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Original Article

Multi-locus sequence typing of *Escherichia coli* isolated from clinical samples in Jordan

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Abstract

Introduction: *Escherichia coli* (*E. coli*) is the major cause of extraintestinal infections in the urinary tracts and bloodstream in humans in the community and health care institutions. Several studies on the genetic characterization of *E. coli* among clinical and environmental isolates were performed and revealed a wide diversity of sequence types (STs). In Jordan, phenotypic and genetic features of *E. coli* were extensively studied but there is still a need to identify the STs that inhabit the community.

Methodology: In this study, multi-locus sequence typing (MLST) was performed on archived clinical *E. coli* isolates collected from different hospitals in Jordan and the identified STs were extensively analyzed.

Results: Genotyping of 92 *E. coli* isolates revealed 34 STs and 9 clonal complexes. The frequencies of STs ranged between 1 to 23 observations. The most frequent STs among *E. coli* isolates were ST131 (n = 23), ST69 (n = 19), ST998 (n = 7), ST2083 (n = 5), and ST540 (n = 4). These five ST accounted for up to 60% of the 92 *E. coli* isolates. Based on the MLST database, the STs reported in this work were world widely recognized in humans, animals, and in the environment.

Conclusions: This study has elaborated more knowledge about the genotypes of *E. coli* in Jordan, with recommendations for future studies to correlate its genotypes with virulence and resistance genes.

Key words: *Escherichia coli*; Jordan; UTI; MLST; Sequence type.

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Introduction

Escherichia coli is a universal inhabitant of the gut microbiome of mammals [1]. This intestinal acclimated bacterium is a rod-shaped, Gram-negative *Enterobacteriaceae* member and is acquired shortly after birth [2].

Escherichia coli (E. coli) is an opportunistic pathogen that can cause a wide spectrum of diseases, ranging from self-limiting to life-threatening intestinal infections [1]. Various symptoms caused by different serotypes of *E. coli* were characterized, including abdominal, pulmonary, skin, and soft tissue infection symptoms, in addition to newborn meningitis and bacteremia [2].

Urinary tract infection (UTI) caused by uropathogenic *E. coli* (UPEC) is the most frequent laboratory-confirmed bacterial infection in medical practice, particularly among sexually active young females [1,3]. Moreover, UPEC is responsible for 70-95% of endogenous community-acquired UTI and 50% of all hospital-acquired UTI [1]. In some cases, UTI due to *E. coli* can progress to bacteremia that might be associated with a significant mortality rate [2].

E. coli has a pliable genome that is easily altered or manipulated naturally or artificially. This genetic plasticity confers a huge diversity of the bacterium, in terms of bacterium-host relationships, virulence, and antimicrobial resistance [4]. Accordingly, several genotyping schemes were developed and applied to

describe the molecular epidemiology of *E. coli* in different regions and among various communities. The genotyping schemes include BOX PCR fingerprinting [5], pulsed-field gel electrophoresis [6], restriction fragment length polymorphism [7], 16S RNA sequencing [8] next-generation sequencing [9], and whole genome sequencing [10].

Multilocus sequence typing (MLST) of *E. coli* was guaranteed as a powerful discriminatory approach to study and understand the population biology of *E. coli* [5]. It is a sequencing-based method that analyzes seven housekeeping genes and joins them into an allelic profile assigned as sequence type (ST) via an electronic online database [11]. The genetic relatedness between the identified STs can be electronically compared with each other, as well as, with other STs identified worldwide and closely related STs can be grouped as clonal complexes [12,13].

The clinical value of MLST was confirmed as a promising molecular epidemiological tool [5]. Its discriminatory ability among bacteria of the same species, ease of operation, and low cost give this approach a favorable advantage over other genotyping methods [11]. In addition to its ability to compare STs retrieved from different countries easily through the online MLST database. Furthermore, PubMLST, which is a public database that contains data on allele sequences and profile definitions for MLST schemes for various microbial genome hosted on various sites, enables the ability of rapidly comparing STs over the Internet. This procedure overcame the need to ship bacterial isolates and to run sequencing experiments again which consumes time and resources [14]. Several studies have inspected the STs of *E. coli* isolated from human, environmental, and animal samples and tracked their existence in various communities [15-17].

In Jordan, *E. coli* was studied extensively; its antibiogram, resistance, and virulence genes were recognized among populations, in the environment, food, and animals [18-22]. Few data are available about the molecular epidemiology of *E. coli* in this endemic country. Therefore, this is the first study that applied MLST for the genetic characterization of *E. coli* isolated from different types of clinical samples in Jordan.

Methodology

Source of Clinical E. coli isolates

A total of 113 *E. coli* clinical isolates were provided by the faculty of medicine/ The Hashemite University. More than 90% of those bacterial isolates were collected from urine samples, while the remaining were collected from blood, sputum, and wound samples. These isolates were previously collected since February 2017 archived in glycerol tubes and stored under deep freeze (-70 °C). Only a single isolate from each patient was obtained to preserve the assumption of independence of observations and to avoid repetition. *E. coli* isolates were identified previously by standard microbiological procedures including culture on MacConkey agar, Gram stain, and manual biochemical tests including citrate, indole, methylred, and Voges-Proskauer tests. Species confirmation was carried out using Vitek 2 compact system using a Gram-negative identification card (BioMerieux, France) [23].

Reactivation of E. coli

In this study, the archived bacteria were reactivated by subculturing onto MacConkey's agar (Oxoid Ltd., UK) and were incubated overnight in at standard atmosphere at 37 ºC. Out of 113 stored isolates, 92 isolates were recovered. Using a wire loop, 3-5 *E. coli* colonies were collected from each agar plate and were sub-cultured into 10 ml of Muller-Hinton broth (Oxoid Ltd., UK) and were incubated overnight at 37 ºC. Dense bacterial suspensions were achieved and were centrifuged for 1 minute; the supernatant was discarded and the bacterial pellet was introduced for DNA extraction.

DNA extraction

DNA was extracted from bacterial cell pellets using DNeasy tissue kit (Qiagen Germany) following the manufacturer's protocol. DNA purity and concentration were assessed for every sample using NanoDrop UV-Vis spectrophotometer (Thermo Scientific Wilmington, DE).

Multi-locus sequence typing (MLST)

Polymerase chain reaction (PCR) was applied to perform Achtman MLST scheme that uses the following housekeeping genes: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate synthetase), and *recA* (ATP/GTP binding motif). The sequences of forward and reverse primers and their details were described previously [24] and are available at enterobase online MLST database accessible at https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html. PCR primer sequences are available at http://web.mpiib-berlin.mpg.de.

The amplification was carried out using QIAamplier thermocycler (Qiagen, Germany) in 50 µL

amplification reaction mixture that comprised 2 µL of 40ng/µL of DNA, 4 µL of each primer (25 pmol/µL) (Macrogen Inc., Rockville, MD), 25 µL 2 × PCR master mix with standard buffer (One Taq, Quick -Load) and 15 µL nuclease-free water (Bioline Ltd UK). The reaction conditions were an initial denaturation step at 94 °C for 2 minutes, followed by 30 cycles of the following thermal conditions: denaturation at 94 °C for 1 minute, 1 minute primer annealing at 54–60 °C, and extension at 72 °C for 2 minutes, with a final extension step at 72 °C for 5 minutes. The presence of the correct size PCR product was confirmed by agarose gel electrophoresis attached to the gel-documentation system. Amplicons from seven housekeeping genes were purified and sequenced via a commercially available service (Macrogen Inc., Rockville, MD).

Raw data processing

An alignment of the forward and/or reverse primers raw products was done against reference sequences of *E. coli* MG1655 using Bioedit sequence alignment editor software version 7.2.5. This reference strain is known to be genetically stable and has a few mutation rates [25]. For each isolate, the locus number, sequence profile, and the corresponding ST were designated by functional options of the MLST websites: https://pubmlst.org/organisms/escherichia-spp; https://enterobase.warwick.ac.uk/species/index/ecoli).

Table 1. Sequence types data.

Sequence type	*Clonal complex	Number of isolates	Sample source
10	ST10 Cplx	1	Urine
38	ST38 Cplx	1	Urine
43	ST10 Cplx	1	Urine
58	ST155 Cplx	1	Urine
69	ST69 Cplx	19	17 urine, 2 blood
90	ST23 Cplx	1	Urine
92		1	Urine
131	ST131 Cplx	23	19 urine, 2 blood, 2 wound
162	ST469 Cplx	1	Urine
405	ST405 Cplx	3	2 urine, 1 sputum
410	ST23 Cplx	1	Urine
424		1	Urine
450		1	Urine
469	ST469 Cplx	2	Urine
485		1	Urine
540		4	3 urine, 1 blood
597	ST69 Cplx	1	Urine
998		7	6 urine, 1 blood
1136		1	Urine
1485	ST648 Cplx	1	Urine
1664		3	Urine
2020		1	Urine
2083		5	Urine
2085		1	Urine
2278	ST131 Cplx	1	Urine
2432		1	Urine
3429	ST38 Cplx	1	Urine
3471		1	Urine
3877		1	Urine
4148		1	Urine
4553	ST648 Cplx	1	Urine
7560		1	Urine
8188		1	Urine
11111		1	Urine

*Clonal complexes: Clonal complexes are STs that match a central ST at four or more loci unless they more closely match another central ST.

Descriptive statistical quantities

Basic statistical quantities such as the number of alleles, the number of variable polymorphic sites per allele, and the rate of single nucleotides polymorphism (SNPs) in each locus were calculated and displayed using functional options in the online analysis plugin of PubMLST for *Escherichia* spp. available at: https://pubmlst.org/organisms/escherichia-spp.

Phylogenetic analysis

Relatedness among isolates was estimated based on two principles: differences in allelic profiles using PhyloViz 2.0 software, which applies goeBURST algorithm [26], and differences in the concatenated sequence of alleles at all loci using molecular evolutionary genetics analysis version-X (MEGA X) software [27]. All STs were uploaded into PhyloViz software to display the relatedness among the STs obtained in this study. Sequences of every allelic profile were joined in the order of loci used to define the allelic profile to achieve a concatenated sequence of 3414 bp. The topology and grouping of all STs retrieved from this study were displayed on the constructed phylogenetic tree using the Maximum Likelihood method and Tamura-Nei model in MEG X software.

Ethics statement

Ethical approval was obtained from the Hashemite University Institutional Review Board (HU-IRB No. 4/5/2020/2021).

Results

Out of 113 samples, 92 bacterial isolates were recovered and genotyped. Thirty-four STs and 9 clonal complexes were identified, with frequencies ranging between 1 to 23 observations. The most frequent STs among *E. coli* isolates were ST131 (n = 23), ST69 (n = 19), ST998 (n = 7), ST2083 (n = 5), and ST540 (n = 4). These five ST accounted for up to 60% of the 92 *E. coli* isolates typed by MLST (Table 1).

Among the obtained STs, the number of alleles per locus varied from 12 to 19. Nucleotide polymorphism was observed at all seven loci, with the number of SNPs ranging from 27 to 98, while the number of variable

Table 2. Basic statistical quantities of the defined loci.

Locus	Analyzed nucleotides No.	[1]Alleles No.	[2]Polymorphic sites	[3]SNPS No.	[4]SNPs frequency
Adk	536	14	75	98	18.2%
fumC	469	17	54	92	19.2%
gyrB	460	19	71	96	20.8%
Icd	518	13	35	57	11.1%
Mdh	452	14	17	27	5.9%
purA	478	13	74	93	19.4%
recA	510	12	55	70	13.7%

[1]Number of different alleles for each locus. [2]Number of individual locations at which SNP occurred. [3]Whole number of all SNPs observed in all alleles for a given locus. [4]Rate of SNPs diversity in relation with locus length (no. of SNP/locus length).

sites within different alleles at the seven loci varied between 17 and 75. The polymorphism rate across all alleles ranged between 5.9% to 20.8% (Table 2). In addition, all STs identified in this study were previously described worldwide from different specimen sources.

Genetic relatedness among studied E. coli STs

All STs were clustered into three major groups that emerged from three major ancestors: ST90, ST10, and ST69 (Figure 1). STs that were identified in 52 isolates were clustered into a single major group and emerged from ST69 which represents the predicted founder. The highest number of STs (n = 11) directly emerged from ST10. Additional subgroup founders were also identified including ST1664, ST38, and ST3471.

The phylogenetic tree of the 3414 bp concatenated nucleotides has revealed genetic relationships among all 34 STs. Most STs formed unique sequences that differed by at least a single nucleotide. When a presumptive axis was drawn vertically along the whole tree to standardize the procedure of grouping of STs clusters, four groups of STs clusters (namely A, B, C, and D) were accomplished that differed in their width and number of STs they enclosed. Three STs (ST405, ST485, and ST2432) were reported as singletons with no shared original node (Figure 2).

Figure 1. PhyloViz diagram representing the relatedness between 34 STs identified in 92 isolates. Resampling for bootstrapping = 10,000; minimum number of identical loci for group definition = 6; minimum number of SLV for subgroup definition = 3.

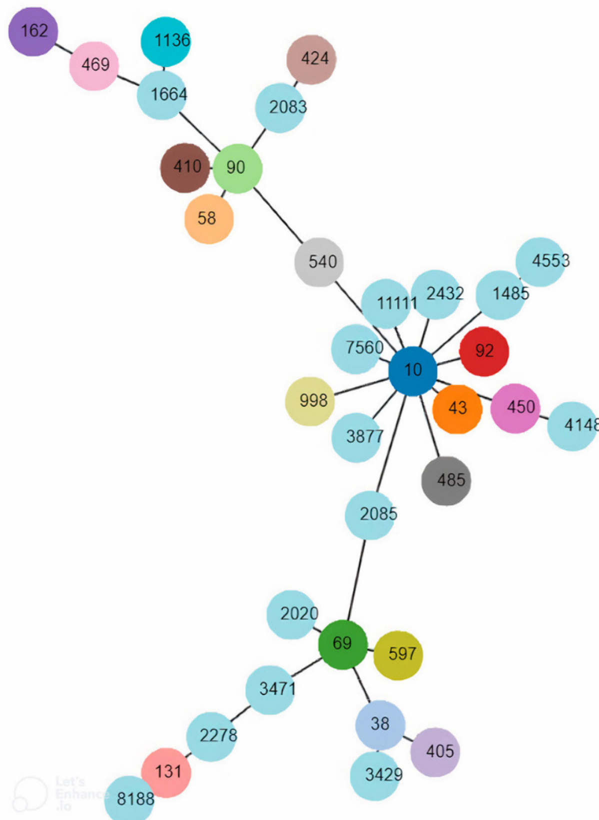
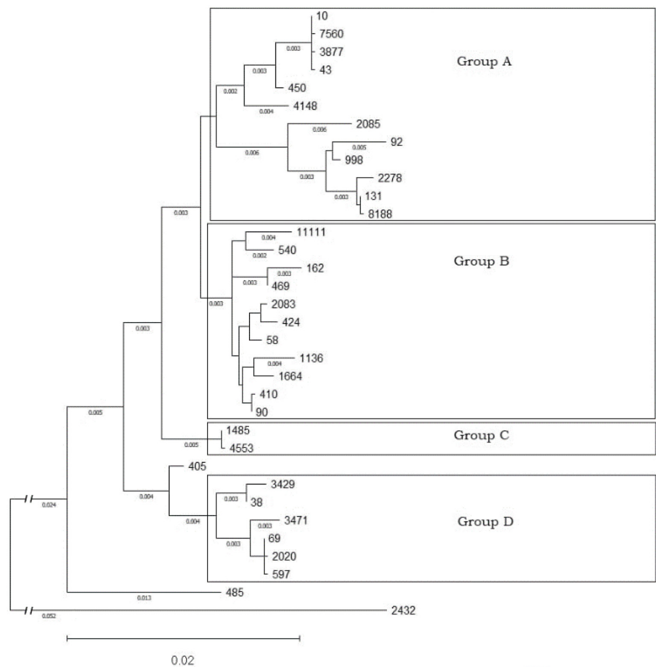


Figure 2. The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model.



The tree with the highest log likelihood (-8887.87) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 34 nucleotide sequences. There were a total of 3414 positions in the final dataset. Evolutionary analyses were conducted in MEGAX.

Several isolates distributed throughout the tree were influenced by the number of contained STs in each cluster and the rate of appearance of each ST among different isolates. Groups A and D harboured the largest number of isolates (40/92) and (25/92), respectively. Groups A and B enclosed more than half of the retrieved STs (24/34).

Discussion

E. coli is an opportunistic pathogen that is responsible for a wide spectrum of diseases involving various anatomical locations in the body with symptoms ranging from self-limiting to life-threatening [28]. Globally, the geographical distribution of clinical *E. coli* genotypes was described in a few countries, despite the recent revolutionary of molecular epidemiology schemes [5]. The population biology of clinical *E. coli* species is poorly understood and was not described enough in Jordan, and thus MLST was applied in this study to uncover the genetic diversity of this clinically significant pathogen.

The majority of the analyzed *E. coli* in this study was isolated from urine specimens. It is well known that community-acquired urinary tract infections are one of the most common bacterial infections encountered in laboratories with *E. coli* as the most common etiology that is responsible for up to 80% of all cases of community-acquired UTIs [28]. In general, urinary tract infections frequently result from endogenous transmission of intestinal *E. coli* into the bladder via the urethra or, to a lesser degree, from exogenous transmission of a distinct subgroup of pathogenic *E. coli* [16]

In the present study, genotyping was performed on clinical *E. coli* isolates using MLST to identify allele sequences and STs to better understand the molecular epidemiology of *Escherichia* spp. by describing their relatedness to each other and to explore the degree of their genotypic diversity among the population in Jordan. MLST of 92 isolates revealed 34 different STs that expressed different frequencies, predomination, and massive allelic heterogeneity. The overall diversity of ST found in the clinical isolates was (0.37 ST/isolate), which is comparable with the diversity ratio of (0.25 ST/isolate) and (0.34 ST/isolate) reported in England [1] and in the United States of America [16], respectively.

Several molecular epidemiology studies on pathogenic and commensal *E. coli* of human and non-human sources reported genotypic diversity with or without predominance of certain genotypes among single population communities of Brazil [29], Nigeria [17], China [30], England [1], USA [16], and Thailand [31].

The diversity and relative frequencies of STs identified in this study may be attributed to the historical introduction and dissemination of different *E. coli* STs among the study population or due to clonal expansion of local STs [1]. In addition, five STs (ST131, ST69, ST998, ST2083, and ST540) accounted for up to 60% of the *E. coli* isolates in this study which refers to the presence of genotypic predomination, this

Table 3. Features of selected sequence types recovered from previous literatures that were similar to those retrieved from this study.

Country	Total no. of isolates/STs	Shared STs	Source	Pathogenic/ Commensal	Reference
Nigeria	30/10	131	Human/animals	Commensal	[17]
		58	Animals	Commensal	
		405	Human/animals	Commensal	
		410	Animals	Commensal	
USA	90/31	10	Human	Pathogenic: UTI, Commensal	[16]
		38	Human	Pathogenic: UTI, Commensal	
		69	Human	Pathogenic: UTI, Commensal	
		131	Human	Pathogenic: UTI, Commensal	
England	88/22	69	Human	Pathogenic: UTI, Bacteremia	[1]
		131	Human	Pathogenic: UTI, Bacteremia	
		410	Human	Pathogenic: UTI	
Thailand	212/66	10	Human, Swine	Commensal	[31]
		131	Human	Commensal	
		410	Swine	Commensal	
Canada	600/52	10	Human/Animal food	Pathogenic: UTI, Commensal	[32]
		38	Human/Animal food	Pathogenic: UTI	
		58	Human/Animal food	Pathogenic: UTI	
		69	Human/Animal food	Pathogenic: UTI, Commensal	
		92	Human	Pathogenic: UTI, Commensal	
		131	Human/Animal food	Pathogenic: UTI	
		405	Human	Pathogenic: UTI	
USA	74/40	131	Animals	Pathogenic: UTI	[33]
Spain	92/56	10	Human	Pathogenic: UTI	[34]
		131	Human	Pathogenic: UTI	

can be attributed to the widespread endemicity of these STs throughout the hospital and community population that become rich areas for infection by these STs [1]. For example, the major predominant STs in this study were ST131 and ST69 which were also endemic in several countries and communities [1,16,17,31,32].

In a study conducted by Woksepp *et al. E. coli* ST131 was recognized as the etiology of nosocomial outbreak in Kalmar County Hospital, Sweden. The isolates were found to have an unusual antibiogram for that geographical region; all having resistance to ciprofloxacin and gentamicin but sensitive to trimethoprim-sulfamethoxazole. The majority of the isolates were collected from urine samples from elderly patients [3].

In accordance with the global MLST database, all STs reported in this study were already identified by previous studies in many geographical regions of various climate conditions and among several population communities of different ethnic groups (Table 3). This finding suggests that commensal *E. coli* global evolutionary mechanisms of allelic profiles are established continually inside the hosts regardless of their locations and features [29]. For example, ST131, ST69, and ST998 were predominantly reported in several countries and from different clinical and environmental sample types, which concur with our findings in this study.

At the molecular level, heterogeneity was assessed by exploring the traits of the entire composition of alleles and loci sequences by checking the SNPs and their features among all 34 STs. The substantial diversity of STs contradicted the limited alleles diversity that proposed an increased recombination replacement incidence relative to substitution mutation in *E. coli* [35,36]. These molecular events would explain the reassortment of existing alleles and the generation of different STs. This diversity of genetic makeup reflects *E. coli* genome dynamicity with generations [35]. Diversity of STs with limited variation of alleles was reported in many MLST studies performed on *E. coli* of different sources [17,31,37].

The established population snapshot on PhyloViz software showed that 11 STs were clustered into a major single group emerged from ST10 and three subgroup founders ST1664, ST38, and ST3471. This pattern might be attributed to numerous single locus variant diversification caused by recombination and point mutation events that yielded different STs [38]. The existence of sub-group founders also reflected minor expansion of ST1664, ST38, and ST3471 that may expect future clonal expansion if more consecutive recombination events occurred. It was postulated that linkage equilibrium between the alleles at altered loci in the population leads to recombination replacement which resulted in clonal and sub-clonal expansion of STs [38].

The evolutionary dendrogram showed high genetic diversity among the concatenated sequences of the 34 *E. coli* STs; majority of the STs formed unique sequences that varied by at least one SNP and therefore were clustered into four groups and a few STs were monotones. Several studies reported clear genetic relatedness between the revealed STs and their gathering into groups with minority of monotones that indicated geographical localization of major STs in the studied area [1,29,33,34,39].

Conclusions

This study has expanded the data for *E. coli* in Jordan and provided insights into its molecular epidemiology. The result of this study concurs with previous reports concluding that *E. coli* STs are prevalent and extremely varying across the population communities. For future studies, this study recommends correlating STs of *E. coli* discovered in Jordan with bacterial virulence and resistance genes.

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Authors' contributions

AMZ led the whole project, analyzed the data, and drafted the manuscript, TM did the molecular part, DSh did bacteriology work, MAT provided the isolates, HMS and AZ helped in manuscript writing, HAB and DAB had collected, identified, and archived the bacterial isolates.

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