C (1 min)	[32]
	sul1	sul1-F sul1-R	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	432 bp	60°C (30 s)	[33]
	sul2	sul2-F sul2-R	TCA ACA TAA CCT CGG ACA GT GAT GAA GTC AGC TCC ACC T	707 bp	55°C (45 s)	[34]
	sul3	sul3-F sul3-R	GGAAGAAATCAAAAGACTCAA CCTAAAAAGAAGCCCATACC	363 bp	53°C (1 min)	[33]
DNA gyrase	*gyrA*	gyrA-F gyrA-R	TGTCCGAGATGGCCTGAAGC TACCGTCATAAGTTATCCACG	347 bp	62°C (45 s)	[35]
	gyrB	gyrB-F gyrB-R	CAAACTGGCGGACTGTCAGG TTCCGGCATCTGACGATAGA	345 bp	62°C (45 s)	[35]
DNA topoisomerase	*parC*	parC-F parC-R	TGTATGCGATGTCTGAACTG CTCAATAGCAGCTCGGAATA	264 bp	55°C (30 s)	[36]

AMP = ampicillin; CHL = chloramphenicol; SXT = trimethoprim-sulphamethoxazole; TET = tetracycline.

Results

1. Distribution of DEC pathotypes and virulent genes among total enterovirulent *E. coli* (*n* = 119).

Based on macroscopic colony characteristics (pink, flat, dry and lactose fermenting colonies with a bordering darker pink area of scurried bile salts) and biochemical tests, a total of 377 *E. coli* isolates were obtained from 599 stool samples. Multiplex and simplex PCR used to target virulent genes enabled the identification of 119 enterovirulent *E. coli* isolates among which 56 (47.05%) were atypical EPEC (*eae* gene only), 44 (36.97%) were EAEC, 12 (10.08%) belonged to STEC, and 7 (5.88) were EIEC. None of the stool specimens was positive for typical EPEC (both *eae* and *bfpA*) and ETEC. The occurrence of DEC among females (52.94%) was slightly higher than in males (47.06%). The participant's average age was 43.42 ± 15.61 years (ranging from 20 to 88 years) for participants in which 119 enterovirulent *E. coli* were detected. DEC was most prevalent in the age group 40 < 50 years (30.25%), with a predominance of EPEC (18.49%) compared with EAEC (7.56%), EIEC (2.52%) and STEC (1.68%). Vomiting and diarrhea, and fever and diarrhea, were observed in 21.85% and 15.13% of patients affected with EPEC, respectively. Patients infected with EAEC (20.17%), EIEC (3.36%) and with STEC (7.56) had abdominal cramps, dysenteric stools/stomach cramps and diarrhea, respectively (Table 3).

The molecular detection of virulence associated genes (Figure 1) showed the presence of the *eae* gene was predominant in 47.06% of the total isolated DEC strains, followed by the *CVD432* gene (33.61%), *aaic* (13.45%), *stx2* (10.08%) and *stx1* (0.84%). The presence of associations *CVD432/aaic* and *stx1/stx2* genes was observed in 10.08% and 0.84%, respectively, whilst the genes *bfpA*, and *eae/bfpA* were absent.

Table 3. Clinical attributes of individuals affected with enterovirulent *E. coli* (*n* = 119).

Clinical Characteristics	Total DEC isolates (*n* = 119)	Enterovirulent *E. coli*, Frequency (%)			
		EPEC (*n* = 56)	EAEC (*n* = 44)	EIEC (*n* = 7)	STEC (*n* = 12)
Sex					
Male	56 (47.06)	28 (23.53)	19 (15.97)	2 (1.68)	7 (5.88)
Female	63 (52.94)	28 (23,53)	25 (21.01)	5 (4.20)	5 (4.20)
Age groups (y)					
20-30	32 (26.89)	14 (11.76)	14 (11.76)	0 (0.00)	4 (3.36)
30-40	22 (18.49)	3 (2.52)	7 (5.88)	0 (0.00)	1 (0.84)
40-50	36 (30.25)	22 (18.49)	9 (7.56)	3 (2.52)	2 (1.68)
50-60	16 (13.45)	6 (5.04)	5 (4.20)	2 (1.68)	3 (2.52)
60-70	19 (15.97)	7 (5.88)	9 (7.56)	1 (0.84)	2 (1.68)
≥ 70	5 (4.20)	4 (3.36)	0 (0.00)	1 (0.84)	0 (0.00)
Symptoms					
Abdominal cramps	24 (20.17)	0 (0.00)	24 (20.17)	0 (0.00)	0 (0.00)
Abdominal cramps and diarrhea	6 (5.04)	0 (0.00)	6 (5.04)	0 (0.00)	0 (0.00)
Abdominal pain	2 (1.68)	0 (0.00)	2 (1.68)	0 (0.00)	0 (0.00)
Abdominal pain and vomiting	12 (10.08)	0 (0.00)	12 (10.08)	0 (0.00)	0 (0.00)
Dysenteric stools	5 (4.20)	1 (0.84)	0 (0.00)	4 (3.36)	0 (0.00)
Dysenteric stools and cramps	3 (2.52)	0 (0.00)	0 (0.00)	3 (2.52)	0 (0.00)
Fever and diarrhea	18 (15.13)	18 (15.13)	0 (0.00)	0 (0.00)	0 (0.00)
Fever and vomiting	11 (9.24)	11 (9.24)	0 (0.00)	0 (0.00)	0 (0.00)
Stomach cramps and diarrhea	9 (7.56)	0 (0.00)	0 (0.00)	0 (0.00)	9 (7.56)
Stomach cramps and vomiting	3 (2.52)	0 (0.00)	0 (0.00)	0 (0.00)	3 (2.52)
Vomiting and diarrhea	26 (21.85)	26 (21.85)	0 (0.00)	0 (0.00)	0 (0.00)

EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*.

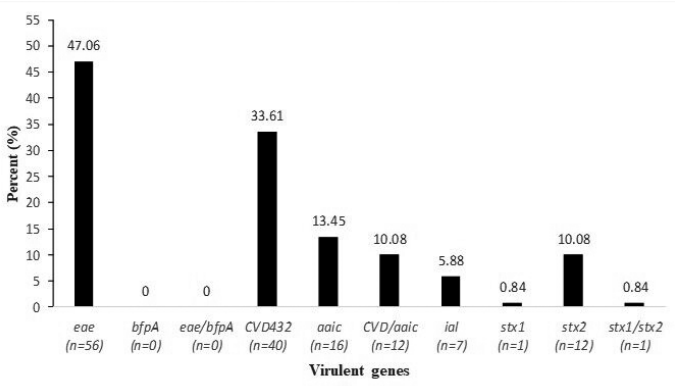

Figure 1. Distribution of virulent genes among total enterovirulent *E. coli* (*n* =119).

2. AMR profile of enterovirulent *E. coli* isolates

The antibiotic resistance patterns of *E. coli* isolates are summarized in Table 4. The *E. coli* isolates exhibited high resistance to antibiotics such as ampicillin (94.64% EPEC, 91.67% STEC, 59.09% EAEC, and 57.14% EIEC; X^2 = 21.94, p = 0.001) and sulfamethoxazole-trimethoprim (100% EPEC and 83.33% STEC, 81.82% EAEC and 71.43% EIEC; X^2 = 12.67, p = 0.005). EPEC (92.86%), STEC (83.33%), EAEC (75.00%) and EIEC (13.64) showed resistance against tetracycline although these results were not statistically significant. *E. coli* isolates showed significant susceptibility to the following antibiotics: ceftazidime, chloramphenicol, ciprofloxacin, gentamicin and norfloxacin. All isolates were 100% susceptible to meropenem.

3. Distribution of the antimicrobial resistant genes according to the pathotype groups

Molecular identification of resistant genes using PCR (Table 5) disclosed the existence of broad spectrum beta lactamase (BSBL) genes *blaTEM* (52.10%), *blaOxa* (9.24%) and *blaPSE-1* (0.84%). The occurrence of each resistant gene was compared with the resistance profile of enterovirulent *E. coli* against the antibiotics. The results showed the *blaTEM* gene was significantly higher (p = 0.001) in ampicillin resistant STEC (75.00%), followed by EPEC (66.07%), EAEC (31.82%) and EIEC (28.57%), whilst blaOxa was significantly higher (p < 0.001) in ampicillin resistant EIEC (28.57%) compared with ampicillin resistant EAEC (20.45%), EPEC (0%) and STEC (0%). The distribution of chloramphenicol resistance genes was 10.08% for both *cat1* and *cat2*, and 0% for both *cmlA* and *floR*. *cat1* and *cat2* genes were significantly higher in chloramphenicol resistant EIEC compared with other chloramphenicol resistant DEC (p < 0.001). Tetracycline resistant genes *tetB*, *tetA* and *tetG*, were detected in 64.71%, 59.94% and 17.65% of the total tetracycline resistant isolates, respectively. *tetB* was significantly higher in tetracycline resistant STEC (p = 0.006) whilst *tetG* was higher in EIEC (p < 0.001) compared with other tetracycline resistant DEC. Sulfonamide resistant genes *sul1*, *sul2* and *sul3* were identified in 16.81%, 71.43% and 5.88% of sulfonamide resistant isolates, respectively, and trimethoprim resistant genes *dfr1A*, *dfrA7* and *dfrA12* were present in 6.72%, 0%, and 0.84% of trimethoprim resistant isolates, respectively. *sul2* was significantly higher in STEC whilst *sul1* was higher in EAEC compared with other *E. coli* pathotypes (p < 0.05).

4. Presence of amino acid mutation in quinolone resistance-determining regions

The mutations in the chromosomal DNA gyrase (*gyrA*, *gyrB*) and DNA topoisomerase (*parC*) carrying the quinolone resistance-determining regions were investigated using simplex PCR followed by sequencing. Six quinolone resistant *E. coli* and 4 sensitive strains were sequenced. No mutation was observed in the *gyrA*, *gyrB* and *parC* protein sequences of all sensitive isolates. For the quinolone resistant *E. coli*, no mutation was observed in the *gyrB* protein sequence of all analyzed isolates. Alignment of the *gyrA* (DNA *GyrAse*) and *parC* (DNA topoisomerase) protein sequence of 1 quinolone resistant *E. coli* isolate is shown in Figures 2 and 3. A double mutation was observed in *gyrA* (accession number: MT334396) at position 83 [S83L (bestows high-level resistance)] and position 87 [D87N (bestows low-level resistance)] for a single isolate resistance to OFX, NOR, CIP and NA, whilst a single mutation in *parC* (accession no.: MT334398) was seen at position 80 (S80I) in the same isolate. There were 33.33% (n = 2) of *E. coli* isolates resistant to NA and susceptible to OFX, NOR and CIP presented a single mutation in *gyrA* (accession no.: MT334397) at position 83 (S83L).

5. Associations between *E. coli* virulent genes and antibiotics susceptibility phenotypes

Significant associations between *E. coli* virulent genes and antibiotic sensitivity phenotypes were observed. Resistance to chloramphenicol was positively associated with *aaic* and *CVD432* (p < 0.05), and negatively associated with *eae* (p < 0.05). Resistance to gentamicin was marked by its positive association with *CVD432* and negative association with *eae* and *aaic* (p < 0.05; Table 6).

6. Associations between virulent and antibiotic resistance genes

There were significant associations between virulent genes and antibiotic resistance genes. Enterovirulent *E. coli* carrying *blaOxa*, *blaTEM*, *cat1*, *cat2*, *sul1*, and *sul2* genes were

Table 4. Antimicrobial resistance profiles of isolated enterovirulent *E. coli* according to pathotype groups.

Antibiotics		Enterovirulent *E. coli*				X^2 (*p*)
		EPEC (*n* = 56)	EAEC (44)	EIEC (7)	STEC (12)	
AMP	S (*n* = 25)	3 (5.36)	18 (40.91)	3 (42.86)	1 (8.33)	21.94 (< 0.001)
	I (*n* = 00)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
	R (*n* = 94)	53 (94.64)	26 (59.09)	4 (57.14)	11 (91.67)	
AZM	S (*n* = 43)	24 (42.86)	16 (36.36)	1 (14.29)	2 (16.67)	10.41 (0.108)
	I (*n* = 73)	32 (57.14)	26 (59.09)	5 (71.43)	10 (83.33)	
	R (*n* = 3)	0 (0,00)	2 (4.55)	1 (14.29)	0 (0.00)	
CAZ	S (*n* = 118)	56 (100.00)	44 (100.00)	7 (100.00)	11 (96.67)	8.99 (0.029)
	I (*n* = 1)	0 (0.00)	0 (0.00)	0 (0.00)	1 (8.33)	
	R (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
CHL	S (*n* = 104)	55 (98.21)	32 (72.73)	5 (71.43)	12 (100.00)	19.77 (0.003)
	I (*n* = 3)	1 (1.79)	2 (4.55)	0 (0.00)	0 (0.00)	
	R (*n* = 12)	0 (0.00)	10 (22.73)	2 (28.58)	0 (0.00)	
CIP	S (*n* = 110)	51 (91.07)	42 (95.45)	6 (85.71)	11 (91.67)	17.32 (0.008)
	I (*n* = 8)	5 (8.93)	2 (4.55)	0 (0.00)	1 (8.33)	
	R (*n* = 1)	0 (0.00)	0 (0.00)	1 (14.29)	0 (0.00)	
CRO	S (*n* = 115)	55 (98.21)	42 (95.45)	7 (100.00)	11 (91.67)	1.77 (0.620)
	I (*n* = 4)	1 (1.79)	2 (4.55)	0 (0.00)	1 (8.33)	
	R (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
CTX	S (*n* = 110)	52 (92.86)	40 (90.91)	7 (100.00)	11 (91.67)	0.7440 (0.862)
	I (*n* = 9)	4 (7.14)	4 (9.09)	0 (0.00)	1 (8.33)	
	R (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
GEN	S (*n* = 106)	56 (100.00)	32 (72.73)	7 (100.00)	12 (100.00)	22.4666 (0.001)
	I (*n* = 10)	0 (0.00)	10 (22.73)	0 (0.00)	0 (0.00)	
	R (*n* = 2)	0 (0.00)	2 (4.55)	0 (0.00)	0 (0.00)	
MEM	S (*n* = 119)	56 (100.00)	44 (100.00)	7 (100.00)	12 (100.00)	0.00 (1.000)
	I (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
	R (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
NA	S (*n* = 95)	49 (87.50)	30 (68.18)	5 (71.43)	11 (91.67)	7.1052 (0.068)
	I (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
	R (*n* = 24)	7 (12.50)	14 (3182)	2 (28.57)	1 (8.33)	
NOR	S (*n* = 118)	56 (100.00)	44 (100.00)	6 (85.71)	12 (100.00)	16.1356 (0.001)
	I (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
	R (*n* = 1)	0 (0.00)	0 (0.00)	1 (14.29)	0 (0.00)	
OFX	S (*n* = 118)	56 (100.00)	44 (100.00)	6 (85.71)	12 (100.00)	16.1356 (0.001)
	I (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
	R (*n* = 1)	0 (0.00)	0 (0.00)	1 (14.29)	0 (0.00)	
SXT	S (*n* = 12)	0 (0.00)	8 (18.18)	2 (28.57)	2 (16.67)	12.6746 (0.005)
	I (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	
	R (*n* = 107)	56 (100.00)	36 (81.82)	5 (71.43)	10 (83.33)	
TET	S (*n* = 13)	4 (7.14)	6 (13.64)	1 (14.29)	2 (16.67)	10.9165 (0.091)
	I (*n* = 5)	0 (0.00)	5 (11.36)	0 (0.00)	0 (0.00)	
	R (*n* = 101)	52 (92.86)	33 (75.00)	6 (13.64)	10 (83.33)	

AMP = ampicillin; CAZ = ceftazidime; CHL = chloramphenicol; CIP = ciprofloxacin; CRO = ceftriaxone; CTX = cefotaxime; EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; GEN = gentamicin; I = intermediate; MEM = meropenem; NA = nalidixic acid; NOR = norfloxacin; R = resistant; S = sensitive; SXT = trimethoprim-sulphamethoxazole; TET = tetracycline.

Table 5. Percentage of *E. coli* antimicrobial resistant genes and their distribution according to pathotype groups.

Antibiotics & resistant genes			Enterovirulent *E. coli*				X^2 (*p*)
			EPEC (*n* = 56)	EAEC (*n* = 44)	EIEC (*n* = 7)	STEC (*n* = 12)	
AMP	*BlaOxa*	Yes (*n* = 11)	0 (0.00)	9 (20.45)	2 (28.57)	0 (0.00)	16.6348 (< 0.001)
		No (*n* = 108)	56 (100.00)	35 (79.55)	5 (71.43)	12 (100.00)	
	BlaTEM	Yes (*n* = 62)	37 (66.07)	14 (31.82)	2 (28.57)	9 (75.00)	15.7073 (0.001)
		No (*n* = 57)	19 (33.93)	30 (68.18)	5 (71.43)	3 (25.00)	
	BlaPSE-1	Yes (*n* = 1)	0 (0.00)	1 (2.27)	0 (0.00)	0 (0.00)	1.7190 (0.632)
		No (*n* = 118)	56 (100.00)	43 (97.73)	7 (100.00)	12 (100.00)	
CHL	*cat1*	Yes (*n* = 12)	0 (0.00)	10 (22.73)	2 (28.57)	0 (0.00)	18.0219 (< 0.001)
		No (*n* = 107)	56 (100.00)	34 (77.27)	125 (71.43)	12 (100.00)	
	cat2	Yes (*n* = 12)	0 (0.00)	10 (22.73)	2 (28.57)	0 (0.00)	18.0219 (< 0.001)
		No (*n* = 107)	56 (100.00)	34 (77.27)	125 (71.43)	12 (100.00)	
	cmlA	Yes (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0.0000 (1.000)
		No (*n* = 119)	56 (100.00)	44 (100.00)	7 (100.00)	12 (100.00)	
	floR	Yes (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0.0000 (1.000)
		No (*n* = 119)	56 (100.00)	44 (100.00)	7 (100.00)	12 (100.00)	
TET	*tetA*	Yes (*n* = 63)	33 (58.93)	21 (47.73)	1 (14.29)	8 (66.67)	6.3917 (0.094)
		No (*n* = 56)	23 (41.07)	23 (52.27)	6 (85.71)	4 (33.33)	
	tetB	Yes (*n* = 77)	35 (62.50)	31 (70.45)	0 (0.00)	11 (91.67)	17.4088 (0.006)
		No (*n* = 42)	21 (37.50)	13 (29.55)	7 (100.00)	1 (8.33)	
	tetG	Yes (*n* = 21)	5 (8.93)	7 (15.91)	6 (85.71)	3 (25.00)	25.7832 (< 0.001)
		No (*n* = 98)	51 (91.07)	37 (84.09)	1 (14.29)	9 (75.00)	
SXT	*dfr12*	Yes (*n* = 1)	0 (0.00)	0 (0.00)	0 (0.00)	1 (8.33)	8.9922 (0.029)
		No (*n* = 118)	56 (100.00)	44 (100.00)	7 (100.00)	11 (91.67)	
	dfr7	Yes (*n* = 00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0.0000 (1.000)
		No (*n* = 119)	56 (100.00)	44 (100.00)	7 (100.00)	12 (100.00)	
	dfr1a	Yes (*n* = 8)	5 (8.93)	3 (6.82)	0 (0.00)	0 (0.00)	1.8046 (0.613)
		No (*n* = 111)	51 (91.07)	41 (93.18)	7 (100.00)	12 (100.00)	
	sul1	Yes (*n* = 20)	1 (1.79)	18 (40.91)	1 (14.29)	0 (0.00)	29.7739 (< 0.001)
		No (*n* = 99)	55 (98.21)	26 (59.09)	6 (85.71)	12 (100.00)	
	sul2	Yes (*n* = 85)	46 (82.14)	23 (52.27)	5 (71.43)	11 (91.67)	13.4697 (0.003)
		No (*n* = 34)	10 (17.86)	21 (47.73)	2 (28.57)	1 (8.33)	
	sul3	Yes (*n* = 7)	3 (5.36)	2 (4.55)	2 (28.57)	0 (0.00)	7.4289 (0.059)
		No (*n* = 112)	53 (94.64)	42 (95.45)	5 (71.43)	12 (100.00)	

AMP = ampicillin; CHL = chloramphenicol ; EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; SXT = trimethoprim-sulphamethoxazole; TET = tetracycline.

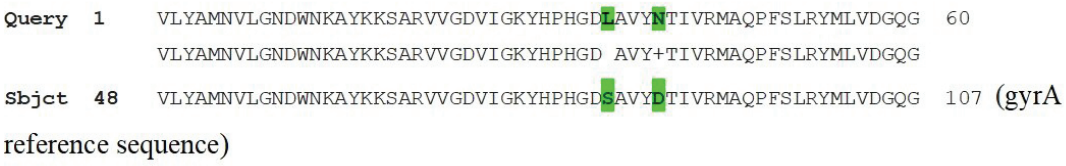

Figure 2. Sequence alignment of the gyrA [DNA gyrase (accession no.: MT334396)] protein sequence. The substitution was seen at position 83 [S83L (confers high-level resistance)] and 87 [D87N (confers low-level resistance)]. The gyrA reference sequence was obtained from the NCBI database.

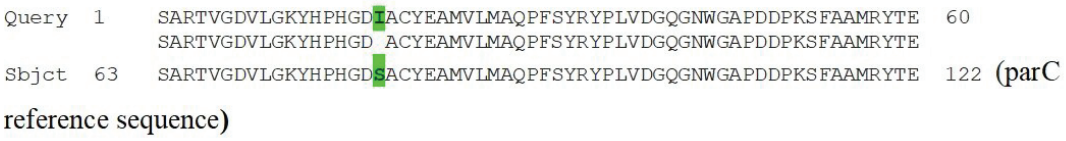

Figure 3. Sequence alignment of the parC [DNA topoisomerase (accession no.: MT334398)] protein sequence. The substitution was seen at position 80 (S80I). The parC reference sequence was obtained from the NCBI database.

Table 6. Association between *E. coli* virulent genes and antimicrobial susceptibility phenotypes ($p < 0.05$).

Virulent genes (%)	Antibiotics (% resistance, % sensitive)													
	AMP (78.99, 21.01)	AZM (2.52, 36.13)	CAZ (0.84, 99.16)	CHL (10.08, 87.39)	CIP (0.84, 92.44)	CRO (0.00, 96.64)	CTX (0.00, 92.44)	GEN (1.69, 89.83)	MEM (0.00, 100.00	NA (24.17, 79.83)	NOR (0.84, 99.16)	OFX (0.84, 99.16	SXT (89.92, 10.08)	TET (84.87, 10.92)
	% of virulent gene among resistance strains - % of virulent gene among sensitive strains													
	p													
eae (47.07)	56.38 -12.00	0.00 -55.81	0.00 -47.46	0.00 -52.88	0.00 -46.36	00.00 -47.83	00 -47.27	0.00 -52.83	0.00 -47.06	29.17 -51.58	0.00 -47.46	0.00 -47.46	52.34 -0.00	51.49 -30.77
	< 0.001*			0.002**				0.002**					< 0.001*	0.036*
aaic (13.45)	12.77 -16.00	00.00 -6.98	00.00 -13.56	83.33 -4.81	0.00 -14.55	0.00 -13.04	0.00 -12.73	0.00 -6.60	0.00 -13.45	45.83- 5.26	0.00- 13.56	0.00- 13.56	14.02- 8.33	10.89- 23.03
				< 0.001*				< 0.001**		< 0.001*				
CVD432 (33.61)	27.66 -56.66	66.67 -34.88	0.00 -33.90	83.33 -27.88	0.00 -34.55	0.00 -33.04	0.00 -32.73	100.00 -26.42	0.00 -33.61	58.33 -27.37	0.00 -33.90	0.00 -33.90	30.84 -58.33	32.67 -23.08
	0.007**			< 0.001*				< 0.001*		0.004*				
ial (5.88)	4.26 -12.00	33.33 -2.33	0.00 -5.93	16.67 -4.81	100.00 -5.45	0.00 -6.09	0.00 -6.36	0.00 -6.60	0.00 -5.88	8.33 -5.26	100.00 -5.08	100.00 -5.08	4.67 -16.67	5.94 -7.69
					< 0.001*						< 0.001*	< 0.001*		
stx1 (0.84)	1.06 -0.00	0.00 -0.00	0.00 -0.85	0.00 -0.96	0.00 -0.91	0.00 -0.87	0.00 -0.91	0.00 -0.94	0.00 -0.84	0.00 -1.05	0.00 -0.85	0.00 -0.85	0.93 -0.00	0.99 - 0.00
Stx2 (10.08)	11.70 -4.00	0.00 -4.65	100.00 -9.32	0.00 -11.54	0.00 -10.00	00.00 -9.57	0.00 -10.00	0.00 -10.38	0.00 -10.08	4.17 -11.58	0.00 -10.17	0.00 -10.17	9.35 -16.67	9.90 - 15.38
			0.002*											

AMP = ampicillin; CAZ = ceftazidime; CHL = chloramphenicol; CIP = ciprofloxacin; CRO = ceftriaxone; CTX = cefotaxime; GEN = gentamicin; MEM = meropenem; NA = nalidixic acid; NOR = norfloxacin; SXT = trimethoprim-sulphamethoxazole; TET = tetracycline.
(% resistance, % sensitive); % of virulent gene among resistant strains - % of virulent gene among sensitive strains.
*Positive association.
**Negative association.

Table 7. Association between *E. coli* virulent genes and antimicrobial resistant genes.

Virulence gene	*BlaOxa*	*BlaTEM*	*BlaPSE-1*	*cat1*	*cat2*	*cmlA*	*floR*	*tetA*	*tetB*	*tetG*	*dfr12*	*dfr7*	*dfr1a*	*Sul1*	*Sul2*	*Sul3*
	% of virulent gene among strains carry the resistant gene - % of virulent gene among strains not carry the resistant gene *p*															
eae	0.00 -51.85 0.001**	59.69 -33.33 0.004*	0.00 -47.46	0.00 -52.34 < 0.001**	0.00 -52.34 < 0.001**	0.00 -47.06	0.00 -47.06	52.38 -41.07	45.45 -50.00	23.81 -52.04 0.018**	0.00 -47.46	0.00 -47.06	62.50 -45.95	5.00 -55.56 < 0.001**	54.12 -29.41 0.014*	42.86 -47.32
aaic	72.73 -7.41 < 0.001**	4.84 -22.81 < 0.001**	100.00 -12.71 0.010*	75.00 -6.54 < 0.001*	83.33 -5.61 < 0.001*	0.00 -13.45	0.00 -13.45	4.76 -23.21 0.003**	14.29 -11.90	19.05 -12.24	0.00 -13.56	0.00 -13.45	0.00 -14.41	55.00 -5.05 < 0.001*	4.71 -35.29 < 0.001**	0.00 -14.29
CVD432	81.82 -28.70 < 0.001*	22.58 -45.61 0.007**	0.00 -33.90	75.00 -28.97 0.001*	83.33 -28.04 < 0.001*	0.00 -33.61	0.00 -33.61	33.33 -33.93	37.66 -26.19	28.57 -34.69	0.00 -33.90	0.00 -33.61	37.50 -33.33	85.00 -23.23 < 0.001*	24.71 -55.88 0.001**	28.57 -33.903
ial	18.18 -4.63	3.23 -8.77	0.00 -5.93	16.67 -4.67	16.67 -4.67	0.00 -5.88	0.00 -5.88	1.59 -10.71 0.034**	0.00 -16.67 < 0.001**	28.57 -1.02 < 0.001*	0.00 -5.93	0.00 -5.88	0.00 -6.31	5.00 -6.06	5.88 -5.88	28.57 -4.46 0.008*
stx1	0.00 -0.93	0.00 -1.75	0.00 -0.85	0.00 -0.93	0.00 -0.93	0.00 -0.84	0.00 -0.84	1.59 -0.00	1.30 -0.00	0.00 -1.02	0.00 -0.85	0.00 -0.84	0.00 -0.90	0.00 -1.01	1.18 -0.00	0.00 -0.89
Stx2	0.00 -11.11	14.52 -5.26	0.00 -10.17	0.00 -11.21	0.00 -11.21	0.00 -10.08	0.00 -10.08	12.70 -7.14	14.29 -2.38 0.039*	14.29 -9.18	100.00 -9.32 0.002*	0.00 -10.08	0.00 -10.81	0.00 -12.12	12.94 -2.94	0.00 -10.71

Chi-square association output of *E. coli* virulent genes and antimicrobial resistant genes.
*Positive association.
**Negative association. Figure titles and legends

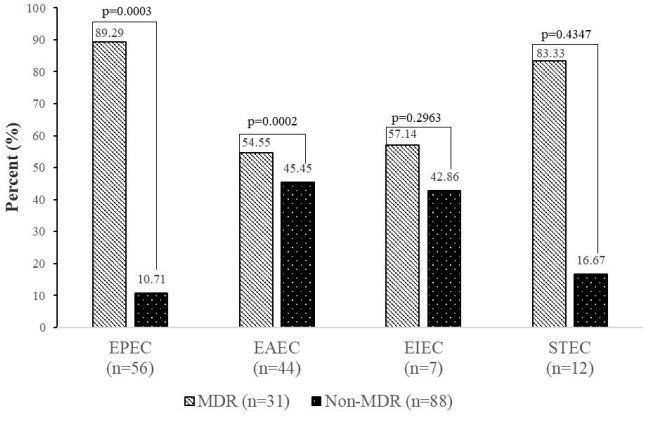

Figure 4. The features of the *E. coli* pathotypes according to multi drug resistance status.
EAEC = enteroaggregative *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*.

distinguished by their associations. Each of the 3 virulent genes (*eae*, *aaic*, *CVD432*), and the *cat1* and *cat2* gene, were each positively associated to *aaic* and *CVD432* gene ($p < 0.05$), and negatively associated to the *eae* gene ($p < 0.05$; Table 7). Concerning chloramphenicol resistance, the associations obtained genetically are in agreement with those obtained phenotypically to virulent genes.

7. Multi-drugs resistant profile

The features of the *E. coli* pathotypes according to MDR status are presented in Figure 4. EPEC and EAEC were more likely ($p < 0.05$) to be MDR (89.29%, 54.55%) than non-MDR (10.71%, 45.45%), respectively.

Discussion

The diversity, as well as the pathogenicity of the *E. coli* strains are due to the insertion of genetic material, whether in the form of transposon, bacteriophage or plasmid [39]. This incorporated genetic material may contain genes involved in virulence and resistance against antimicrobial drugs [40].

Enterovirulent *E. coli* has been identified as a major cause of diarrhea in developing countries [41]. These pathogens are not routinely sought in developing countries due to deficiency of infrastructures such as advanced molecular techniques. Consequently, the precise burden of enterovirulent *E. coli* among adult patients throughout Cameroon, notably the Bamboutos Division, is uncertain.

In the present study, stool samples were collected from patients with enteric diseases, being treated in Mbouda, Adlucem and district hospitals, in the Bamboutos Division, West region of Cameroon. This is the first in-depth study of enterovirulent *E. coli* in the population of Mbouda. The occurrence of atypical EPEC was higher compared with EAEC, STEC and EIEC. None of the stool specimens were positive for typical EPEC (both *eae* and *bfpA*) and ETEC. Similar results obtained in Mangalore, India showed that, the most prevalent DEC was atypical EPEC, followed by EAEC and STEC [42], contrary to that of Spano, Brazil [43].

The occurrence of EPEC was higher in patients with symptoms of vomiting and diarrhea, and fever and diarrhea, while patients infected with EAEC, EIEC and STEC reported abdominal cramps, dysenteric stools/stomach cramps and diarrhea, respectively. It has been shown that, various *E. coli* pathotypes contribute to diarrheal episodes by inhibiting the serotonin transporter, although the mechanism is poorly understood [6]. Among all enterovirulent *E. coli*, EPEC was observed to be the most widespread pathotype for diarrheal infection in Mbouda.

The molecular detection of virulent associated genes revealed that, the occurrence of the *eae* gene in total screened isolates was high compared with *CVD432*, *aaic*, *stx1* and *stx2*. The association of the *CVD432*/*aaic* and *stx1*/*stx2* genes was observed in 10.08% and 0.84%, respectively, whilst the other genes (*bfpA*, *eae*/*bfpA*) were absent. These findings are comparable to those obtained in a study reported in India [42]. The distribution of *E. coli* pathotypes and virulent genes depends on geographical and socio-economic features of each region. In Tehran, Shahrokhi et al showed that, *ST* was the most frequent toxin type followed by *LT* and *LT*/*ST* [44]. Dominance of the *ST*-expressing ETEC has been documented in Egypt, Bangladesh, and Iran [44,45].

Highly significant resistance rates were observed for ampicillin and sulfamethoxazole-trimethoprim in this study. EPEC (92.86%), STEC (83.33%), EAEC (75.00%) and EIEC (13.64) showed non-significant resistance to tetracycline. These results are comparable to the global general trend of fecal *E. coli* isolates [3]. Nevertheless, resistance rates fluctuate by country. In comparison, the rates observed in this study for Cameroon are higher than those registered in 6 European countries including Russia [46], and lower than those from Nigeria [47]. The spectrum of antibiotics used in Cameroon for prophylaxis, therapy, growth promotion and auto-medication can directly affect AMR of endogenous bacteria. In addition, the environment can also be a source for resistant microorganisms and resistant genes for humans [48].

BSBL genes *blaTEM* and *blaOxa*, were detected in a wide number of isolates, contrary to an existing report [49]. This may have been the cause of resistance to ampicillin in the study isolates. The prevalence of chloramphenicol resistant genes *cat1* and *cat2*, was higher in *E. coli* isolates resistant to chloramphenicol compared with the *cmlA* and *floR* genes. This indicates non-enzymatic or plasmid chloramphenicol resistant mechanisms of study isolates [50]. Tetracycline genes *tetB*, *tetA*, and *tetG*, were detected in 64.71%, 59.94% and 17.65% of the total tetracycline resistant isolates, respectively, with a predominance among tetracycline resistant STEC for *tetB* and EIEC for *tetG*. These results show that, study isolates express resistance via an efflux mechanism [51]. *sul1*, *sul2* and *sul3* (dihydropteroate synthase genes) genes were detected in 16.81%, 71.43% and 5.88% of trimethoprim-sulphamethoxazole resistant isolates, respectively. These genes play a major role in sulfonamide resistance and are remarkably related to integrons and transposons. High occurrence of the *sul2* gene observed in this study is in accordance with the results obtained by Wu et al in 2010 [52]. The genes *dfr1A*, *dfrA7* and *dfrA12* were detected in 6.72%, 0.00%, and 0.84% of trimethoprim resistant isolates, respectively, *dfr1A* was the most commonly identified. The occurrence of antibiotic resistance fluctuates with respect to each country. These findings are similar to those from a report in Iran [53].

A double substitution (S83L; D87N) was seen in the *gyrA* protein sequence for a single isolate resistant to OFX, NOR, CIP and NA whilst a single mutation in *parC* was seen at position 80 (S80I). 33.33% *E. coli* isolates resistant to NA and susceptible to OFX, NOR and CIP presented a single mutation in *gyrA* at position 83 (S83L). Many factors such as animal-to-human transfer of resistant bacteria have been reported to be the main contributor to the appearance of highly quinolone-resistant bacteria, mainly due to mutations in the DNA gyrase and topoisomerase IV genes [54].

Many associations between virulent factors and AMR were identified. The most remarkable was chloramphenicol resistance that was positively associated with *aaic* and *CVD432*, and negatively associated with *eae*, and that of gentamicin which was distinguished by its positive association with *CVD432* and negative association with *eae* and *aaic*. The combination of *E. coli* virulent factors and antibiotic resistance has been reported although the molecular pathways underlying the association between resistance and virulence is not clearly understood.

Other associations were observed between virulent genes and antimicrobial resistant genes. Enterovirulent *E.*

coli carrying the *blaOxa*, *blaTEM*, *cat1*, *cat2*, *sul1*, and *sul2* genes, were distinguished by their association with each of the 3 virulent genes (*eae*, *aaic*, *CVD432*). *cat1* and *cat2* genes were each positively associated with *aaic* and the *CVD432* genes, and negatively associated with the *eae* gene. Concerning chloramphenicol resistance, the associations obtained genetically are in agreement with those obtained phenotypically to virulent genes.

These findings are of huge public health prominence since the virulent and resistant genes of enterovirulent *E. coli* are not routinely assessed in hospital-based laboratories in Mbouda, Cameroon. However, the study population in this present study was limited to patients aged ≥ 20 years. In addition, the focus of this work was on enterovirulent *E. coli* isolates. Even though non-enterovirulent *E. coli* isolates were obtained, no further molecular studies were performed. Enterovirulent *E. coli* is reputed to carry resistant genes on plasmids, transposons, and integrons. These mobile genetic materials can be transferred between microorganisms of the same species or different genera across horizontal gene transfer [40]. Thus, additional studies are needed to evaluate the risks associated with *E. coli* which carry antibiotic resistance genes, and the capability of transferring these genes to other bacteria, including commensal *E. coli* and other enteric bacterial pathogens which could have a prejudicial effect on public health.

Conclusion

This study, the first in Mbouda, Cameroon to determine the virulence and antibiotic resistance of enterovirulent *E. coli* isolates from a sample of the Mbouda adult population with enteric diseases. The presence of atypical EPEC, EAEC, STEC, and EIEC was observed. A high antibiotic resistance phenotype was noted, with resistance to commonly used antibiotics including ampicillin, sulfamethoxazole-trimethoprim, and tetracycline. Significant susceptibility to antibiotics such as ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, norfloxacin and meropenem, was equally observed. BSBL genes blaTEM and blaOxa, chloramphenicol resistant genes (*cat1* and *cat2*), tetracycline resistant genes (*tetB*, *tetA* and *tetG*), dihydropteroate synthase genes (*sul1*, *sul2* and *sul3*) and trimethoprim resistant genes (*dfr1A*, *dfrA7* and *dfrA12*) were detected in the respective antibiotic resistant enterovirulent *E. coli*. Many associations between virulent factors and antimicrobial resistant phenotype, and between virulent genes and antimicrobial resistant genes, were noted. The detection of virulent and highly antibiotic resistant genes in fecal *E. coli* in Mbouda adults suggested that measures should be taken to reduce the potential harm to public health, such as the appropriate etiological diagnosis and reasonable use of antimicrobial agents.

Appendix

Supporting files are available at https://www.kcdcphrp.org.

Supporting file1 (.xls): Raw data of the study.

Supporting file2 (.doc): Figure S1. Nucleotide sequence of the *gyrA* DNA sequence (accession number: MT334396) form sample 60J whose a Compositional matrix adjust analyses show A double Mutation in *gyrA* protein sequence at position 83 (S change to L= S83L) and 87 (D change to N= D87N);Figure S2. Nucleotide sequence of the *gyrA* DNA (accession number: MT334398) sequence form sample 90J3 whose a Compositional matrix adjust analyses show A single Mutation in *gyrA* protein sequence at position 83 (S change to L= S83L); Figure S3. Nucleotide sequence of the *parC* DNA sequence (accession number: MT334397) fragment form sample 60J whose a Compositional matrix adjust analyses show A single Mutation in *parC* protein sequence at position 80 (S change to I= S80I).

1. Data availability

All data generated or analyzed during this study are included in this published article and the supporting file. Sequences have been deposited in NCBI GenBank under the accession numbers MT334396; MT334398 and MT334397.

Conflicts of Interest

The authors have no conflicts of Interest to declare.

Acknowledgments

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