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ORIGINAL STUDY

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Detection of Newly Identified Phylogenetic Lineages in Uropathogenic *Escherichia coli* Isolates in Iraq

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Abstract

Background: *Escherichia coli* (*E. coli*) strains have been classified into eight distinct phylogenetic clusters as per a novel quadruplex PCR method. However, the exact phylogenetic relationship among these bacterial lineages is still uncertain.

Objectives: The current study employed the Clermont phylotyping method to enhance the understanding of phylogenetic clusters of E. coli and evaluate antibiotic resistance in uropathogenic *E. coli* (UPEC) strains in Iraq.

Materials and Methods: A total of forty-two UPEC isolates were evaluated for antibiotic susceptibility using a disk diffusion test, while the innovative Clermont phylotyping method was employed for the phylogenetic classification of the isolates.

Results: The results showed that different phylogroups were found at the hospitals in Babylon Governorate, Iraq. Phylogroup B2 was the most common, accounting for 47.61% of the samples. It was followed by Clade I (14.28%), B1 (11.90%), A (9.52%), D (4.76%), C (2.38%), and an unidentified phylogroup (9.52%). Furthermore, 37 (88.09%) and 5 (11.90%) of the 42 uropathogenic E. coli isolates under study exhibited multidrug resistance (MDR) and extensive drug resistance (XDR), respectively. There are many MDR and XDR UPEC isolates within phylogroup B2, as evidenced by the fact that MDR and XDR strains within the group accounted for 17 out of 37 cases (45.24%) and 3 out of a total of 5 cases (60%), respectively. Additionally, two new phylogroups were discovered, called C and clade I, which are connected to cryptic E. coli and E. coli sensu stricto, respectively.

Conclusion: Therefore, more research must be done to better understand the characteristics of antibiotic resistance as well as the prevalence of various phylogroups in Iraq.

Keywords: *Escherichia coli*, Phylogeny, Phylogroups, Quadruplex PCR, UTIs

1. Introduction

Urinary tract infections (UTIs) are a group of bacterial infections caused by antibiotic-resistant uropathogens, such as Escherichia coli (E. coli), Klebsiella pneumoniae (K. pneumoniae), Proteus mirabilis (P. mirabilis), Enterococcus faecalis (E. faecalis), and Staphylococcus saprophyticus (S. saprophyticus) [1]. Escherichia coli remains the predominant species responsible for these infections [2], comprising approximately 80% of community-acquired urinary tract infections [3].

Urinary tract infections (UTIs) are currently a significant worldwide health issue, and many countries consider them an ongoing problem that poses a threat to public health [4]. Although UTIs typically do not show any symptoms, multiple studies have established a connection between UTIs and

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various pathological outcomes, including end-stage kidney disease. The mainstay of the conventional treatment approach for urinary tract infections is antibiotic therapy, which can be given either as targeted antimicrobial chemotherapy or as empirical antibiotic chemotherapy. In order to evaluate antibiotic resistance and effectiveness against the infecting microorganism, potential antibiotics are examined after the specific causative agent has been identified in customized antimicrobial therapy [5, 6]. The commonly prescribed antibiotics for both UTIs acquired in the community and in the hospital include β-lactam antibiotics, fluoroquinolones, cotrimoxazole, nitrofurantoin and fosfomycin [7, 8].

Escherichia coli strains classified as uropathogenic (UPEC) are distinct from commensal strains of the same genus because they affect extraintestinal tissues pathologically [9]. It is known that UPEC strains have a variety of virulent traits, such as extra genetic material that increases pathogenecity [10]. As the primary cause of UTIs, UPEC strains are responsible for complicated and non complicated cases [11]. Presently, there is increasing evidence that UTI-associated UPEC isolates are becoming more resistant to commonly prescribed antibiotics [12–15].

The primary, overarching theme in the biological sciences is evolution. Given that medicine is founded on fundamental biological principles, it is unquestionably an exception. It's interesting to note, however, that very few medical research studies offer an evolutionary analysis of the subjects they examine [16]. As such, there is currently a pressing need for information to address biomedical issues [17].

The emergence of new infectious agents has always posed a potentially deadly crisis for humanity [18]. This remains a significant public health concern, placing strain on the global economy and health institutions. Phylogenetics is a field that helps us understand the evolutionary relationships within microbial populations. It does this by developing frameworks based on molecular evolution, deducing patterns of diversification, and tracking evolutionary changes. Phylogenetics is particularly useful for analyzing the emergence and evolution of both existing and new microbial species and subspecies. Phylodynamic analysis utilizes the interaction between epidemiological models and phylogenetic inferences to enhance and broaden our comprehension of disease spread, transmission patterns, and the influence of human activities on the evolution of infectious pathogens. Escherichia coli is a Gram-negative, rod-shaped, anaerobic, flagellated, non-spore-forming, genetically versatile bacterial species [19].

Although most *E. coli* strains are harmless and naturally reside in the gastrointestinal tract as part of the normal microflora, some have developed into pathogenic strains that can cause diseases either within or outside the intestines [20], leading to additional diseases outside the intestines, such as cystitis [21], pyelonephritis [22], pneumonia [23, 24], meningitis [25, 26], neurological infectious lesions [25], urinary tract infections, peritonitis [27], and septicemia [22]. It also causes intestinal diseases, including enterohemorrhagic and diarrheagenic illnesses (such as traveler's diarrhea) [28]. Microbiologists primarily relied on conventional, phenotype-centered methods for classifying bacteria. Since the genomic era began, the method by which evolutionary microbiologists classify microorganisms into taxonomic groups has undergone significant changes [29].

Comparative genomics has shown that molecular phylogenetics, can reveal the molecular differences that identify an organism based on its physical characteristics [30]. E. coli has been categorized both genotypically and phenotypically in recent years [31, 32]. However, a research has shown that phenotypic classification of E. coli is unreliable because it cannot distinguish the cryptic lineages [30]. Nevertheless, cryptic strains have been taxonomized into five distinct monophyletic taxa, known as Escherichia clades I–V [30, 33]. Clermont and associates have been developing a multiplex rapid PCR assay for typing the genus, Escherichia since 2000. To classify E. coli isolates into one of the eight phylogroups A, B1, B2, C, D, E, and F, or E. coli sensu stricto—and clade I, an E. coli cryptic lineage—quadruplex PCR was introduced in 2013 and targets an additional gene called arpA in addition to three other genes, chuA, yjaA, and TspE4.C2 [34].

The phylogroup B2 is primarily responsible for the majority of virulent uropathogenic E. coli (UPEC) isolates that cause pathogenicity in extra-intestinal sites, with group D showing a minor prevalence. On the other hand, most commensal isolates belong to either group A or group B1 [7, 8, 35, 36]. The present study aimed to assess the effectiveness and feasibility of Clermont's novel scheme (2013) in phylogenetic typing of UPEC isolates native to Iraq. This is important because any alteration in phylogenetic typing plays a crucial role in increasing the accuracy of phylotyping, identification of emerging taxa, and development of new therapeutic agents. The current study sought to determine the resistance of the examined isolates to antimicrobial agents as well as to distinguish between the UPEC phylogroups using the novel technique developed by Clermont and colleagues.

Table 1. Phylogenetic markers with their primer sequences, amplicon sizes (bp) and PCR conditions.

The name of primer	Primer sequence (5′-3′)	Product size	Condition		
chuA	F:5-ATGGTACCGGACGAACCAAC-3 R:5-TGCCGCCAGTACCAAAGACA-3	288	94°C 94°C 59°C 72°C 72°C	4 min 5 sec 20 sec 5 min 5 min	1x 30x 1x
yjaA	F:5-CAAACGTGAAGTGTCAGGAG-3 R:5-AATGCGTTCCTCAACCTGTG-3	211	94°C 94°C 59°C 72°C 72°C	4 min 5 sec 20 sec 5 min 5 min	1x 30x 1x
TspE4C2	F:5-CACTATTCGTAAGGTCATCC-3 R:5-AGTTTATCGCTGCGGGTCGC-3	152	94°C 94°C 59°C 72°C 72°C	4 min 5 sec 20 sec 5 min 5 min	1x 30x 1x
arpA	F:5-AACGCTATTCGCCAGCTTGC-3 R:5-TCTCCCCATACCGTACGCTA-3	400	94°C 94°C 59°C 72°C 72°C	4 min 5 sec 20 sec 5 min 5 min	1x 30x 1x
arpA (groupE)	F:5-GATTCCATCTTGTCAAAATATGCC-3 R:5GAAAAGAAAAAGAATTCCCAAGAG	301	94°C 94°C 59°C 72°C 72°C	4 min 5 sec 20 sec 5 min 5 min	1x 30x 1x
trpA (groupC)	F:5-AGTTTTATGCCCAGTGCGAG-3 R:5-TCTGCGCCGGTCACGCCC-3	219	94°C 94°C 57°C 72°C 72°C	4 min 5 sec 20 sec 5 min 5 min	1x 30x 1x

2. Materials and methods

2.1. Diagnosis of bacterial strains

Two hundred male and female UTI patients at the Al-Hashimyah General and Al-Hillah General Teaching Hospitals in the Iraqi province of Babylon provided urine samples between February and June 2017. All of the patients ranged in age from five to sixty-nine. The standard microbial and biochemical protocols as previously described elsewhere were used to identify UPEC isolates [37].

2.2. Antibiotic susceptibility testing

The disk diffusion assay was employed to assess the antibiotic susceptibility patterns in UPEC isolates [8]. We purchased antibiotic discs of various names and concentrations from Conda, a company based in Spain. The following antibiotics were used in the study: TMP-SMX (5.250 μg), gentamicin (10 μg), ofloxacin (5 μg), nitrofurantoin (300 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), levofloxacin (5 mg), amikacin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefpodoxime (30 μg), cefepime (30 μg), doxycyclin (10 μg), imipenem (10 μg), meropenem (10 μg), ceftriaxone (30 μg), piperacillin-tazobactum (100/10 μg), amoxicillin-clavulanic acid (20/10 μg), and nalidixic acid (30 μg). The isolates were cultivated at a temperature of 45°C on Mueller-Hinton agar (MHA). Subsequently, they were transferred onto sterile plates and covered with antibiotic disks using aseptic techniques. Subsequently, the MHA plates were subjected to a 24-hour incubation period at a temperature of 37°C. The inhibition zones were interpreted according to guidelines of Clinical & Laboratory Standard Institute (CLSI) [38].

2.3. DNA extraction

Following the collection of 42 UPEC isolates, DNA was extracted using a commercial kit in accordance with the instructions supplied by the manufacturer, Geneaid (Presto TM Mini gDNA Bacterial Kit Protocol, Bioneer, Korea). Afterwards, specific DNA segments of interest were amplified using Polymerase Chain Reaction (PCR).

2.4. Molecular analysis

The Clermont *et al*. method [35] was utilized to evaluate the phylogroup distribution in UPEC isolates. Table 1 shows the primer sequences (Bioneer, Korea),

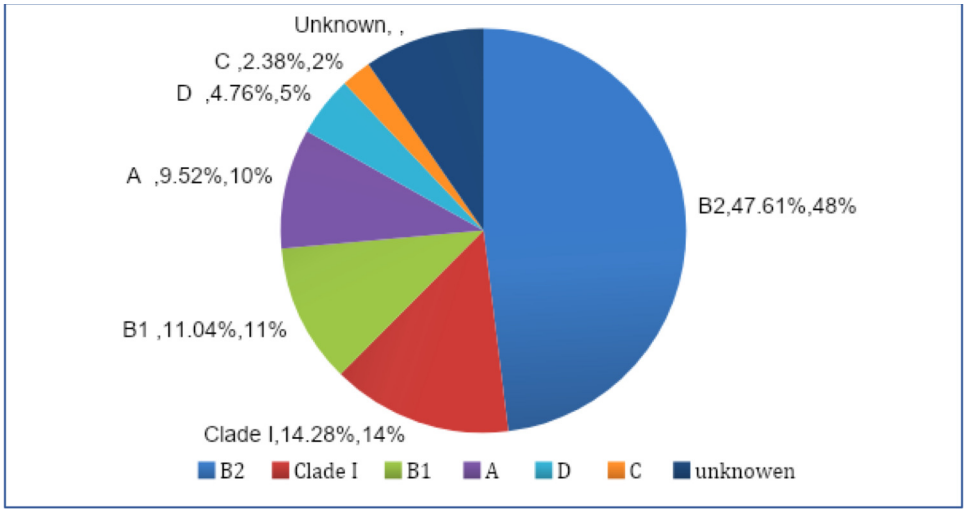

Fig. 1. The frequency of phylogroups in uropathogenic E. coli isolates.

the amplicon size in base pairs (bp), and the PCR conditions.

2.5. Statistical analysis

SPSS version 21 (SPSS Inc., Chicago, IL, USA) was used to statistically analyze the data. The Fisher's exact test and chi-squared test were employed to compare the categorical variables. Any P value < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Molecular analysis

The distribution of phylogroups in 42 UPEC isolates was assessed in the present study using the PCR method described by Clermont and associates [35]. Thirty eight isolates of UPEC were successfully grouped into six phylogenetic clusters, comprising group six, called Clade I, which is associated with E. coli cryptic strain, and D, C, B2, B1, and A, which are recognized as E. coli strain sensu stricto.As was previously mentioned, Clermont and colleagues created a quadruplex PCR by adding an additional gene called arpA to the three genes chuA, yjaA, and TspE4C2—that were utilized in the previous triplex PCR.

Seven phylogroups were produced as a result of this modification: trpA for phylogroup C and arpA for phylogroup E. These were identified by means of allele-specific PCRs [35].

The original Clermont's phylotyping technique is being applied for the first time in Iraq to categorize E. Coli isolates obtained from patient with urinary tract infections. The researchers utilized the tailored PCR technique developed by Clermont and colleagues to assess the distribution of phylogroups in forty-two UPEC isolates [35]. A total of thirty-eight UPEC isolates were effectively categorized into six distinct phylogenetic clusters, namely D, C, B2, B1, and A, which are recognized as E. coli strain sensu stricto. Additionally, there is a group referred to as Clade I, which falls under the E. coli cryptic strain.

Clermont and colleagues developed a quadruplex PCR by adding an additional gene, called arpA, to the three genes (chuA, yjaA, and TspE4C2) used in the previous triplex PCR. As a result of this alteration, two allele-specific PCRs were conducted, one for phylogroup C targeting the trpA gene and another for phylogroup E targeting the arpA gene. This led to the identification of seven phylogroups: F, E, D, C, B2, B1, and A [35]. According to Fig. 1 and Table 2, the most prevalent phylogenetic group among the forty-two UPEC isolates was B2, with a total of 20 isolates, representing 47.61% of the sample. Clade I was the second most abundant group, with 6 isolates, accounting for 14.28%. B1 had 5 isolates, making up 11.90% of the sample. Group A had 4 isolates, constituting 9.52%. Group D had 2 isolates, representing 4.76%. Group C had 1 isolate, accounting for 2.38%. Additionally, there were 4 isolates, representing 9.52% of the sample, that could not be assigned to any specific phylogroup.

The results of our study were consistent with another investigation conducted by [39], which reported the distribution of phylogenetic groups among human UPEC isolates as follows: phylogroup B2 (468), A (38), B1 (46), C (22), D (79), E (10), and two unidentified isolates. In addition, a different laboratory [40] found that phylogroup B2 was the most prevalent (34.0%), followed by phylogroup A. The distribution is as follows: B1 accounts for 12.9%, D accounts for 9.0%, C makes up 3.2%, and E makes up 2.5%,

Table 2. The distribution of different phylogenetic groups of uropathogenic E. coli isolates with specific genes through the extended quadruplex PCR.

No.	*arpA* 400 bp	*chuA* 288 bp	*yjaA* 211 bp	TspE4.C2 152 bp	Phylogroup	Group E	Group C	Phylogroup
1	−	+	+	+	B_2*			
2	−	+	+	+	B_2*			
3	+	−	−	−	A*			
4	−	+	−	+	B_2*			
5	+	−	−	−	A*			
6	+	−	+	−	A or C*		C^+	C
7	+	−	−	+	B_1*			
8	+	−	+	+	unknown			
9	−	+	+	−	B_2*			
10	−	+	−	+	B_2*			
11	+	−	−	+	B_1*			
12	+	+	+	−	E or Clade I	E^-		Clade I *E.coli* cryptic
13	−	+	+	+	B_2*			
14	−	+	−	+	B_2*			
15	+	−	−	+	B_1*			
16	+	−	+	+	unknown			
17	−	+	+	+	B_2*			
18	−	−	+	−	Clade I *E.coli* cryptic			
19	−	+	+	+	B_2*			
20	−	+	−	+	B_2*			
21	−	+	+	+	B_2*			
22	−	+	+	+	B_2*			
23	+	+	−	+	D or E*	E^-		D
24	+	+	+	−	E or Clade I	E^-		Clade I *E.coli* cryptic
25	−	+	+	+	B_2*			
26	+	−	−	+	B_1*			
27	−	+	−	+	B_2*			
28	−	+	+	+	B_2 *			
29	+	+	+	−	E or Clade I	E^-		Clade I *E.coli* cryptic
30	−	+	+	+	B_2*			
31	+	−	+	−	A or C*		C-	A
32	−	−	+	+	unknown			
33	+	+	−	+	D or E*	E^-		D
34	+	+	+	−	E or Clade I	E^-		Clade I *E.coli* cryptic
35	−	+	+	+	B_2*			
36	+	−	+	−	A or C*		C-	A
37	−	+	+	+	B_2*			
38	−	+	+	+	B_2*			
39	+	+	+	−	E or Clade I	E^-		Clade I *E.coli* cryptic
40	−	+	+	−	B_2*			
41	+	−	−	+	B_1*			
42	+	+	+	+	unknown			

*E. coli sensu stricto.

while accounting for 28.7%. In contrast, the current findings were consistent with those of [35], which examined two sets of E. coli samples obtained from fecal samples collected in Australia (373 strains) and France (293 strains) using the quadruplex method. The percentages of phylogroups among the strains in Australia were as follows: Clade I (0.3%), F (6.7%), E (2.9%), D (14.2%), C (0.8%), B2 (38.1%), B1 (18.8%), and A (18.2%). In France, the phylogenetic groups of strains were: A (28.7%), B1 (12.3%), B2 (34.5%), C (3.0%), D (9.2%), E (2.4%), F (9.6%), and Clade I (0.3%).

Furthermore, the results of the current investigation were consistent with those of a separate study [41] that identified the B2 phylogenetic group as the most prevalent, comprising 39.3% of the samples. The second most common group was categorized as unknown, accounting for 27.1%. The subsequent phylogroups were as follows: E, accounting for 9.3%; clade I and C, both comprising 6.4%; B at 5%; F and D, both reported to constitute 2.9%; with A, being the least common, constituting only 0.7%.The quadruplex PCR phylogrouping method provides numerous advantages when compared to the triplex PCR method. While quadruplex PCR phylogrouping has more advantages than disadvantages, it is unable to accurately assign a small fraction of isolates to the correct phylogroup. The reasons are as follows: Unassignable isolates may potentially

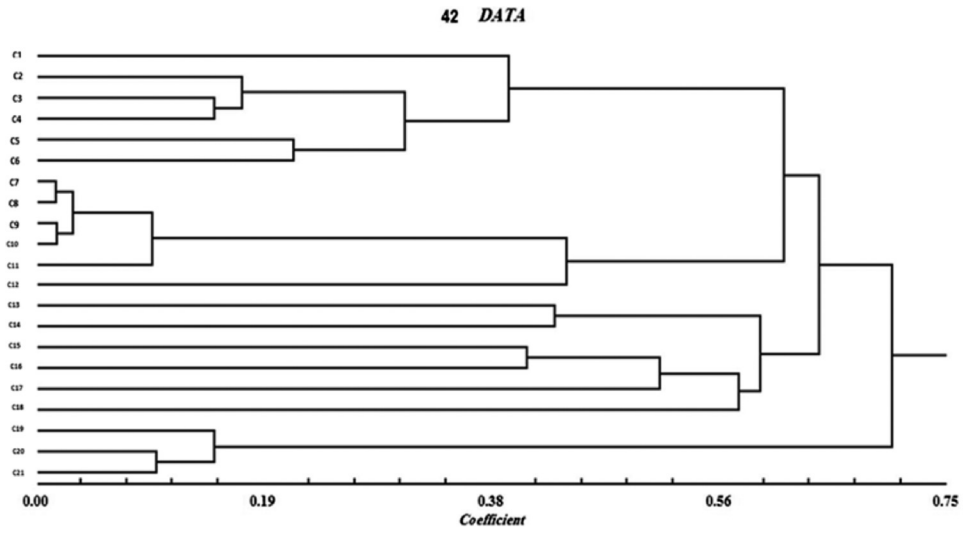

Fig. 2. The dendrogram showing phylogenetic relationships among UPEC isolates.

represent extremely rare phylogroups or result from extensive recombination events between two distinct phylogroups. Furthermore, this occurrence may be attributed to the limited conservativity of the E. coli genome, which is facilitated by the repetitive and cumulative mutations [35, 42].

Nevertheless, the recent findings diverged from the earlier findings reported by [43]. The previous study indicated that the distribution of phylogenetic groups among human isolates was predominantly composed of phylogroup A (54.3%), followed by phylogroup B1 (23.5%) and phylogroup D (13.6%). In contrast, phylogroup B2 was found to be scarce, accounting for only 3.7%. Furthermore, a study conducted by [44] revealed that phylogroup A was the most prevalent group (79.5%) among E. coli isolates. This was followed by phylogroup B1, phylogroup B2, phylogroup D, and phylogroup F, which accounted for 7.5%. 4.1%, 5.8%, and 0.7%, respectively. However, 2.4% of isolates were not classified to any phylogenetic group.

The variations in the proportion of phylogroups observed in this study and other scientific reports can be attributed to a multitude of factors, such as the physiological status and genomic makeup of the host, the host's nutritional style, environmental conditions, socioeconomic factors, geographic variations, and differences in sampling locations [45]. Furthermore, Mokracka and colleagues stated that varying distribution of phylogroups was associated with geographic factors, resistance to antibiotics, and the site of infection [46]. According to Manjarrez-Hernandez and colleagues [47], in Mexico City, a specific group of E. coli called clonal group A is responsible for causing urinary tract infections (UTI) and infections in areas outside the intestines. This group of E. coli plays a significant role in the development of resistance to trimethoprim/sulfamethoxazole. Moreover, clonal group A appears to be a significant constituent of multi-drug resistant (MDR) isolates obtained from patients with urinary tract infections (UTI). The phylogenetic distance between forty-two UPEC isolates was quantified, and the resulting phylogenetic reconstruction is depicted in (Fig. 1). Furthermore, (Fig. 2), (Fig. 3), (Fig. 4), (Fig. 5), (Fig. 6), illustrate the existence of distinct phylogenetic indicators in a total of forty-two UPEC isolates.

Table 3. The distribution of MDR and XDR isolates among different phylogroups in forty-two uropathogenic E. coli isolates.

UPEC phylogroups	No. of MDR (%)	No. of XDR (%)
B2	17 (45.24)	3 (60)
Clade I	6 (16.21)	0 (0.0)
B1	4 (10.81)	1 (20)
A	3 (8.10)	1 (20)
D	2 (5.40)	0 (0.0)
C	1 (2.70)	0 (0.0)
Unknown	4 (10.81)	0 (0.0)
Total	37 (100)	5(100)

3.2. Distribution of MDR and XDR among uropathogenic E. coli phylogroups

Table 3 displayed the distribution of XDR and MDR UPEC isolates across various phylogenetic groups. The prevalence of MDR isolates was highest in the B2 phylogroup (45.24%), followed by Clade I (16.21%), B1 (10.81%), unknown (10.81%), A (8.10%), D (5.40%), and C (2.70%). The distribution of XDR isolates was as follows: phylogroup B2 accounted for 60% of the isolates, followed by B1 with 20%, and A with 20%. This aligns with a study [63] that confirmed phylogroup

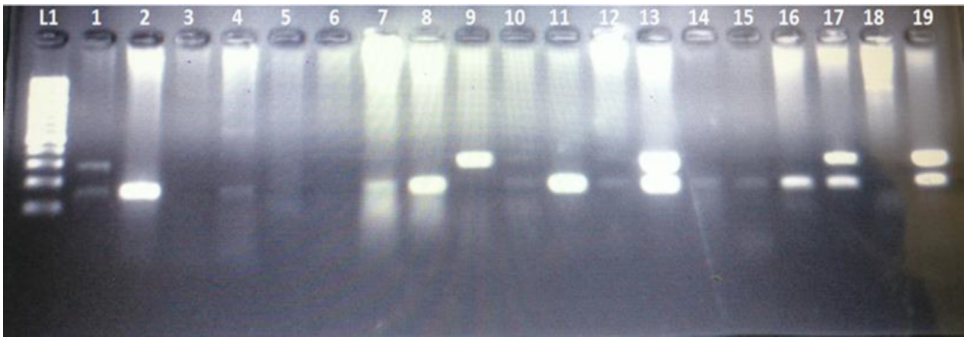

Fig. 3. Agarose gel electrophoresis was conducted at 70 volts within 50 minutes targeting chuA and TspE4C2 sequences, 152 bp.

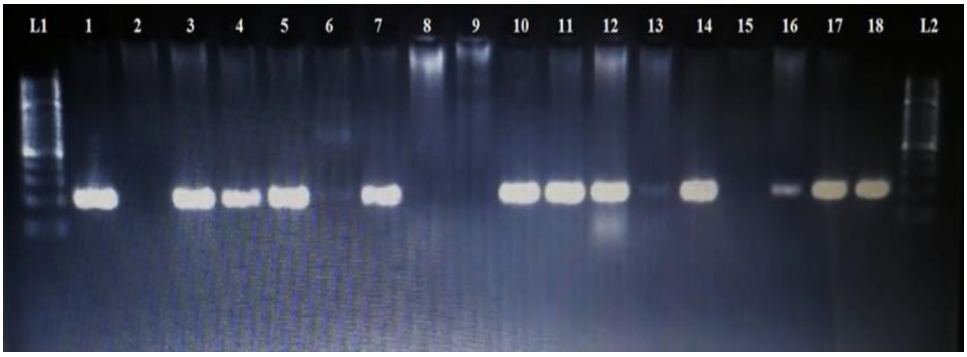

Fig. 4. Agarose gel electrophoresis was conducted at 70 volts for 50 minutes targeting yjaA sequence, 211 bp.

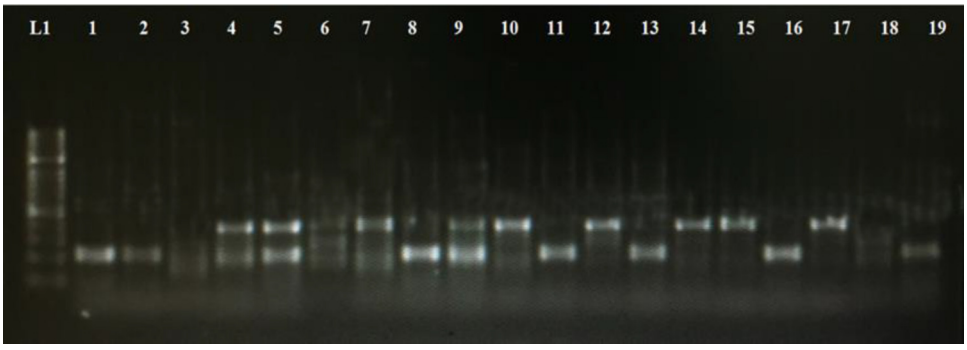

Fig. 5. Agarose gel electrophoresis was carried out at 70 volts within 50 minutes for arpA (400) and trpA (group C), 219 bp.

Fig. 6. Agarose gel electrophoresis was conducted at 70 volts for 50 minutes targeting arpA (group E, 301 bp.

B2 as the predominant group of XDR UPEC isolates in Mexico.

Nevertheless, the current findings contradicted a previous study [48] that documented the prevalence of multidrug-resistant uropathogenic Escherichia coli (MDR UPEC) across three phylogenetic groups as follows: phylogroup D accounted for 54.87%, B2 comprised 39.02%, and A constituted 6.09%. In addition, the proportions of XDR UPEC isolates were as follows: B2 (47.61%), D (42.85%), and A (9.52%).UPEC isolates within phylogenetic group B2 were found to possess both virulence factors and multidrug resistance [49, 50]. Furthermore, the MDR phenotype was observed specifically in phylogenetic groups A and D [51]. A significant rise in the XDR profile displayed by UPEC isolates has been recently reported, which greatly hampers the effectiveness of UTI treatment [52, 53]. The XDR and MDR isolates have been associated with phylogroups A, D, and B2 [51, 54].

4. Conclusion

In the present study, new phylogroups, namely C and clade I, were identified, linked respectively to *E. coli sensu stricto* and cryptic *E. coli*.

Acknowledgements

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Ethical approval

This research was approved ethically by both the Ethics Committee at the College of Medicine, University of Babylon, Iraq, and the Ethics Committee of the health directorate in Babylon governorate.

Conflicts of interest

There is no conflict of interest in this work.

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