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Genotypic analysis of *Escherichia coli* strains that cause urosepsis in the Aegean region

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Background/aim: The aim of this study was to characterize strains genotypically, to determine their phylogenetic relationships, to investigate the presence of the *papG* gene, and to compare their antibiotic susceptibility test results.

Materials and methods: Seventy pathogenic *E. coli* strains were isolated from both urine and blood cultures of patients with the preliminary diagnosis of urosepsis who were referred to the Ege University Faculty of Medicine, Bacteriology Laboratory of Medical Microbiology Department in İzmir. All of these strains were examined for the *pap*G gene and phylogenetic groups with the multiplex polymerase chain reaction technique. Pulsed-field gel electrophoresis and multilocus sequence typing (MLST) were used for epidemiologic analysis.

Results: Phylogenetically, it was found that 16 belonged to group B2, 31 belonged to group D, 15 belonged to group A, and 7 belonged to group B1. One strain was not identified as belonging to a group. *pap*G genes were found in 26 of 70 *E. coli* strains. Thirty urosepsis pathogenic *E. coli* strains were analyzed with MLST. Twenty-two strains were identified as new STs.

Conclusion: These findings are extremely important for Turkey and these new 22 strains should be investigated in more detail because they are new and have the potential to lead to infections.

Key words: *E. coli*, *pap*G, urosepsis, phylogenetic grouping, PFGE

1. Introduction

E. coli strains that cause diseases in systems other than the intestinal tract, such as the urinary or circulatory systems, are called extraintestinal pathogenic *E. coli* (ExPEC), and these strains can be divided into four groups: uropathogenic *E. coli* (UPEC), neonatal-meningitis–causing *E. coli*, avian pathogenic *E. coli* (APEC), and sepsis pathogenic *E. coli* (1–4). Although ExPEC strains are the most common cause of urinary tract infections, they might be the pathogens of sepsis, neonatal meningitis, and pneumonia. APEC strains that lead to respiratory tract infections and septicemia in birds and poultry, as well as other ExPEC strains, cause serious economic losses (5).

Most of the genes encoding the virulence factors of ExPEC strains are located in DNA regions called pathogenicity islands and are not found in commensal *E. coli* strains (6,7). ExPEC strains may colonize the urinary tract due to these virulence factors and can lead to serious infections, such as sepsis, by overcoming the host's defense. Among these virulence factors, the most studied adhesive organelles are type 1 and P pili, which are encoded by many UPEC strains. The expression of P pili is often associated with pyelonephritic UPEC isolates. A specific adhesin protein called *pap*G, which is localized at the distal tip of the P pilus, mediates bacterial adhesion to host cells (3,6,7).

Molecular epidemiological studies based on the multilocus sequence typing (MLST) have shown that *E. coli* strains are composed of quite diverse individual strains dispersed into different phylogenetic groups. Multilocus enzyme electrophoresis analyses have shown that most of the ExPEC strains belong to group B2 and some of them to group D, while most of the intestinal pathogenic and commensal *E. coli* strains belong to phylogroups A and B1 (8–11). MLST, which is considered the gold standard and is currently the best method for strain analysis, is widely used. MLST data also indicate that phylogroup B2, which comprises the majority of ExPEC isolates, represents the oldest lineage among the evolutionary species (11).

Bacteremia occurs when bacteria enter the circulatory system. If these bacteria in the circulatory system begin to reproduce, bacteremia progressively develops into septicemia. Sepsis, also known as systemic inflammatory response syndrome, is a serious clinical picture triggered

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by infection of the bloodstream (12,13). Urinary tract infections are common and as a result of inadequate treatment, the disease progresses, and more serious illnesses such as sepsis can occur (14,15).

Unless urgent, coordinated action is taken, the world is heading towards a postantibiotic era, in which common infections and minor injuries, which have been treatable for decades, can once again kill. Thus, the need for new drugs capable of blocking different mechanisms related to pathogenicity is increasing (16). In addition to virulence factors and pathogenic clones of ExPEC, strains can also vary regionally (17). To develop new drugs, pathogenic microorganisms should be comprehensively analyzed at the molecular level by taking geographical conditions into consideration. Due to the aforementioned reasons, this present study investigated the phylogenetic and genotypic diversity of *Escherichia coli* strains isolated from blood samples of urosepsis patients from different cities who were referred to the Ege University Faculty of Medicine, Bacteriology Laboratory of Medical Microbiology Department in Izmir and who were phenotypically identified in the same laboratory using the pulsed-field gel electrophoresis (PFGE), multiplex polymerase chain reaction (PCR), and MLST methods. The study investigated the presence of the *pap*G gene, an important virulence factor of uropathogenic *E. coli*, and compared the obtained findings to the antibiotic susceptibility results of the strains.

2. Material and methods

In the present study, 70 *E. coli* strains were isolated from both urine and blood cultures of patients with a preliminary diagnosis of urosepsis who were referred to Ege University Faculty of Medicine, Bacteriology Laboratory of Medical Microbiology Department in İzmir in 2013 and 2014 and who were phenotypically identified with automated systems (VITEK 2, bioMérieux, Marcy-l'Étoile, France) and subjected to more advanced molecular tests.

Of these patients, there were 51 from İzmir, 5 from Manisa, 5 from Balıkesir, 2 from Aydın, 2 from Muğla, 2 from Kütahya, 1 from Isparta, 1 from Denizli, and 1 from Elazığ.

2.1. DNA isolation

DNA isolation was performed as suggested by Liu et al. (2000) and the DNA was stored at –20 °C until further investigation (18).

2.2. Phylogenetic analysis

Phylogenetic grouping of all isolates was determined by a modified version of the multiplex PCR method as described previously (9). As distinct from the method used by Clermont et al. (9), we used different TSPE4C.2 primers (19). The primer sequences, the size of the amplified fragment (base pair), and the annealing temperature are shown in Table 1. Multiplex PCRs were performed using Fermentas Taq polymerase (Waltham, MA, USA). These assays were carried out according to the manufacturer's instructions.

2.3. Detection of the *pap*G gene

Flanking *pap*G primers were used to investigate the presence of the *pap*G gene (Table 1). *pap*G was detected by multiplex PCR with flanking primers as previously described (19,20).

2.4. MLST analysis

Based on the results of antibiotic susceptibility tests performed on the 70 *E. coli* strains, 30 *E. coli* strains were selected for the MLST analysis. Of these 30 selected strains, 25 tested positive for extended-spectrum beta lactamase (ESBL) and 5 were fluoroquinolone-resistant. The present study was carried out by modifying the standard protocol for *E. coli* (http://mlst.warwick.ac.uk/mlst/dbs//documents/primerscoli_html). Annealing temperatures and primer pairs used for these genes are listed in Table 1. For each gene, 2.5 µL of 10X reaction buffer, 2.5 µL of (each 2 mM) dNTP mix, 1.5 µL of the forward and reverse primer, 3 µL of $MgCl_2$, 0.25 µL of Taq polymerase (Fermentas) and 5 µL of DNA, for a total volume of 25 µL, were added.

Products of conventional and multiplex PCRs were electrophoresed in 1.5% and 2% agarose gel with 0.5 µg/mL ethidium bromide, respectively. Gels were photographed by using an ultraviolet transilluminator and digital capture system (DNr Bio-Imaging Systems Ltd., Jerusalem, Israel). The sizes of amplicons were determined by comparing them with a 100-bp DNA ladder (ABM Inc., Richmond, BC, Canada).

2.5. DNA sequence analysis

Prior to the DNA sequence analysis, PCR products were purified using Sephadex and Spin columns. PCR products cleaned with the Sephadex column were measured with the NanoDrop Spectrophotometer (Wilmington, DE, USA) in amounts suitable for DNA sequencing.

Then cycle sequencing was performed using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Waltham, MA, USA). In order to achieve this, 10 µL of mixture was prepared by adding 2 µL of DNA, 1 µL of primer, and 1 µL BigDye terminator v1.1, v3.1 5X Sequencing buffer to the BigDye Terminator v3.1 Cycle Sequencing Kit. For each gene, sequencing was performed with forward and reverse primers. The sequencing conditions were 1 min at 96 °C, 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C.

After being sequenced, the PCR products were cleaned again and transferred to a 96-well microplate and were analyzed using the ABI 3130 XL (ThermoFisher). Sequence results were entered into the MLST database and sequence type (ST) numbers were determined.

Table 1. List of the primers used in the study.

Genes	Primer sequences	Annealing	Size of product	References
adk F	5'-ATTCTGCTTGGCGCTCCGGG-3'	54 °C	583 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
adk R	5'-CCGTCAACTTTCGCGTATTT-3'			
fumC F	5'-TCACAGGTCGCCAGCGCTTC-3'	60 °C	806 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
fumC R	5'-GTACGCAGCGAAAAAGATTC-3'			
icd F	5'-ATGGAAAGTAAAGTAGTTGTTCCGGCACA-3'	60 °C	878 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
icd R	5'-GGACGCAGCAGGATCTGTT-3'			
gyrB F	5'-TCGGCGACACGGATGACGGC-3'	60 °C	911 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
gyrB R	5'-ATCAGGCCTTCACGCGCATC-3'			
mdh F	5'-ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG-3'	68 °C	932 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
mdh R	5'-TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT-3'			
purA F1	5'-TCGGTAACGGTGTTGTGCTG-3'	64 °C	816 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
purA R	5'-CATACGGTAAGCCACGCAGA-3'			
recA F	5'-CGCATTCGCTTTACCCTGACC-3'	60 °C	780 bp	http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html
recA R	5'-TCGTCGAAATCTACGGACCGGA-3'			
chuA F	5'-GACGAACCAACGGTCAGGAT-3'	59 °C	279 bp	Clermont et al., 2000
chuAR	5'-TGCCGCCAGTACCAAAGACA-3'			
YjA F	5'-TGAAGTGTCAGGAGACGCTG-3'	59 °C	211 bp	Clermont et al., 2000
yjA R	5'-ATGGAGAATGCGTTCCTCAAC-3'			
TSPE4. C2F	5'-AGTAATGTCGGGGCATTCAG-3'	59 °C	151 bp.	Clermont et al., 2000
TSPE4.C2 R	5'-TCGCGCCAACAAAGTATTACG-3'			
PapG I F	5'-CTGTAATTACGGAAGTGATTTCTG-3'	63 °C	1140 bp	Johnson et al., 1998
PapG I R	5'-TCCAGAAATAGCTCATGTAACCCG-3'			
PapG IIF	5'-CTGTAATTACGGAAGTGATTTCTG-3'	63 °C	1070 bp	Johnson et al., 1998
PapG II R	5'-ACTATCCGGCTCCGGATAAACCAT-3'			
PapG III F	5'-CATGGCTGGTTGTTCCTAAACAT-3'	63 °C	1070 bp	Johnson et al., 1998
PapG III R	5'-ACTATCCGGCTCCGGATAAACCAT-3'			

2.6. PFGE

PFGE was performed according to Durmaz et al. (21). Electrophoresis conditions were set as follows: 5 s initial pulse duration, 30 s ending pulse duration, 120° striking angle, 6 V/cm^2 current, 14 °C temperature, and 20 h duration. After electrophoresis, the gel was put into 500 mL of water containing 10 mg/mL EtBr, stained for 20 min and viewed under UV light (21,22).

2.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility and ESBL testing of 70 *E. coli* strains were performed with an automated method (VITEK 2, bioMérieux). The following drugs were tested: cefalotin (30 mg), imipenem (10 mg), cefepime (30 mg), ciprofloxacin (5 mg), ampicillin (10 mg), ceftazidime (30 mg), amikacin (30 mg), meropenem (10 mg), cefuroxime (30 mg), gentamicin (10 mg), amoxicillin/clavulanic acid

(30 mg), and trimethoprim/sulfamethoxazole (25 mg) (Oxoid, Basingstoke, UK).

3. Results

3.1. Phylogenetic grouping

Phylogenetic grouping was determined as described by Clermont et al. (9) and was as follows: 16 strains belonged to group B2, 31 belonged to group D, 15 belonged to group A, 7 belonged to group B1, and one strain was not identified as belonging to a group.

3.2. Detection of the *pap*G gene

*pap*G was detected by PCR in 26 of the 70 strains (Figures 1 and 2).

3.3. MLST

In the present study, 30 urosepsis pathogenic *E. coli* strains (25 ESBL-resistant and 5 fluoroquinolone-resistant) were analyzed with MLST. Of these 30 strains, 7 were identified as recognized STs (4 as ST131, 1 as ST405, 1 as ST476, and 1 as ST973). One strain was not registered in the database because it was allelically quite different. Because 22 strains did not resemble any of the STs entered into the database previously, they were identified as new STs and were registered into the database with new numbers. As a result of the MLST analysis, a minimum spanning tree (MST) indicating the relationship among the 29 samples was constructed using the pubMLST online system and is shown in Figure 3 and Table 2.

3.4. PFGE

In the PFGE analysis, the DNA of the *E. coli* isolates was cleaved with the XbaI enzyme and profiles having different numbers of fragments, ranging from 13 to 25, were identified. The bands were compared using the BIO-PROFILE Bio 1D ++ program. Based on the fact that the 70 *E. coli* strains were similar by more than 80% in the PFGE results, 65 different pulsotypes were obtained. Strains E81 and E82 have the same band profile and belong to the LIV pulsotype.

Figure 1. *pap*G gene results for E1–E53. K12: negative control, 2H16: positive control for *pap*G II, J96: positive control for *pap*G I and II, M: marker.

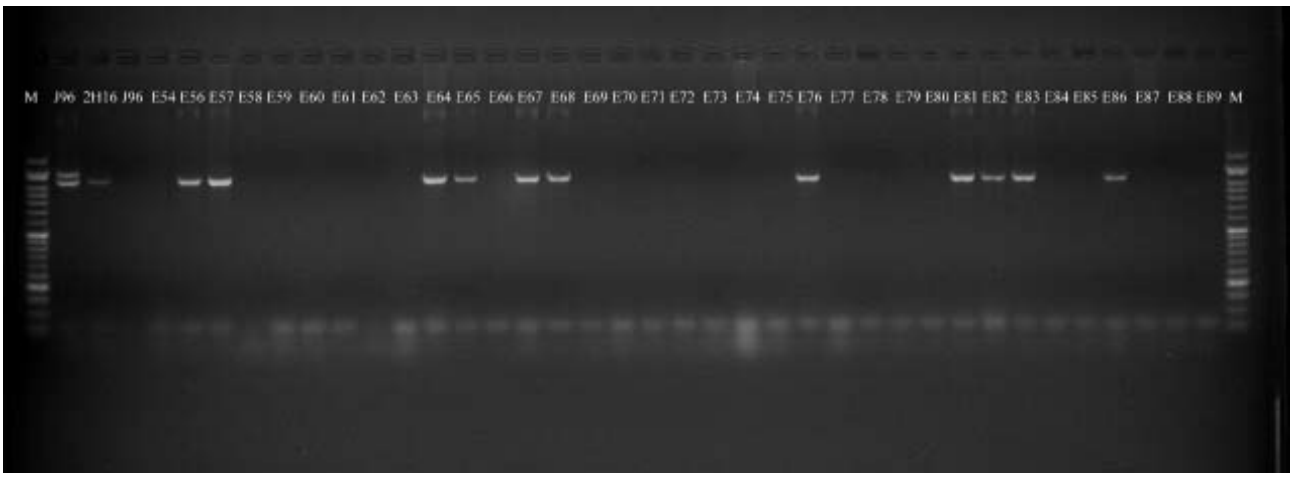

Figure 2. *pap*G gene results. M: marker, K12: negative control, 2H16: positive control for *pap*G II, J96: positive control for *pap*G I and III. E54–E89.

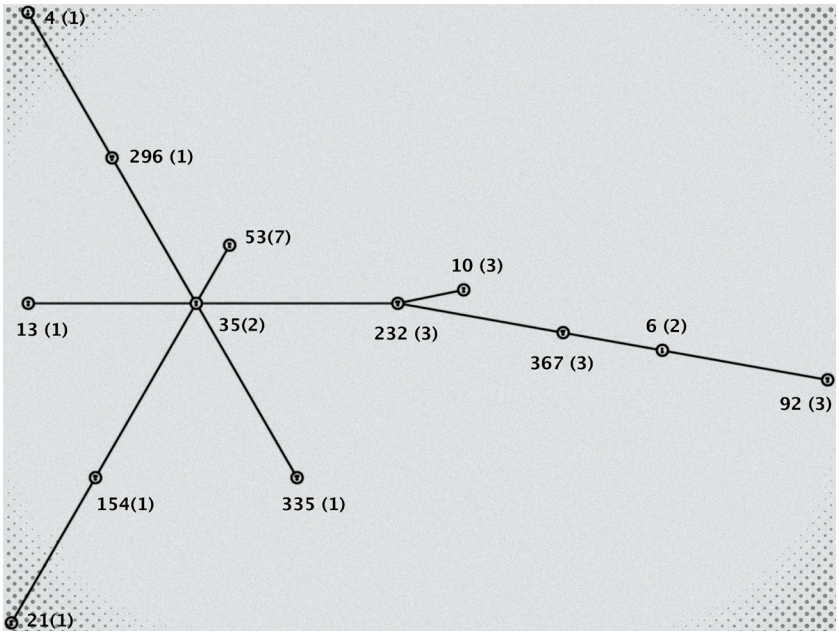

Figure 3. The minimum spanning tree of *E. coli* strains.

Type/subtype profiles and dendrograms obtained through the PFGE data analysis are shown in Figure 4.

3.5. Antimicrobial susceptibility test results

Of the 70 *E. coli* strains, 52 were resistant to ampicillin, 37 to trimethoprim/sulfamethoxazole, 25 to cefuroxime, 27 to ciprofloxacin, and 18 to amoxicillin/clavulanic acid. In addition, 25 strains were identified to produce ESBL.

4. Discussion

Phylogenetic analysis showed that intestinal *E. coli* and ExPEC strains were classified into four main groups: A, B1, B2, and D (23,24). ExPEC strains mostly belong to group B2 and, to a lesser extent, to group D, whereas intestinal and commensal *E. coli* strains belong to groups A and B1 (9,10,24–28). A UPEC strain whose clonal group was designated as A in the 1990s was then considered a dominant pathogen of multidrug-resistant urinary tract infections belonging to the phylogenetic group D in the United States (29,30). Yumuk et al. isolated 3108 ESBL-positive *E. coli* isolates from patients with urinary tract infections in Turkey, and after analyzing them phylogenetically they reported that 35% of the strains belonged to group B2, 35% to group D, and 35% to group A (31). Koga et al. reported that 10 *E. coli* strains isolated from human blood cultures belonged to group B1 (32). In the present study, of the urosepsis pathogenic *E. coli* strains, 44% belonged to group D, 24% to group B2, 23% to group A, and 9% to group B1, which is consistent with the findings of the aforementioned studies. The fact that some of the isolates in the present study belonged to groups A and B1 indicates that the strains in these groups can cause systemic infections.

P fimbriae are one of the most important virulence factors of ExPEC strains, which are common particularly in uropathogenic *E. coli* strains (33,34). In studies carried out to date, P fimbriae have frequently been detected in *E. coli* strains causing pyelonephritis. If pyelonephritis is not appropriately treated, the organism enters the bloodstream and can lead to sepsis and even more complicated diseases (35). Therefore, the presence of P fimbriae and all of its molecular properties should be investigated, and drugs specific to it should be developed if the disease is to be treated successfully (16). Johnson determined the presence of the *pap*G gene in 76% of the *E. coli* strains isolated from 75 sepsis patients (35). Moreno et al. investigated the presence of *pap*G and other virulence genes in *E. coli* strains, and determined that the *pap*G gene was present in 98 of the 150 isolates (65%) (36). Ramos et al. investigated the presence of the *pap*G gene in 143 *E. coli* strains isolated from sepsis and urosepsis patients with the primers specific to *pap*G II and *pap*G III alleles and determined the presence of *pap*G III in 80% of the 143 patients and *pap*G II in 40% (10).

In Turkey, Arısoy et al. investigated the presence of *pap*, *afa*, *sfa*, *hly*, *cnf*, and *aer* genes in pediatric *E. coli* isolates, and determined that the *pap*G gene was present in 23% of the isolates (37). In the present study, the presence of the *pap*G gene was determined in 26 of the 70 *E. coli* strains (37%). These results are different from the results of studies conducted abroad but are consistent with those of studies conducted in Turkey. Therefore, the difference in the number of virulence genes is thought to stem from regional variations.

Table 2. ST numbers of isolates and ST complex.

Isolate	ST	ST Cplx
E4	ST131	ST131 Cplx
E29	ST405	ST 405 Cplx
E38	ST476	ST12 Cplx
E42	ST5101	No ST Cplx
E43	ST5100	No ST Cplx
E48	ST5102	No ST Cplx
E50	ST131	ST 131 Cplx
E59	ST5105	No ST Cplx
E60	ST5106	No ST Cplx
E61	ST131	ST131 Cplx
E62	ST5104	No ST Cplx
E63	ST5103	No ST Cplx
E66	ST5107	No ST Cplx
E67	ST5108	No ST Cplx
E69	ST5109	No ST Cplx
E70	ST5110	No ST Cplx
E71	ST5111	No ST Cplx
E72	ST973	None
E73	ST5112	No ST Cplx
E74	ST5113	No ST Cplx
E75	ST5114	No ST Cplx
E77	ST5115	No ST Cplx
E78	ST5116	No ST Cplx
E79	ST5117	No ST Cplx
E84	ST5118	No ST Cplx
E85	ST5119	No ST Cplx
E86	ST5120	No ST Cplx
E87	ST131	ST131 Cplx
E88	ST5121	No ST Cplx

In order to interpret DNA fragments and to convert the interpretation into epidemiologically useful data, microbiologists should understand how PFGE patterns are compared and how random genetic events can change these patterns. Ideally, the PFGE patterns of isolates representing any pandemic strain should not be distinguishable from each other and should be significantly different from epidemiologically unrelated strains (38). Methods such as PFGE, which are based on electrophoretic band patterns, have some disadvantages, since their interlaboratory repeatability and reliability are limited. Laboratory results can be compared with improved analysis programs under standardized electrophoresis conditions. However, pulsotype database regarding the method is fairly limited. Given the results, MLST is a more reliable method than PFGE because it is based on sequence analyses. Its interlaboratory repeatability is higher. However, its cost is higher than PFGE (39). While PFGE reveals the relationship between the strains in the same area in detail, MLST can detect strains isolated from different regions and reveal the relationship between organisms. The concomitant use of PFGE and MLST helps us understand how bacteria species change (40). Therefore, in many studies performed so far, PFGE and MLST have been used together (31,41–43). In their study conducted in Iran, Anvarinejad et al. analyzed 90 *E. coli* strains isolated from children with urinary tract disease using PFGE and obtained 65 different PFGE profiles (44). Moulin-Schouleur et al. worked on 22 ExPEC strains of human origin and 16 ExPEC strains of animal origin with PFGE and determined 37 different PFGE band profiles. In addition, the PFGE profiles clearly revealed the difference between the animal and human ExPEC strains (45). Yumuk et al. determined 13 different electrophoretic patterns from 17 isolates using PFGE analysis (31). In the molecular epidemiologic study by Tartof et al., 19 of the 22 STs and 69 UPEC strains yielded identical results for all 7 of the gene regions analyzed with the MLST method, but these 19 strains showed 14 different band patterns with PFGE (42). Usein et al. analyzed 90 *E. coli* strains isolated from the vaginas of adult women using PFGE and found 59 different profiles (46). In Turkey, molecular studies conducted on ExPEC strains are very few. In one of these very few studies, Görgeç et al. analyzed 76 nosocomial *E. coli* strains producing ESBL with PFGE and determined 69 different genotypic profiles (47). Similar to these studies, in the present study, 70 urosepsis pathogenic *E. coli* strains themselves underwent diversification, and 65 different PFGE patterns were obtained from these 70 *E. coli* strains. In most of the PFGE analysis of ExPEC strains conducted so far, it has been observed that strains themselves diversified and had differences, which suggests that *E. coli* strains undergo quite a rapid evolution in terms of adaptation to their niches. In Turkey, our search for studies in which ExPEC strains were analyzed with MLST revealed a gap in the literature; there are no STs previously registered in the database from Turkey (http://mlst.warwick.ac.uk/mlst/dbs/ecol). In the present study, 22 new STs were identified with MLST. In Figure 4, the differences between the strains are clearly seen, and the species that have the same features in terms of the *adk* gene are cited in the same node. Even

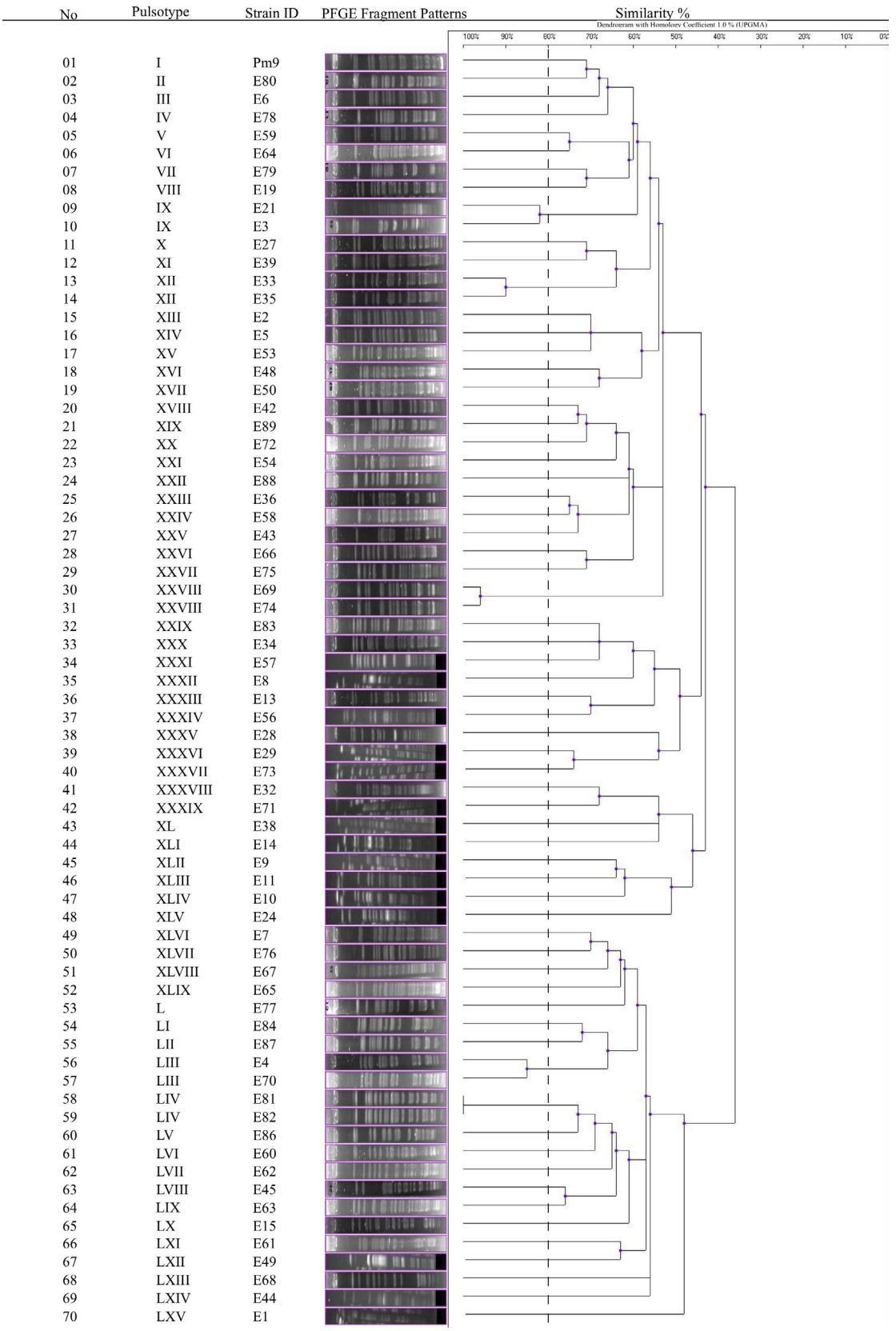

Figure 4. PFGE results and pulsotypes.

if the species having the same *adk* allele were different STs, they were placed in the same node. According to the MST, evolutionary distances between strains emerge depending on the allelic variations of genes other than *adk*. Because there are no studies on the MST in Turkey, the results were not compared regionally. However, while ESBL-positive strains are usually detected in ST131, this rate in Turkey is 13%, and 73% of them have been identified as new STs. This finding is extremely important for Turkey and these 22 strains should be investigated in more detail because they are new and have the potential to lead to infections.

In several studies carried out to date, a relationship has been observed between phylogenetic groups and resistance to quinolones (nalidixic acid, pipemidic acid), fluoroquinolones (ciprofloxacin, norfloxacin), trimethoprim/sulfamethoxazole, beta-lactams (ampicillin), extended-spectrum cephalosporins and cephamycins, and bacterial virulence factors.

These antibiotic-resistant strains have been reported to mostly belong to phylogenetic groups other than group B2 and to have virulence factors to a lesser extent (19,48–50). In this study, an investigation of the relationship between the *pap*G gene and ciprofloxacin and trimethoprim/sulfamethoxazole revealed that of the 26 *pap*G-positive strains, 7 were resistant to trimethoprim/sulfamethoxazole, and 5 were resistant to ciprofloxacin. Given the relationship between the *pap*G gene and ESBL positivity, only 3 of the 26 strains produced ESBL. Therefore, if the rate of virulence factors is lower in a strain, it develops antibiotic resistance; otherwise, it is less resistant to antibiotics. The results obtained in this present study are consistent with this view. The accelerating arms race between microbes and human beings threatens humankind more than it threatens microbes.

The proliferation of microorganisms that cannot be killed by even the most powerful antibiotics has the potential to return humankind to medieval conditions in terms of the fight against infectious diseases. Therefore, the antibiotic age should come to an end for the long-term, and the development of vaccines and new drugs should take priority. In order to achieve all these, genome maps of pathogenic microorganisms should be studied in detail, the details of virulence factors should be clearly defined, and typing and epidemiological distribution of virulence factors should be determined. The present study was carried out with this purpose in mind and is of importance because it may form the basis for future studies.

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