Iranian Journal of Microbiology
Volume 16 Number 2 (April 2024) 176-186

ORIGINAL ARTICLE

Sequence-subtype association of multi-drug-resistant diarrheagenic *Escherichia coli*

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Received: August 2023**, Accepted:** January 2024

ABSTRACT

Background and Objectives: Multi-drug-resistant pathogens pose a significant threat as they can rapidly spread, leading to severe healthcare-associated invasive infections. In developing countries, diarrheagenic *Escherichia coli* (DEC) is a major bacterial pathogen responsible for causing diarrhea. However, the outbreak of resistant strains has made the treatment of DEC infections much more challenging. This study aimed to investigate the relationship between antibiotic resistance genes and other virulence categories in *E. coli* strains that cause diarrhea, particularly DEC.

Materials and Methods: The phylogenetic grouping was defined using PCR and multi-locus sequence type (MLST) methods.

Results: Among the isolates analyzed, 14 were identified as resistant and were classified into eight distinct sequence types: ST3, ST53, ST77, ST483, ST512, ST636, ST833, and ST774, indicating genetic diversity among the resistant strains. Certain sequence types, notably ST512 and ST636, were found to be associated with multiple antibiotic resistance in DEC. Regarding antibiotic susceptibility, strains showed the highest resistance to amoxicillin, suggesting that this antibiotic may not be effective in treating DEC infections. On the other hand, the isolates demonstrated susceptibility to amikacin and chloramphenicol, implying that these antibiotics could be more suitable treatment options for DEC infections.

Conclusion: The findings underscore the importance of promptly identifying antibiotic resistance patterns and their correlation with specific pathogenic virulence categories, as this knowledge can aid in selecting the most appropriate antibiotics for treating DEC infections. Considering the antibiotic resistance profiles and associated resistance genes is crucial in managing and containing diarrheal outbreaks and in selecting effective antibiotic therapies for DEC infections.

Keywords: *Escherichia coli*; Multilocus sequence typing; Multidrug resistant; β-lactams; Virulence factors

INTRODUCTION

Escherichia coli is a Gram-negative bacterium that is commonly found as a harmless commensal intestinal flora in the human gastrointestinal tract. However, it is one of the most common human pathogens in a variety of pathotypes, primarily causing urinary tract infections, bloodstream infections, neonatal sepsis, skin structure infections, traveler's diarrhea, and bacteremia. The incidence of bacteremia is increasing globally, and it is one of the leading causes of death (1). The emergence and spread of antimicrobial resistance is a worldwide source of concern for public health issues. Antibiotic resistance is increasing among *E. coli* strains, and this opportunistic pathogen can cause a variety of intestinal and extraintestinal diseases. It causes at least 30% of pathogenic bacteremia (1, 2). Enteroaggregative *E.*

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coli (EAEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Shiga-toxin-producing *E. coli* (STEC), Enteroinvasive *E. coli* (EIEC), and Enterohemorrhagic *E. coli* (EHEC) are the six DEC pathotypes. The epidemiological monitoring of *E. coli* strains was based on a variety of strain-specific criteria. Among these methods, the most important are serotyping, phylogenetic group determination, and multi-locus sequence typing (MLST). Serotyping was the first method used to classify *E. coli*, and it was later refined into standardized procedures (2). Phylogenetic analyses revealed that *E. coli* could be classified into four distinct groups (A, B1, B2, and D). Human extra-intestinal pathogenic *E. coli* (ExPEC) strains were classified into two groups: B2 and D. The *TspE4.C2*, *chuA*, and *yjaA* genes can be used to distinguish *E. coli* phylogroups. Commensal and less virulent strains, on the other hand, belonged to the A or B1 groups (3, 4). Antimicrobial-susceptible strains are frequently found in phylogenetic group B and have been linked to virulence genes (5). Antimicrobial resistance in *E. coli* virulent isolates has been related to virulence-associated genes, according to research (6, 7). Furthermore, *E. coli* genomes allow for the estimation of genetic distance between strains based on nucleotide polymorphism as a highly reproducible applied method to genotype *E. coli*. The MLST genotyping method defines clonal diversity by sequencing multiple housekeeping genes. This genetic association analysis method is regarded as the gold standard for DNA sequence-based typing of bacterial pathogens (8). Furthermore, MLST data permits the determination of phylogenetic relationships between lineages (9). The definition of MLST and sequence types (STs) is based on allelic variations in 6-11 "housekeeping genes" (8). MLST is a reliable sequence-based technique for revealing *E. coli* genetic diversity and variability by describing the genetic structure of bacterial populations (8). ST and a numerical designation are assigned to *E. coli* strains according to two widely accepted schemes. Three standard MLST schemes for *E. coli* have been introduced, each relying on a different set of 7-8 genes (10). To assess species diversity, the BIGSdb software (https://pubmlst.org/software/database/bigsdb/) can be used (11, 12). The pan-ge- nome can be used to type bacterial genomes (this is known as the Pasteur scheme) (13, 14). Clustering based on the presence or absence of genes allows for a more precise analysis of different strain isolates.

Eight housekeeping genes were sequenced: *trpA* (tryptophan synthase subunit A), *uidA* (beta-glucuronidase), *dinB* (DNA polymerase), *pabB* (p-aminobenzoate synthase), *putP* (proline permease), *polB* (polymerase PolII), and *trpB* (tryptophan synthase subunit B). The internal parts of these genes were compared to the database available at https://bigsdb.pasteur.fr/ (10). Every distinct sequence in a locus was assigned a unique allele number, and each dis- tinct allele combination was also assigned a specific sequence type (ST). A new ST was defined as a new allele number described in the database. The genes for extended-spectrum -lactamases (ESBLs) are fre- quently encoded on transferable plasmids. Such gene acquisition by commensal or pathogenic isolates may result in multidrug-resistant (MDR) pathogens. Be- cause of the cost and scarcity of antibodies, prelimi- nary identification by serotyping is difficult, particu- larly in developing countries. It is thus preferable to replace it with a direct assessment of the existence of virulence-associated genes (15).

In addition, multidrug-resistant *E. coli* has been found in several of countries. Resistance to cephalosporins, for example, has caused serious concern in the treatment of diseases caused by *E. coli*. The production of plasmid-borne, extended-spectrum -lactamases (ESBLs) was thought to be the dominant mechanism underlying beta-lactam antibiotic resistance in *E. coli*. ESBL genes are frequently encoded on transferable plasmids. The acquisition of such resistant genes by commensal or pathogenic isolates may result in MDR pathogens. The current study for human pathogenic *E. coli* strains examined the potential relationship between antimicrobial resistance and molecular characterization.

MATERIALS AND METHODS

Sample collection, isolation, and identification. The stool samples were collected from patients suffering from diarrhea at various reference hospitals in Tehran. All of the specimens were transported under standard conditions to the Pasteur Institute of Iran, Molecular Biology Department. The stool samples were cultured overnight at 37°C on MacConkey agar (MAC; MERCK, Darmstadt, Germany), from which lactose-fermenting (pink) colonies were selected, and biochemical characteristics were cultured on triple sugar iron (TSI, MERCK, Darmstadt, Germany) and

tryptone soya agar (TSA, MERCK, Darmstadt, Germany). Molecular tests confirmed the presence of *E. coli* strains (16-27). In a previous study (28), diarrheagenic *E. coli* (DEC) isolates were identified. From the culture, a single colony of *E. coli* strains was picked. *E. coli* pure subcultures were prepared and stored at 70°C. Antibiotic resistance phenotypes of *E. coli* isolates.

The antimicrobial susceptibility of *E. coli* strains was determined using the Kirby Bauer disk diffusion method, as described in the Clinical and Laboratory Standards Institute (CLSI) Guidelines (19). In 3 mL of Mueller-Hinton broth (MERCK, Darmstadt, Germany), a bacterial colony was suspended, and the suspension concentration was adjusted to 0.5 McFarland. The bacterial suspension was cultured on Mueller-Hinton agar (MERCK, Darmstadt, Germany) and subsequently disks (BD BBL, USA) containing amikacin (30 g); amoxicillin (10 g); ceftazidime (10 g); chloramphenicol (30 g); ciprofloxacin (CIP) (5 g); cefotaxime (CTX) (5 g); gentamicin (GM) (10 g);imipenem (IMP) (10 g); kanamycin (30 g); streptomycin (STM) (10 g); tetracycline (30 g) were placed. The zone diameter was used to interpret the results, which were "susceptible" (S), "intermediate" (I), or "resistant" (R) against the tested antibiotic as recommended by the manufacturer, while multi-drug resistance was defined as resistance (R) against more than three antimicrobial categories, with *E. coli* ATCC25922 used as the control strain. The presence of ESBL was confirmed by using the disk diffusion method suggested by CLSI guidelines to assess ESBL production among the isolates. ESBLs were detected using a combined disc assay with ceftazidime (30 g) and cefotaxime (30 g) discs in combination with clavulanic acid (10 g). The production of ESBL was determined by the combined discs' zone diameters increasing by 5 mm when compared to ceftazidime and cefotaxime zones (15, 21).

DNA extraction. DNA was extracted using a QIAamp DNA extraction kit (QIAgen®, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA extracts was measured using Nanodrop (NanoDropTM 2000/c Spectrophotometers) after electrophoresis on 0.8% agarose gels. The DNA samples were kept at -20 degrees Celsius until they were used for gene amplification.

Molecular pathotype gene detection. Genomic DNAs were isolated using a polymerase chain reaction (PCR) template preparation kit (Roche, Germany). DEC-PCR assay as approved EPEC (*eae*), EHEC (*eae*, *stx1*, and *stx2*), STEC (*stx1* or *stx2*), ETEC (*elt* or *est*), EIEC (*ipaH*), and EAEC (*aggR*) (Table 1) was used. The kit protocol contains a total mixture (20µL) of 10µL master mix (2×) (Fermatas, Catalog No. K0171 (Thermo Scientific) including Taq DNA polymerase (0.05U/µL), reaction buffer (10×), 4mM $MgCl_2$, and 0.4mM of each dNTP and 7µL deionized water, 2µL of each forward, reverse primers (Table 1), and 1µL template DNA was used. Finally, PCR was done in 20µl final volume. The PCR was performed in a thermocycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 20s, annealing at 60°C for 30s, extension at 72°C for 45s, and a final extension at 72°C for 5min. Reference strains were obtained from the collection of the Molecular Biology Department of the Pasteur Institute, Tehran. EPEC serotype E2348-69, ETEC serotype H10407, EAEC serotype O42, and STEC serotype EDL933 were used as positive controls, and *E. coli* DH5α as a negative control.

***E. coli* phylogenetic grouping and serotypes.** PCR performed with a standard protocol described by Clermont et al. (18). The applied primer pairs are presented in Table 1. *E. coli* serotypes O26, O45, O55, O83, O103, O119, O111, O121, O128, O145, and O157 as the most frequent O-antigen types of clinical *E. coli* strains were considered (19). Primer sequences were selected to detect O groups (Table 2). The annealing temperature of each PCR reaction was selected according to the Tm (Melting temperature) of each primer pair (20, 23).

Multi-locus sequence typing (MLST). The phylogeny of clonal isolates was investigated as a sequence type (ST) of *E. coli* isolates. Eight selected housekeeping genes, including *dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA*, were amplified for phylogenetic assessment of strains (Table 3). Briefly, all PCR amplifications were performed in 25µL reaction mixtures containing 2.5µL 10× PCR buffer, 0.3µL of each primer (Table 1), 1µL of template DNA (approximately 150 ng), 0.4µL of dNTPs, 0.7µL of $MgCl_2$, and 0.2µL Taq DNA polymerase. The reaction mixtures were cycled in an automated thermal cycler (Eppendorf, Hamburg, Germany) under the

Table 1. Primers of pathotype genes

Analysis	Marker	Sequences (5'→3')	Product length (bp)	Ref.
Pathotyping	*ipaH* (EIEC)	F:CTCGGCACGTTTTAATAGTCTGG	933	(19, 20)
		R:GTGGAGAGCTGAAGTTTCTCTGC		
	eae (EPEC)	F:AGGCTTCGTCACAGTTG	570	
		R:CCATCGTCACCAGAGGA		
	aggR (EAgEC)	F:ACGCAGAGTTGCCTGATAAAG	400	
		R:AATACAGAATCGTCAGCATCAGC		
	Stx1 (STEC)	F:GGCGTTCTTATGTAATGACTGC	250	
		R:ATCCCACGGACTCTTCCATC		
	Stx2 (STEC)	F:CGTTTTGACCATCTTCGTCTG	325	
		R:AGCGTAAGGCTTCTGCTGTG		

Table 2. Primers of phylogenetic grouping and serotypes

Analysis	Marker	Sequences (5'→3')	Product length (bp)	Ref.
Conventional phylogenetic grouping	*ChuA*	F:GACGAACCAACGGTCAGGAT	279	(8)
		R:TGCCGCCAGTACCAAAGACA		
	YjaA	F:TGAAGTGTCAGGAGACGCTG	211	
		R:ATGGAGAATGCGTTCCTCAAC		
	TspE4C2.1	F:GAGTAATGTCGGGGCATTCA	152	
		R:CGCGCCAACAAAGTATTACG		
Serotyping	O26	*wzx*-F:TAAATTGCGGGGAAAGAATG	256	(19)
		wzx-R:GACTTCATGGGTACCGCCTA		
	O45	*wzx*-F:CCGGGTTTCGATTTGTGAAGGTTG	527	
		wzx-R:CACAACAGCCACTACTAGGCAGAA		
	O55	*wzx*-F:AATGGAACATTGCAACAGCA	150	
		wzx-R:TGTGGATTCCAGAAAAGCAA		
	O83	*wzx*-F:GTACACCAGGCAAACCTCGAAAG	362	
		wzx-R:TTCTGTAAGCTAATGAATAGGCACC		
	O111	*wzx*-F:ATGGTATTAACAGTGAAA	1263	
		wzx-R:ATAGACATTTTTCGCTCT		
	O103	*wzx*-F:TTGGAGCGTTAACTGGACCT	321	
		wzx-R:GCTCCCGAGCACGTATAAG		
	O119	*wzx*-F:GTTAACAATCAGCTCGATAAAC	650	
		wzx-R:TTTGCAAGTAAACACCCTAAAC		
	O121	*wzx*-F:AGGCGCTGTTTGGTCTCTTA	310	
		wzx-R:TCGCTACCGCTAATGATTCC		
	O128	*wzx*-F:TCTTGCTTATAGCCAGAATT	1353	
		wzx-R:AATAAACCGACACCGAAA		
	O145	*wzx*-F:ACTGGGATTGGACGTGGATA	222	
		wzx-R:AGGCAAGCTTTGGAAATGAA		
	O157	*wzx*-F:TCAGCGGCTAAGTTGATT	861	
		wzx-R:ATTTGCTCCCATGTCTCC		

following conditions: initial denaturation at 94ºC for 4 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing for 45 s (10), extension at 72ºC for 1min based on size of the expected fragment (1 min/kb), and a final extension at 72ºC for 10 min. The amplified genes were detected by electrophoresis in a 1.8% agarose gel.

The sequenced genes were assembled, trimmed, and aligned using CLC sequence viewer 8 (http://www.clcbio.com). All the nucleotide se-

Table 3. Primers of phylogenetic grouping and serotypes

Analysis	Marker	Sequences (5'→3')	Product length (bp)	Ref.
	dinB	F:GTTTTCCCAGTCACGACGTTGTATGAGAGGTGAGCAATGCGT R:TTGTGAGCGGATAACAATTTCCGTAGCCCCATCGCTTCCAG	450	
	icdA	F:GTTTTCCCAGTCACGACGTTGTAATTCGCTTCCCGGAACATTG R:TTGTGAGCGGATAACAATTTCATGATC GCGTCACCAAAYTC	516	
MLST	*pabB*	F:GTTTTCCCAGTCACGACGTTGTAAATCCAATATGACCCGCGAG R:TTGTGAGCGGATAACAATTTCGGTTCC AGTTCGTCGATAAT	468	
	polB	F:GTTTTCCCAGTCACGACGTTGTAGGCGGCTATGTGATGGATTC R:TTGTGAGCGGATAACAATTTCGGTTGG CATCAGAAAACGGC	447-450	(10)
	putP	F:GTTTTCCCAGTCACGACGTTGTACTGTTTAACCCGTGGATTGC R:TTGTGAGCGGATAACAATTTCGCATCG GCCTCGGCAAAGCG	444-456	
	trpA	F:GTTTTCCCAGTCACGACGTTGTAGCTACGAATCTCTGTTTGCC R:TTGTGAGCGGATAACAATTTCGCTTTCATCGGTTGTACAAA	561	
	trpB	F:GTTTTCCCAGTCACGACGTTGTACACTATATGCTGGGCACCGC R:TTGTGAGCGGATAACAATTTCCCTCGT GCTTTCAAAATATC	594	
	uidA	F:GTTTTCCCAGTCACGACGTTGTACATTACGGCAAAGTGTGGGTCAAT R:TTGTGAGCGGATAACAATTTCCCATCAGCACGTTATCGAATCCTT	595-606	

quences of the MLST loci were submitted and analyzed. STs were compared with the database at https://bigsdb.pasteur.fr/.

Statistical analysis. For statistical analysis, the current study used SPSS software version 21.0 (IBM Corporation, New York, USA). MEGA 7, eBRUST V. 3 built a phylogenetic tree to generate the population snapshot.

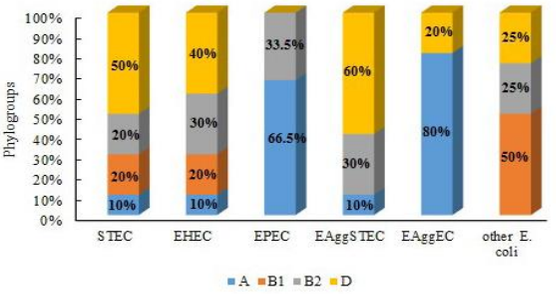

Fig. 1. Distribution of phylogenetic groups among isolated *E. coli* strains

RESULTS

Phenotypic antimicrobial resistance and multi-drug resistance. Fifty isolates of resistant *E. coli* were studied. Antimicrobial susceptibility profile and zone of inhibition using antibiotic discs with *E. coli* strains were measured. Most isolates showed resistance to amoxicillin (92%) and susceptibility to amikacin (97%) and chloramphenicol (92%). In the current research, the isolated *E. coli* strains predominantly belonged to phylogroup D (40.5%), followed by phylogroup B2 (23.8%), B1 (14.3%), and A (21.4%) (Fig. 1).

The multi-drug resistance (MDR) profiles of antibiotic-resistant *E. coli* isolates are presented in Table 4. They exhibited 83.7% and 59.5% resistance to amoxicillin (AX) and tetracycline (TE), respectively. They belonged to the β-lactam group. The next most frequent antibiotic resistance (streptomycin (S)) was 47.6%, as an aminoglycoside (Table 4). Up to 27.6% of resistant strains showed resistance to three and four antibiotics. The multi-drug resistance profile groups of isolates showed that 6.9% and 4.6% of isolates have four and five resistance markers, respectively (Table 4).

Phylogenetic characterization by multi-locus sequence typing (MLST). The housekeeping genes of the isolates' PCR products were sequenced. MLST analysis of *E. coli* strains identified those with STs of defined strain types (Table 5). For example, enterohemorrhagic *E. coli* (EHEC) was identified as ST563, except for the *polB* and *trp* genes, whose sequences are incompatible with any of the genes in the Pasteur databases. The *uidA, pabB,* and *putP* gene sequences in some isolated strains have deletions or inser-

tions, as well as base differences that correspond to ST3 strains. ST77, ST833, and ST563 isolates were all sensitive strains (Table 5). EPEC and STEC, which have different virulence-associated genes, were ST3 despite having different antibiotic susceptibility results with different genes. Allele sequences and STs were available on the MLST website at http://www.pasteur.fr/mlst.

ST483, ST636, ST833, and ST512 isolates were assigned to group B1; these are associated with clinical conditions.

The eBurst algorithm identified 12 sequence types (STs) from MLST analysis of 14 different *E. coli* strains. Two new STs were discovered and submitted to the Pasteur Institute website using the IDs 1353 and 1361. MEGA 7 software was used to create a phylogenic tree (Fig. 2). The Maximum Likelihood method evolutionary analysis of diarrheagenic *E. coli* sequence types (STs) produced the allelic profiles of housekeeping genes (Fig. 3).

Table 4. Multi drug resistance profile groups of isolates

Antibiotic profile	No. of MDR	Percentage (%)	Total
AMX /CAZ/CIP/GM/IMP/S/TE	7	5%	5%
AMX /CTX/CIP/ GM/C/TE	6	2.5%	2.5%
AMX/AK/CAZ/IMP/ K/S	5	5%	15%
AMX/CAZ/C/TE	5	2.5 %	
AMX /CTX /S/TE	5	7.5%	
AMX/CTX/TE	4	5%	10%
AMX/C/TE	4	5%	
AMX /GM/K	3	5%	35%
AMX/TE/S	3	12.5%	
CIP/TE/S	3	17.5%	

AK: Amikacin; AMX: Amoxicillin; CAZ: Ceftazidim; C: Chloramphenicol; CIP: Ciprofloxacin;
CTX: Cefotaxime; GM: Gentamicin; IMP: Imipenem; K: Kanamycin; S: Streptomycin; TE: Tetracycline.

***E. coli* diagnosis via virulence gene and serotype determination.** Serotypes O45, O111, O103, O119, O121, O128, and O145 were also identified as serogroups responsible for the majority of human STEC illnesses (Ludwig et al. 2020). They were not found in the infectious isolates, on the contrary. ST3 and ST53 isolates are classified as B2, whereas ST77 and ST774 isolates are classified as A (Table 5).

DISCUSSION

The widespread prevalence of multi-drug resistance (MDR) among various pathotypes of DEC is a major public health concern. Severe diarrheal infections remain a significant public health concern among both children and adults in developing countries. DEC is

Table 5. MLST Phylogroups, pathotypes, serogroups profiles of clinical *E. coli* isolates

Phylogroup	Pathotype	Serogroup	Resistance profile	ST	ID**	Allelic profile*
B2	EPEC	-	C, TE, AX	3	1352	3,8,5,11,8,3,5,3
B2	other *E. coli*	-	CIP, S, TE	53	1357	1,7,1,9,20,20,1,6
B1	STEC	-	S, TE	483	1358	21,3,3,133,16,31,134,2
B1	other *E. coli*	O26	S, TE, AX	636	1359	7,3,3,68,74,7,4,5
B1	EHEC	-	S, AX	New	1353	5,2,4,10,7,8,2,2
B1	EHEC	O26	TE	833	1354	8,2,7,3,186,1,98,2
B2	STEC	-	AX	3	1360	3,8,5,11,8,3,5,3
B1	STEC	-	CIP	New	1361	8,2,7,3,7,1,98,2
B1	other *E. coli*	O26	GN, S, TE, AX	512	1362	25,37,4,10,84,7,4,2
A	EAggEC	O83	AX	77	1363	3,43,31,33,8,3,5,11
A	EPEC	-	TE, AX	774	1355	8,2,7,3,7,1,179,2
A	STEC	-	C, CIP, GN, TE, AX	-	-	8,262, -,3,186,1, -,2
A	EAggSTEC	-	TE, AX	-	-	8,28, -,3,7,1, -,2
B2	EHEC	O26	AX	563	1356	11,72,134,52,25,145,18,2

* Allelic profile based on MLST of 8 housekeeping genes (*dinB, icdA, pabB, polB, putP, trpA, trpB,* and *uidA*), ** Pasteur institute ID

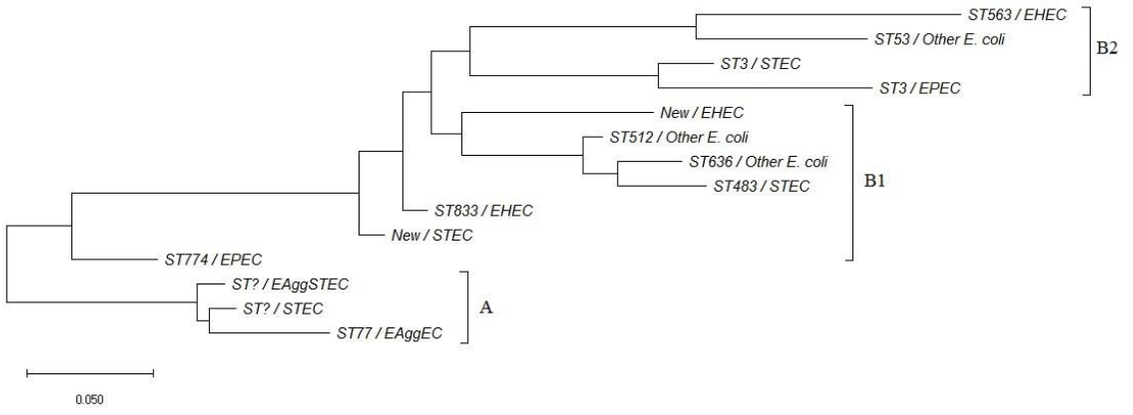

Fig. 2. Diarrheagenic *Escherichia coli* Relationship of sequence types (STs) with different pathotypes generated from the allelic profiles of housekeeping genes by the neighbor-joining method.

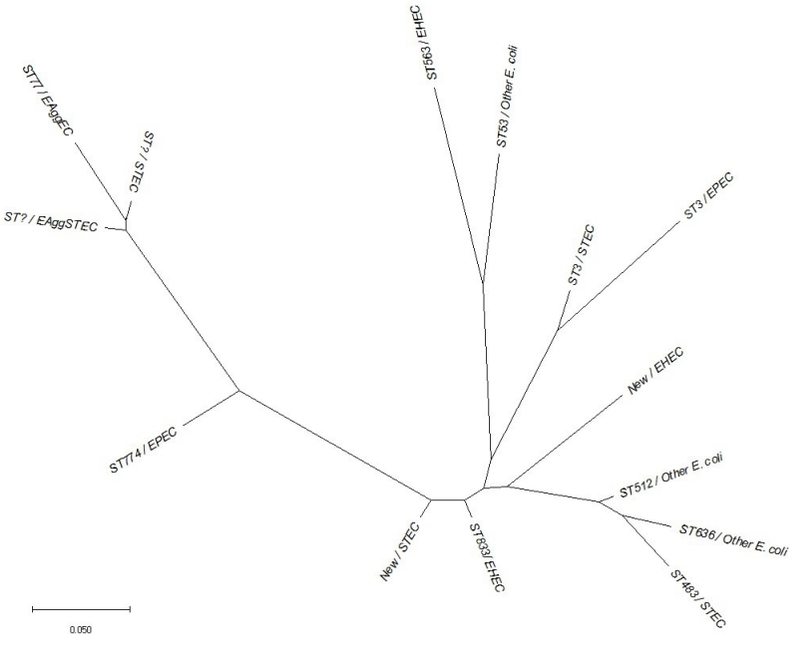

Fig. 3. Evolutionary analysis of diarrheagenic *E. coli* sequence types (STs) generated from the allelic profiles of housekeeping genes by Maximum Likelihood method.

a prominent global pathogen, and its genetic variation has garnered increased attention worldwide. Notably, Iran experienced one of the most significant outbreaks of diarrheagenic *E. coli*, with EPEC being the most prevalent pathotype, accounting for 21.4% of cases (23-28). Previous studies in developing countries have shown that *stx1* and *stx2*, which are associated with certain types of *E. coli* infections, are rarely isolated from patients with diarrhea (25-27). Among the pathotypes, STEC has the highest frequency (35.4%), followed by ETEC (14.0%) and EPEC (13.1%) (28). Additionally, there are *E. coli* isolates comprising EAEC (4.3%) and EIEC (0.3%), which are associated with STEC and EHEC infections, respectively. These findings highlight the significance of DEC as a major contributor to severe diarrheal infections in developing countries.

In the present research, the most common isolates were identified as belonging to phylogroups D (40.5%) and B1 (14.3%). Notably, phylogenetic analysis revealed that the majority of MDR isolates from patients were associated with the D group. Among

the different pathotypes, the highest prevalence of phylogenetic groups A, B1, B2, and D was observed in EAggEC (80%), other *E. coli* strains (50%), EPEC (33.5%), and EAggSTEC (60%), respectively. The phylogenetic analysis of the isolates revealed interesting associations between specific pathotypes and phylogroups. For instance, the A phylogroup was predominantly associated with EAggEC, highlighting their genetic relatedness and potential for causing severe diarrheal infections. On the other hand, the D phylogroup showed the highest prevalence among MDR isolates, suggesting its potential role in the dissemination of antibiotic resistance among DEC strains. In terms of serotype distribution, O26 was the most common serotype identified in this study, which aligns with previous findings of O26 being associated with highly pathogenic strains of *E. coli*. The detection of new clones of EHEC with a high pathogenic potential, specifically O26, in other regions (Europe and the United States) further emphasizes the global concern associated with this serotype (29). In the mentioned study, the study conducted by Alikhani and colleagues reported a prominent occurrence of several Shiga toxin-producing *Escherichia coli* (STEC) serogroups, namely O55, O26, O111, O125, O128, O114, O86, O142, O119, O127, and O126, in the STEC strains analyzed in their investigation (23). In terms of sequence types (STs), the study identified ST3 and ST53 as being classified under the B2 phylogroup, while ST77 and ST774 isolates were classified under the A phylogroup. In the previous study's analysis of DEC isolates, phylogenetic group A was found to be the most prevalent, with a frequency of 37.1% for the A1 type and 12.9% for the A0 type. This differs from previous studies that have reported varying frequencies for each phylogenetic group. These findings suggest that phylogenetic group A may be more commonly associated with DEC infections. In contrast to previous reports, the current study did not find a significant association between group B2 *E. coli* strains and healthy individuals' fecal samples. Previous studies have suggested that group B2 *E. coli* strains are relatively rare in fecal samples of healthy individuals. However, the results of this study may indicate that the prevalence of group B2 *E. coli* strains in healthy individuals' fecal samples may be higher than previously thought or that other factors may contribute to the presence of these strains in fecal samples. Further research is needed to better understand the relationship between group B2 *E. coli* strains and healthy individuals' fecal samples (30, 31). The frequent MLST types observed in susceptible strains were ST833, ST37, and ST563. In contrast, resistant (MDR) isolates exhibited the presence of ST53, ST483, ST636, ST512, and ST774.

The high resistance frequency observed for amoxicillin (92%) in this study is particularly alarming, as amoxicillin is a commonly used antibiotic for treating diarrheal diseases. This finding highlights the challenges in selecting an appropriate antibiotic therapy for DEC infections, considering the limited options available due to high resistance rates. It is worth noting that the lowest resistance frequency was observed for amikacin (3%), indicating its potential as a more effective treatment option. Incorporating the use of alternative antibiotics like amikacin, which show lower resistance rates, could be crucial in managing severe diarrheal infections caused by DEC. In the previous study, the most commonly observed multidrug resistance (MDR) pattern among *Escherichia coli* isolates was resistance to ampicillin, ciprofloxacin, amikacin, and tetracycline.

The discussion of the article highlights the prevalence of severe diarrheal infections caused by DEC in developing countries. It also mentions the genetic variation of the pathogen, the distribution of different DEC pathotypes and their phylogenetic groups, the resistance patterns observed in the isolates and the implications for treatment. The most commonly observed multidrug resistance (MDR) pattern among *E. coli* isolates in the previous study was resistance to ampicillin, ciprofloxacin, amikacin, and tetracycline. The frequencies of this MDR pattern were 37%, 8%, 5%, and 37%, respectively. This indicates that a significant proportion of *E. coli* isolates in this study were resistant to multiple antibiotics commonly used in clinical settings, which poses a major challenge in the treatment of bacterial infections caused by these organisms. The high frequency of MDR *E. coli* isolates highlights the need for continued surveillance and the development of new antibiotics to combat antimicrobial resistance (32-35). This study demonstrates that the *uidA*, *pabB*, and *putP* gene sequences have deletions or insertions. Therefore, multidrug-resistant-bacteria defined according to a standard acquired non-susceptibility to at least one agent in three or more antimicrobial categories based on an international the European Centre for Disease Prevention Control (ECDC) and the Centers for Disease Control and Prevention (CDC) definition (36).

In this study, the resistance to chloramphenicol observes among the A and B2 phylogroups, which belong to STEC and EPEC Pathotypes. The association between sequence types (STs) and different pathotypes is an important finding of this study. For instance, ST3 was predominantly found among isolates of both EPEC and STEC pathotypes, indicating their genetic similarity and potential for shared antibiotic resistance mechanisms. These associations strengthen our understanding of the genetic diversity and epidemiology of DEC strains, and may contribute to improved diagnostics and targeted treatment strategies in the future. In addition, ST3 isolates belong to the B2 phylogroup. A previous study showed that ST10 was the most predominant in isolates (37). In the current study, two new isolates (ID1353 and ID1361) in EHEC and STEC were found that belong to the B1 phylogroup with different resistance profiles. The new STEC is similar to ST833 EHEC, while the new EHEC is the cluster in evolutionary analysis.

In this study, the most frequent MDR resistance marker is 35% among isolates with three resistance markers with amoxicillin and tetracycline in common. The less frequent MDR resistance marker is 2.5% for six antibiotic resistances. The prevalence of MDR among DEC isolates underscores the urgent need for appropriate antibiotic stewardship and infection control measures. Developing practical antibiotic therapy guidelines based on the knowledge of prevalent MDR isolates can help clinicians in selecting effective treatment options while minimizing the spread of antibiotic resistance. Furthermore, surveillance programs should be established to monitor the emergence and spread of MDR DEC strains, enabling timely intervention and prevention of severe diarrhea outbreaks. The most dominant serotype is O26 which belongs to the EHEC and other *E. coli* among B1 and B2 phylogroups. The multiple antibiotic-resistant strains prevalence elevated, making it a public health problem. Finally, the classified pathogenic bacteria definition with recognized MDR in diarrheagenic *E. coli*, assists in outbreaks treatment in the future.

CONCLUSION

In conclusion, this study provides valuable insights into the multi-drug resistance and genetic diversity of clinical DEC isolates from different phylogroups and pathotypes. The high prevalence of antibiotic resistance, particularly in the β-lactam group, presents significant challenges in treating severe diarrheal infections caused by DEC. The associations between specific STs and pathotypes highlight the importance of understanding the genetic profiles of DEC strains for targeted treatment strategies. By implementing appropriate antibiotic stewardship, infection control measures, and surveillance programs, we can effectively address the public health risks associated with MDR DEC and mitigate the further spread of antibiotic resistance.

ACKNOWLEDGEMENTS

We thank the Institute Pasteur, MLST, and whole genome MLST teams databases for curating the data and making them publicly available at https://bigsdb.pasteur.fr/.

REFERENCES

1. Dobrindt U. (Patho-) Genomics of *Escherichia coli*. *Int J Med Microbiol* 2005; 295: 357-371.
2. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* 2013; 5: 58-65.
3. Johnson JR, Russo TA. Extraintestinal pathogenic *Escherichia coli*: "the other bad *E. coli*". *J Lab Clin Med* 2002; 139: 155-162.
4. Croxen MA, Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat Rev Microbiol* 2010; 8: 26-38.
5. Cooke NM, Smith SG, Kelleher M, Rogers TR. Major differences exist in frequencies of virulence factors and multidrug resistance between community and nosocomial *Escherichia coli* bloodstream isolates. *J Clin Microbiol* 2010; 48: 1099-1104.
6. Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, Lim H, et al. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Appl Environ Microbiol* 2005; 71: 6753-6761.
7. Wang X-M, Jiang H-X, Liao X-P, Liu J-H, Zhang W-J, Zhang H, et al. Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiol Lett* 2010; 306: 15-21.

8. Maiden MC. Multilocus sequence typing of bacteria. Annu Rev Microbiol 2006; 60: 561-588.
9. Feil EJ. Small change: keeping pace with microevolution. *Nat Rev Microbiol* 2004; 2: 483-495.
10. Jaureguy F, Landraud L, Passet V, Diancourt L, Frapy E, Guigon G, et al. Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. *BMC Genomics* 2008; 9: 560.
11. Chaudhuri RR, Henderson IR. The evolution of the *Escherichia coli* phylogeny. *Infect Genet Evol* 2012; 12: 214-226.
12. Clermont O, Gordon D, Denamur E. Guide to the various phylogenetic classification schemes for *Escherichia coli* and the correspondence among schemes. *Microbiology (Reading)* 2015; 161: 980-988.
13. Rouli L, Merhej V, Fournier P-E, Raoult D. The bacterial pangenome as a new tool for analyzing pathogenic bacteria. *New Microbes New Infect* 2015; 7: 72-85.
14. McNally A, Oren Y, Kelly D, Pascoe B, Dunn S, Sreecharan T, et al. Combined analysis of variation in core, accessory and regulatory genome regions provides a super-resolution view into the evolution of bacterial populations. *PLoS Genet* 2016; 12(9): e1006280.
15. Hien BT, Scheutz F, Cam PD, Serichantalergs O, Huong TT, Thu TM, et al. Diarrheagenic *Escherichia coli* and Shigella strains isolated from children in a hospital case-control study in Hanoi, Vietnam. *J Clin Microbiol* 2008; 46: 996-1004.
16. Thakur N, Jain S, Changotra H, Shrivastava R, Kumar Y, Grover N, et al. Molecular characterization of diarrheagenic *Escherichia coli* pathotypes: Association of virulent genes, serogroups, and antibiotic resistance among moderate-to-severe diarrhea patients. *J Clin Lab Anal* 2018; 32(5): e22388.
17. Eklund M, Scheutz F, Siitonen A. Clinical isolates of non-O157 Shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. *J Clin Microbiol* 2001; 39: 2829-2834.
18. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 2000; 66: 4555-4558.
19. Sánchez S, Llorente MT, Echeita MA, Herrera-León S. Development of three multiplex PCR assays targeting the 21 most clinically relevant serogroups associated with Shiga toxin-producing *E. coli* infection in humans. *PLoS One* 2015; 10(1): e0117660.
20. DebRoy C, Roberts E, Fratamico PM. Detection of O antigens in *Escherichia coli*. *Anim Health Res Rev* 2011; 12: 169-185.
21. Bardbari AM, Arabestani MR, Karami M, Keramat F, Aghazadeh H, Alikhani MY, et al. Highly synergistic activity of melittin with imipenem and colistin in biofilm inhibition against multidrug-resistant strong biofilm producer strains of *Acinetobacter baumannii*. *Eur J Clin Microbiol Infect Dis* 2018; 37: 443-454.
22. Ludwig JB, Shi X, Shridhar PB, Roberts EL, DebRoy C, Phebus RK, et al. Multiplex PCR assays for the detection of one hundred and thirty seven serogroups of Shiga toxin-producing *Escherichia coli* associated with cattle. *Front Cell Infect Microbiol* 2020; 10: 378.
23. Alikhani MY, Hashemi SH, Aslani MM, Farajnia S. Prevalence and antibiotic resistance patterns of diarrheagenic *Escherichia coli* isolated from adolescents and adults in Hamedan, Western Iran. *Iran J Microbiol* 2013; 5: 42-47.
24. Canizalez-Roman A, Flores-Villaseñor HM, Gonzalez-Nuñez E, Velazquez-Roman J, Vidal JE, Muro-Amador S, et al. Surveillance of diarrheagenic *Escherichia coli* strains isolated from diarrhea cases from children, adults and elderly at northwest of Mexico. *Front Microbiol* 2016; 7: 1924.
25. Khan A, Yamasaki S, Sato T, Ramamurthy T, Pal A, Datta S, et al. Prevalence and genetic profiling of virulence determinants of non-O157 Shiga toxin-producing *Escherichia coli* isolated from cattle, beef, and humans, Calcutta, India. *Emerg Infect Dis* 2002; 8: 54-62.
26. Flaherty KE, Grembi JA, Ramachandran VV, Haque F, Khatun S, Rahman M, et al. High-throughput low-cost nl-qPCR for enteropathogen detection: A proof-of-concept among hospitalized patients in Bangladesh. *PLoS One* 2021; 16(10): e0257708.
27. Harada H, Fujimori Y, Gomi R, Ahsan MN, Fujii S, Sakai A, et al. Pathotyping of *Escherichia coli* isolated from community toilet wastewater and stored drinking water in a slum in Bangladesh. *Lett Appl Microbiol* 2018; 66: 542-548.
28. Eybpoosh S, Mostaan S, Gouya MM, Masoumi-Asl H, Owlia P, Eshrati B, et al. Frequency of five *Escherichia coli* pathotypes in Iranian adults and children with acute diarrhea. *PLoS On*e 2021; 16(2): e0245470.
29. Delannoy S, Mariani-Kurkdjian P, Bonacorsi S, Liguori S, Ison SA, Fach P. Draft genome sequences of human-pathogenic *Escherichia coli* O26:H11 strains carrying the *stx2* gene only and circulating in France. *Genome Announc* 2015; 3(4): e00852-15.
30. Duriez P, Clermont O, Bonacorsi S, Bingen E, Chaventre A, Elion J, et al. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. *Microbiology (Reading)* 2001; 147: 1671-1676.
31. Blyton MD, Cornall SJ, Kennedy K, Colligon P, Gordon DM. Sex-dependent competitive dominance of phylogenetic group B2 *Escherichia coli* strains within human hosts. *Environ Microbiol Rep* 2014; 6: 605-610.
32. Spano LC, da Cunha KF, Monfardini MV, de Cássia Bergamaschi Fonseca R, Scaletsky ICA. High prevalence of diarrheagenic *Escherichia coli* carrying tox-

in-encoding genes isolated from children and adults in southeastern Brazil. *BMC Infect Dis* 2017; 17: 773.
33. Jarocki VM, Reid CJ, Chapman TA, Djordjevic SP. *Escherichia coli* ST302: Genomic analysis of virulence potential and antimicrobial resistance mediated by mobile genetic elements. *Front Microbiol* 2020; 10: 3098.
34. Shakya P, Barrett P, Diwan V, Marothi Y, Shah H, Chhari N, et al. Antibiotic resistance among *Escherichia coli* isolates from stool samples of children aged 3 to 14 years from Ujjain, India. *BMC Infect Dis* 2013; 13: 477.
35. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; 18: 268-281.
36. Yu F, Chen X, Zheng S, Han D, Wang Y, Wang R, et al. Prevalence and genetic diversity of human diarrheagenic *Escherichia coli* isolates by Multilocus sequence typing. *Int J Infect Dis* 2018; 67: 7-13.
37. Aworh MK, Kwaga JKP, Hendriksen RS, Okolocha EC, Thakur S. Genetic relatedness of multidrug resistant *Escherichia coli* isolated from humans, chickens and poultry environments. *Antimicrob Resist Infect Control* 2021; 10: 58.