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SHEDDING LIGHT ON GENETIC RELATIONSHIPS, PLASMID PROFILES AND ANTIBIOTIC RESISTANCE PATTERNS OF CLINICAL *ESCHERICHIA COLI* STRAINS: PULSED-FIELD GEL ELECTROPHORESIS PERSPECTIVE

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ABSTRACT

In the present work, 47 *Escherichia coli* isolates collected from various clinical samples were elucidated with regard to genetic relationship, antibiotic resistance and plasmid profile. The genetic diversity of the isolates was defined by the pulsed field gel electrophoresis (PFGE) method. With a degree of similarity of 80%, the results from PFGE separated the *E. coli* strains into 39 different groups representing four subtypes. Antibiotic resistance patterns and extended spectrum of beta lactamase (ESBL) producing properties of the strains were determined by using a disk diffusion and double disc synergy method, respectively. According to the susceptibility test results, 36 distinct resistance profiles (**resisto type**) were observed among clinical strains. The strains were mostly resistant to ampicillin (100%) and this has been followed by cephalothin and ticarcillin/clavulanic acid with the ratio of 89.36%, cefuroxime with the ratio of 87.24%, tetracycline and cefotaxime with the ratio of 80.85%. It was observed that 24 of the strains (51.06%) were defined as ESBL positive. When *E. coli* strains were evaluated according to plasmid size, it was determined that 27 of 47 strains (57.44%) carried plasmids and the sizes of the determined plasmids were ranging between 77.1 kb and 1.6 kb. It was concluded that plasmids of *E. coli* strains are randomly distributed and no significant correlation was found between antibiotic resistance patterns and plasmids.

Keywords: Antibiotic resistance, ESBL, *Escherichia coli*, PFGE, Plasmid

INTRODUCTION

Escherichia coli, a bacterium that is still the focus of many studies today, is a model microorganism for genetic studies. We suppose that this may be one of the reasons why *E. coli* is studied so often. The ability to successfully colonize such a wide range of environments relies in large part on its ability to acquire and integrate genetic cargo from lateral sources and acquire new genetic materials such as plasmids, genetic islands, that affect *E. coli* evolution. After this genetic material transfer new pathotypes can develop by the changing of outer membranes, metabolism and invasion factors such as toxin and adhesion. Warm-blooded mammals and avian species consists *E. coli* in their intestinal systems and commensal lifestyle can be seen in that systems **(Ludden *et al.*, 2014)**. If *E. coli* leaks from the intestinal system, it causes several infections such as urinary tract infections, diarrhea, dysentery, hemolytic uremic syndrome, bladder and kidney infection worldwide. It can also be transferred to the circulatory system and lead to blood poisoning. There are several pathogenic *E. coli* strains such as Entero hemoragic *E. coli* (EHEC), Entero toxinogenic *E. coli* (ETEC), Entero aggregative *E. coli* (EAEC), Entero pathogenic *E. coli* (EPEC), Entero invasive *E. coli* (EIEC) etc. The presence of *E. coli* in drinking water indicates the fecal contamination and gives an information about water quality **(Uysal *et al.*, 2013)**.
Another concern with *E. coli* is an increasing antibiotic resistance levels which is global problem worldwide. The indiscriminate use of antibiotics is one of the most important factors in increasing antibiotic resistance. New data from EARS-Net showed that approximately 33000 people in Europe died from the bacterial infections caused by bacteria resistant to antibiotics and its cost to the healthcare systems of European Union countries is nearly 1.1 billion Euro **(ECDC, 2019)**. Multiple resistance mechanisms (mobile genetic elements encoding resistance) can be acquired (such as the production of extended spectrum beta-lactamases (ESBLs) and carbapenemases) or mutations on bacterial genes occurs (fluoroquinolone resistance) thereby limiting the treatment of infections becomes more burdensome and problematic. In fact, the main reasons behind the high levels and spread of antimicrobial resistance are indiscriminate use of antibiotics and transmission of resistant bacteria to antibiotics between organisms and environment. Hence monitoring of antibiotic resistance levels became the most important phenomenon in surveillance studies.
Determination of genetic relations of bacterial strains by molecular technics is an important way to definition of clonal relations in outbreaks. There are lots of molecular analysis such as Random Amplification of Polymorphic DNA (RAPD), Multi locus sequence typing (MLST), Ribotyping, Pulsed field gel electrophoresis (PFGE) etc. to identifying of clonal relations of microorganisms. But PFGE is known to be gold standard method for genotyping of microorganisms in an outbreak. So PFGE studies became the most popular genotyping method in molecular analyses **(Uysal & Durak, 2012)**.
Therefore, this study is designed i) to definition of clonal relations of 47 *E. coli* strains isolated from clinical samples by PFGE method, ii) to determination of their antibiotic resistance levels against antibiotic groups and by disc diffusion technic, iii) profiling of their plasmid sizes and iv) to reveal extended spectrum of beta lactamases (ESBL) production by double disc synergy method.

MATERIAL AND METHODS

Sample collection

Forty-seven clinical samples, including blood, urine, wound, were collected from Selçuk University Faculty of Medicine Hospital in Konya city between March 2009 and July 2010. Isolation of the strains were performed on blood and EMB agar.

Identification of *E. coli* isolates by the API 20E test

The API 20E test was carried out in accordance with the manufacturer's protocol (BioMérieux, Marcy l'Etoile, France). The 20 biochemical test reactions on the strip were converted into an octal profile number. Each profile number was then decoded by using the Analytical Profile Index. Apiweb™, was used to identify species belonging to Enterobacteriaceae and to identify percentages.
Finally, Chromocult TBX (Tryptone Bile X-glucuronide) agar medium (Merck, Darmstadt, Germany) was used to verify of identification. Bluish green colonies were determined as *E. coli* **(Uysal & Durak, 2012)**.

Pulsed-field gel electrophoresis (PFGE) method for genotyping of *E. coli* isolates

PFGE was conducted to assess the genetic diversity of *E. coli* isolates in order to analyze genetic similarities between clinical samples as well as among the different sources. Isolates were subtyped based on PFGE patterns of *Xba*I-digested genomic DNA fragments in accordance with the standard protocol established by the Centers for Disease Control and Prevention (PulseNet; Centers for Disease Control

and Prevention, Atlanta, GA 2008) and by **Durmaz *et al.* (2009)**, with some modification.

The DNA band profiles were analysed by GelCompar II software (version 6.5; Applied Maths, Sint-Martens-Latem, Belgium). DNA profiles for each gel were normalized using the external reference strains (*E. coli* ATCC 25922). Fingerprints were clustered by using the Dice coefficient evaluated by the unweighted-pair group method (UPGMA). A tolerance and optimization of 0.5% was allowed to account for gel-to- gel differences. Isolates that had ≥80% pattern similarity were considered highly closely related. According to the criteria of **Tenover *et al.* (1997)**, the strains were categorized as either of the following: indistinguishable, closely related, possibly related or different.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing (AST) of *E. coli* isolates against 15 antimicrobial agents (Table 1) was performed using Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) according to Clinical and Laboratory Standards Institute (2012). Bacterial suspension with turbidity equivalent to 0.5 McFarland standards was evenly dispensed on the surface of MHA plates using a sterile cotton swab. Antibiotic discs were placed on the surface of MHA agar plates and incubated at 37 °C for 18 to 20 hours. The zone of inhibition was measured to the nearest millimetre and interpreted based on the guidelines of CLSI (2012). *E. coli* ATCC 25922 was used as a control strain. In addition, multiple antibiotic resistances (MAR) indexing of *E. coli* strains were determined according to **Krumperman (1983)**.

Table 1 Concentrations and diffusion zone breakpoints for resistance for antimicrobial agents tested in this study, sorted by class of antimicrobial agent

Antimicrobial agent	Disk drug concen. (µg)	Diffusion zone breakpoints (mm)		
		Resistant	Intermediate	Susceptible
Beta lactams				
Ampicillin (AM)	10	≤13	14-16	≥17
Amoxicillin/clavulanic acid (AMC)	20/10	≤13	14-17	≥18
	75/10	≤14	15-19	≥20
Ticarcillin/calvulanic acid (TIM)	100/10	≤17	18-20	≥21
Piperacillin/tazobactam (TZP)	30	≤14	15-16	≥17
	10	≤12	13-14	≥15
Amino glycosides	30	≤12	13-14	≥15
Amikacin (AK)				
Gentamicin (CN)	30	≤14	15-17	≥18
Netilmisin (NET)	30	≤14	15-22	≥23
Cephalosporins	30	≤14	15-17	≥18
Cephalothin (KF)	30	≤14	15-17	≥18
Cefotaxime (CTX)				
Ceftazidime (CAZ)	30	≤12	13-17	≥18
Cefuroxime (CXM)				
Phinicols	5	≤15	16-20	≥21
Chloramphenicol (C)				
Quinolones	30	≤14	15-18	≥19
Ciprofloxacin (CIP)				
Tetracyclines	10	≤13	14-15	≥16
Tetracycline (TE)				
Carbapenems				
Meropenem (MEM)				

(Uysal & Durak, 2012)

All samples were screened for the production of an ESBL by the double disc synergy test (DDST) as described by **Uysal *et al.* (2018)**. The strains were pre-incubated in brain heart infusion broth (BHIB) at 37°C and the optimal density of 0.5 McFarland standards. This bacterial suspension was swabbed with sterile cotton on to a Mueller-Hinton agar medium. The antagonistic tests were conducted with antibiotic discs of amoxicillin/clavulanic acid (20/10 µg.disk^{-1}) and cefotaxime (30 µg.disk^{-1}), ceftazidime (30 µg.disk^{-1}) and ceftriaxon (30 µg.disk^{-1}) were placed at a distance of about 4 cm apart from each other and incubated. After incubation a clear extension of the edge of the inhibition zone of any of the antibiotics towards the disk containing clavulanic acid-CA (10 µg.disk^{-1}) was interpreted as positive for ESBL production.

Plasmid analysis

Extraction of plasmid DNA was done using the alkaline lysis method of **Bimboim and Doly (1979)** The samples were processed using gel electrophoresis to identify the number of plasmid copies present in different isolates. For this purpose, an agarose gel of 0.8% (w/v) was prepared. Crude DNA extract solutions were subjected to electrophoresis using a horizontal apparatus the Submerged Agarose Gel Electrophoresis (AE-6125, Atto; Tokyo, Japan) and a constant voltage (100V) power source for ~1.5 h as described by **Aladağ *et al.* (2009)**. DNA bands were visualized using a 366 nm UV transilluminator and photographed with UVP, GelDoc It™ Imaging System. Plasmid molecular masses were estimated by electrophoresis with plasmids of known molecular mass from *E. coli* V517.

RESULTS

Typing of *E. coli* isolates by PFGE

In the present study, PFGE method recommended by **Durmaz *et al.* (2009)** was applied with some modifications. In PFGE analysis, in order to generate DNA fragments; *Xba*I restriction enzyme obtained from *Xanthomonas badrii* bacteria and cut the DNA of *E. coli* isolates by recognizing 5'...T/CTAGA...3' nucleotide sequence was used. Electrophoresis conditions were set to be 10 sec, end pulse time 50 sec, pulse angle 120°, current 200 V (6 V/cm2), temperature 14 °C, electrophoresis duration 20 hours. The stated conditions were maintained sequentially between the electrodes for 20 hours. The gel images obtained by staining after electrophoresis and it was seen that the strains formed bands ranging from 10 to 23. The obtained band profiles were evaluated using GelCompar II (version 6.5) software to determine possible clonal relationship between strains. A tolerance of 0.5% was selected for the calculation. The dendogram was created using the UPGMA method and cluster analysis was performed. The relationship between strains was determined using the Dice similarity coefficient. By taking 80% as the cut-off value, genotypes (Pulso types) and their subtypes were determined **(Akçimen & Köksal, 2010; Carrico *et al.*, 2005).**

The dendogram obtained as a result of the analysis based on the band profiles of these strains is given in Figure 1.

Among the clinical strains, 39 different pulso types were determined (A-AN). Between the 39 groups, only four groups were divided into subtypes (Figure 1). The group with the highest number of subtypes is the AK group with six subtypes (AK1-AK6). A 91.3% clonal similarity was detected between the subtypes of the group, DSK 6 (AK4) and DSK 8 (AK5). These two strains were thought to be possibly related strains. A similar relationship was observed between subtypes AK1, AK2 and AK3. It was found that between strains DSK 9 and DSK 10 there was a genetic similarity of 88%. DSK 7 numbered strain (AK3) was related to these two strains with a rate of 87.4%; In the evaluation made based on these ratios, it was determined that these three strains were possibly related strains. The five strains mentioned above were found to be clonally related at a rate of 85% (Figure 1). The last subtype associated with the AK pulso type is AK6, which is formed by the DSK 11 strain. When this strain was compared with other members of the group, it was determined that there was 83.7% genetic similarity between them. All strains were identified as possibly related strains. It was revealed that DSK 6 and DSK9 strains were isolated from urinary tract infection and DSK 10 was isolated from drainage fluid. DSK strains 7, 8 and 11 were isolated from blood.

According to the dendrogram, the group forming another subtype was found to be the U group (Ü1, Ü2). It was observed that the genetic similarity between DSK 18 (Ü1) and DSK 20 (Ü2) strains belonging to this group was 83.3%, and these two strains isolated from urinary tract infection were likely related isolates. T pulso type with strains DSK 30 and DSK 33 also showed two subtypes (T1, T2). Of these two strains with 85.9% genetic affinity; It was determined that DSK 30 (T1) was isolated from throat infection and DSK 33 (T2) was isolated from urinary tract infection, while they were possibly related strains. The last group with subtype in clinical samples is M pulso type. The genetic similarity between the DSK 34 (M1) and DSK 35 (M2) strains related to this type is 88.1%; It was determined that both strains were possibly related strains and isolated from urinary tract infection. The remaining strains were determined as unrelated isolates. Although many were isolated from similar infections, no closely related or identical strains were observed among clinical strains. The genetic diversity of the strains was found to be high and their clonal relationship weak (Figure 1).

Antibiotic susceptibility results of *E. coli* isolates

Antibiotic susceptibility results of 47 *E. coli* strains isolated from various clinical sources were given in Table 2.

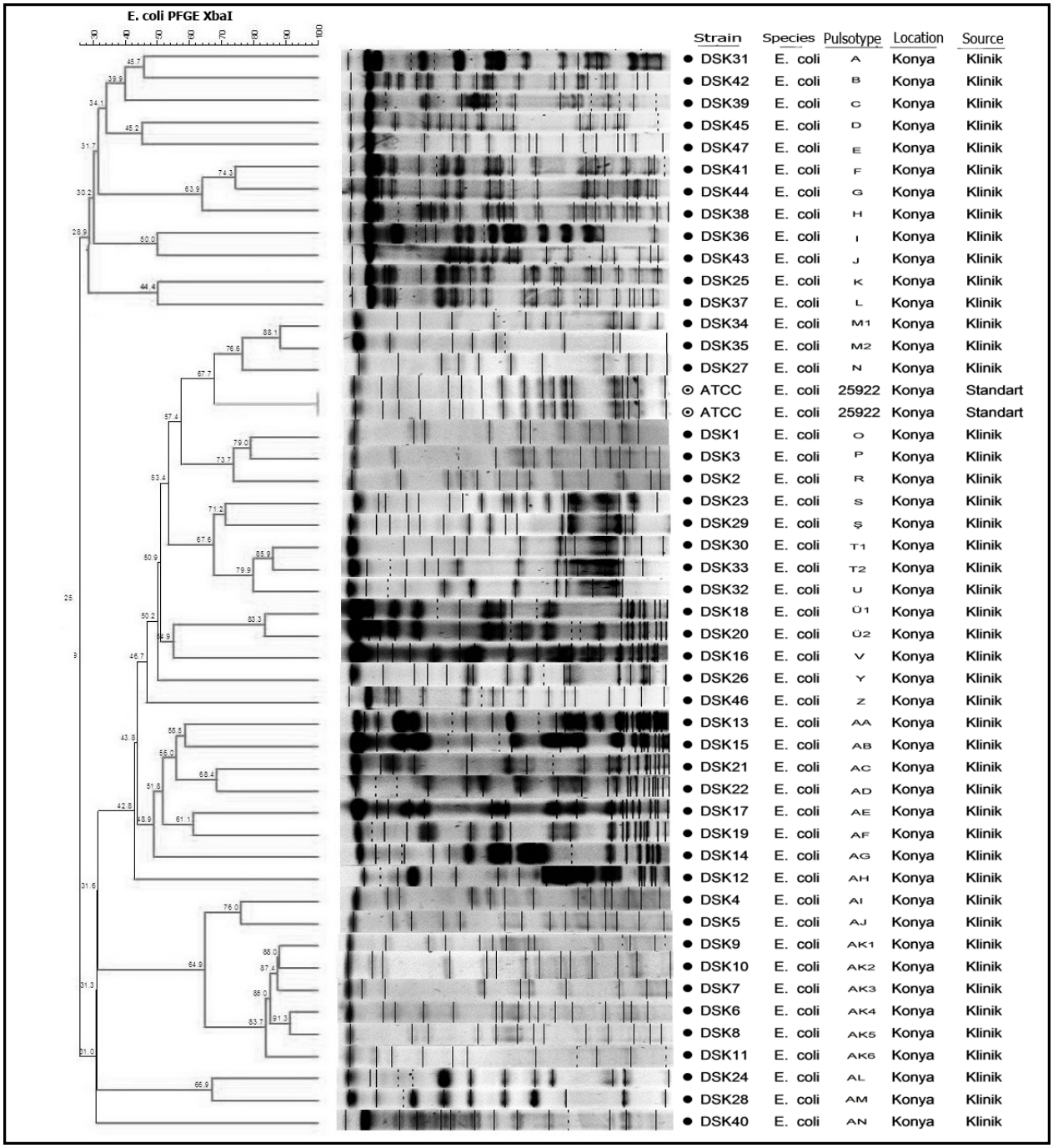

Figure 1 PFGE *Xba*I digestion patterns and clonal analysis of 47 *E. coli* isolates obtained from clinical samples. The dendogram was constructed with the use of Dice similarity coefficient (0.5% tolerance) and UPGMA clustering method by comparison of *Xba*I PFGE patterns. Numbers represent the distance values between the respective isolates.

Table 2 Antibiotic susceptibility patterns of *E. coli* isolates

	R% (n)	I % (n)	S % (n)
TZP	12.76 (6)	36.17 (17)	51.07 (24)
TE	80.85 (38)	8.51 (4)	10.64 (5)
CTX	80.85 (38)	2.12 (1)	17.03 (8)
AM	100 (47)	0	0
CIP	57.45 (27)	2.12 (1)	40.43 (19)
TIM	89.36 (42)	2.12 (1)	8.52 (4)
AMC	46.81 (22)	21.28 (10)	31.91 (15)
KF	89.36 (42)	2.12 (1)	8.52 (4)
MEM	0	0	100 (47)
C	23.40 (11)	4.25(2)	72.35 (34)
NET	4.25 (2)	6.38 (3)	89.37 (42)
AK	2.12 (1)	2.12 (1)	95.76 (45)
CN	55.32 (26)	2.12 (1)	42.55 (20)
CXM	87.24 (41)	2.12 (1)	10.64 (5)
CAZ	38.29 (18)	17.03 (8)	44.68 (21)

S – susceptibility,**I–** Intermediate, **R –** resistance

Among the strains, the highest resistance against beta-lactam penicillin was shown to AM with a rate of 100%, and none of the strains were found to be sensitive to this antibiotic. In terms of resistance percentage, TIM was the second with 89.36%. While only one strain (2.12%) was found to be intermediate to this antibiotic, the number of susceptible strains was determined as four (8.52%). While 22 of the strains (46.81%) were resistant to AMC, 10 strains (21.28%) were found to be intermediate, and 15 strains (31.91%) were found to be susceptible. Among the penicillin groups, the least resistance was shown to TZP with 12.76%. 17 strains (36.17%) were found to be intermediate to this agent, and 24 strains (51.07%) were found to be susceptible (Table 2).

When cephalosporin group were evaluated; the highest resistance was observed against KF with 42 strains (89.36%). Only one of the strains was found to be intermediate and four (8.52%) was susceptible. Although 41 (87.24%) of the strains were found to be resistant to CXM, a second generation cephalosporin, one strain was found to be intermediate and five strains (10.64%) were susceptible. It was revealed that 38 strains (80.85%) were resistant to CTX, one strain was intermediate and eight strains (17.03%) were susceptible. Among the cephalosporin, it was observed that the least resistance was against CAZ with 38.29% (18), while the strains were intermediate at a rate of 17.03% (8) and susceptible at a rate of 44.68% (21) (Table 2).

The most resistance against aminoglycosides were seen for CN (55.32%), while the number of susceptible strains was 20. Only one strain (2.12%) against AK and

two strains (4.25%) against NET were found to be resistant. The number of strains susceptible to these antibiotics was determined as 45 (95.76%) and 42 (89.37%), respectively.

All strains were found to be susceptible to MEM. Thirty-eight (80.85%) of the strains were resistant to TE, four strains (8.51%) were intermediate, and five strains (10.64%) were susceptible. 27 strains (57.45%) were found to be resistant and 19 strains (40.43%) were susceptible to CIP. It was found that 11 (23.40%) of the strains were resistant and 34 (72.35%) were susceptible to C. The percentages of resistance, intermediate and susceptibility status of the strains are given in Table 2.

Resistance profile and MAR indexes of clinical strains were calculated (Table 3). According to the data obtained from the susceptibility test, 36 different resistance profiles (**resisto type**) were observed among clinical strains. Among these, the most common profiles are as follows: The resistance profile against eight different antibiotics (**Resisto type XIX:** AM, CTX, TE, TIM, AMC, KF, C, CXM) was observed in three strains (6.38%). DSK strains 9, 12, 28 showing this profile were found to have a MAR index of 0.533. Another profile was evaluated against nine antibiotics (**Resisto type XXVII:** AM, CTX, TE, CIP, TIM, KF, CN, CXM, CAZ) with a rate of 6.38% in three strains (DSK 18, 26, 39). The MAR index of these strains was 0.600. Finally, the distribution of the profile shown against 11 antibiotics (**Resisto type XXXV:**AM, CTX, TE, CIP, TIM, AMC, KF, CN, C, CXM, CAZ) among strains was 6.38%, and it was determined in three strains (DSK 10, 11, 19). The MAR index for these strains was 0.733 (Table 3).

Table 3 Antibiotic resistance patterns and ESBL production of *E. coli* isolates

Resisto type	Resistance profiles	Number of isolates	%	Strains	ESBL	MAR index *
I	AM**	1	2.13	DSK 36		0.067
II	AM, KF, CXM	1	2.13	DSK 46		0.200
III	AM, TE, TIM	2	4.26	DSK 41, 43		0.200
IV	AM, TE, CIP, C	1	2.13	DSK 17		0.267
V	AM, TE, CIP, KF, CXM	1	2.13	DSK 45		0.333
VI	AM, TE, CIP, TIM, C, CN	1	2.13	DSK 42		0.400
VII	AM, CTX, TE, TIM, KF, CXM	1	2.13	DSK 44		0.400
VIII	AM, CTX, TIM, KF, CN, CXM	2	4.26	DSK **31, 15**	**ESBL(+)**	0.400
IX	AM, CTX, AMC, KF, CXM, TZP	1	2.13	DSK **47**	**ESBL(+)**	0.400
X	AM, CTX, TIM, KF, C, CXM	1	2.13	DSK **38**	**ESBL(+)**	0.400
XI	AM, CTX, TIM, AMC, KF, CXM	1	2.13	DSK 32		0.400
XII	AM, CTX, TE, TIM, KF, CXM	1	2.13	DSK **29**	**ESBL(+)**	0.400
XIII	AM, CTX, TE, TIM, KF, CXM, CN	1	2.13	DSK **2**	**ESBL(+)**	0.467
XIV	AM, CTX, TE, TIM, KF, CXM, CAZ	1	2.13	DSK **6**	**ESBL(+)**	0.467
XV	AM, CTX, TE, CIP, TIM, KF, CXM	1	2.13	DSK **23**	**ESBL(+)**	0.467
XVI	AM, TE, CIP, TIM, AMC, KF, C	1	2.13	DSK 30		0.467
XVII	AM, CTX, TE, TIM, KF, CN, CXM	1	2.13	DSK 33		0.467
XVIII	AM, CTX, TE, TIM, KF, CN, CXM, CAZ	1	2.13	DSK **7**	**ESBL(+)**	0.533
XIX	AM, CTX, TE, CIP, TIM, KF, CN, CXM	3	6.38	DSK **9, 12**, 28	**ESBL(+)**	0.533
XX	AM, CTX, TE, TIM, AMC, KF, C, CXM	1	2.13	DSK 14		0.533
XXI	AM, CTX, TIM, AMC, KF, CN, CXM, CAZ	1	2.13	DSK 21		0.533
XXII	AM, CTX, CIP, TIM, AMC, KF, CXM, CAZ	1	2.13	DSK 22		0.533
XXIII	AM, CTX, TE, TIM, AMC, KF, CN, CXM	1	2.13	DSK **37**	**ESBL(+)**	0.533
XXIV	AM, CTX, TE, TZP, TIM, AMC, KF, CXM	1	2.13	DSK **25**	**ESBL(+)**	0.533
XXV	AM, CTX, TE, CIP, TIM, AMC, KF, CXM, CAZ	2	4.26	DSK **3,** 16	**ESBL(+)**	0.600
XXVI	AM, CTX, TE, TZP, TIM, AMC, KF, CXM, CAZ	1	2.13	DSK 13		0.600
XXVII	AM, CTX, TE, CIP, TIM, KF, CN, CXM, CAZ	3	6.38	DSK 18, 26, **39**	**ESBL(+)**	0.600
XXVIII	AM, CTX, TE, CIP, TIM, AMC, KF, CN, CXM	2	4.26	DSK **34**, 35	**ESBL(+)**	0.600
XXIX	AM, CTX, TE, CIP, TIM, KF, NET, CN, CXM	1	2.13	DSK **40**	**ESBL(+)**	0.600
XXX	AM, CTX, TE, CIP, TIM, AMC, KF, CN, CXM, CAZ	2	4.26	DSK **4, 5**	**ESBL(+)**	0.667
XXXI	AM, CTX, TE, CIP, TIM, KF, C, CN, CXM, CAZ	1	2.13	DSK **8**	**ESBL(+)**	0.667
XXXII	AM, TZP, TE, CIP, TIM, AMC, KF, NET, AK, CXM	1	2.13	DSK 20		0.667
XXXIII	AM, CTX, TE, CIP, TIM, AMC, KF, C, CN, CXM	1	2.13	DSK **24**	**ESBL(+)**	0.667
XXXIV	AM, CTX, TE, CIP, TIM, AMC, KF, CN, TZP, CXM, CAZ	1	2.13	DSK 1		0.733
XXXV	AM, CTX, TE, CIP, TIM, AMC, KF, CN, C, CXM, CAZ	3	6.38	DSK 10, **11, 19**	**ESBL(+)**	0.733
XXXVI	AM, CTX, TE, CIP, TIM, AMC, KF, CN, C, CXM, CAZ, TZP	1	2.13	DSK **27**	**ESBL(+)**	0.800

*__MAR:__ Multiple antibiotic resistance

**__AM:__ Ampicillin, TE: Tetracycline, AMC: Amoxicillin/clavulanic acid, KF: Cephalothin, TIM: Ticarcillin/clavulanic acid, CAZ: Ceftazidime, CXM: Cefuroxime, CTX: Cefotaxime, CN: Gentamicin, CIP: Ciprofloxacin, C: Chloramphenicol

It was defined that only one strain (DSK 36) was resistant to a single antibiotic. The DSK 27 numbered strain manifested resistance to the highest number of antibiotics (**Resisto type XXXVI**), and it was found to be resistant to 12 antibiotics. MAR index was determined as 0.800 and this index was the highest value observed among clinical strains. When the total results were considered, one antibiotic covering one (**Resisto type I**), two resistance profiles (**Resisto types II and III**) covering three antibiotics, one resistance profile (**Resisto type IV**) covering four antibiotics, one resistance profile covering five antibiotics (**Resisto type V**), seven resistance profiles covering six antibiotics (**Resisto types VI to XII**), five resistance profiles (**Resisto types XIII to XVII**) covering seven antibiotics, seven resistance profiles (**Resisto types XVIII to XIV**) covering eight antibiotics, five resistance profiles (**Resisto types XXV to XIX**) covering nine antibiotics, four resistance profiles (**Resisto types: XXX to XXXIII**) covering 10 antibiotics, two resistance profiles (**Resisto types XXXIV and XXXVI**) covering 11 antibiotics and one resistance profile (**Resisto type XXXVI**) covering 12 antibiotics were revealed (Table 3).

The ESBL enzyme production potential of *E. coli* strains were examined using the double disc synergy method. As a result, it was observed that 24 of the strains (51.06%) were ESBL positive and 23 (48.94%) of the strains were ESBL negative

(Table 3). Although the numbers of ESBL-producing strains were high (51.06%), these strains were resistant to at least six or more antibiotics, as expected.
Although DSK strains 15, 29, 31, 38 and 47 were resistant to six antibiotics; DSK 2, 6 and 23 numbered strains manifested resistance against to seven antibiotics. It was assigned that ESBL-producing positive DSK strains 7, 9, 12, 25 and 37 were resistant to eight antibiotics; DSK strains 3, 37, 39 and 40 were resistant to nine antibiotics; DSK 4, 5, 8 and 24 strains were resistant to ten antibiotics; DSK strains 11 and 19 were resistant to 11 antibiotics. DSK 27, ESBL-producing positive strain, was reported as the bacterium the most resistant to antibiotics among clinical isolates (Table 3). Consequently, it was concluded that ESBL producing strains in this study were also multidrug resistant strains.

Plasmid profiles

Figure 2a and 2b shows gel image of plasmid bands extracted from *E. coli* isolates. When *E. coli* strains were evaluated according to plasmid size, it was defined that 27 of 47 strains (57.44%) carried plasmids and the remaining 20 strains (42.66%) did not carry plasmids (Table 4). The sizes of the determined plasmids were ranging between 77.1 kb and 1.6 kb. Among the strains, it was elucidated that the strain with the highest number of plasmids was DSK 35 with 11 plasmids. This strain was followed by DSK 2 with eight plasmids, DSK 12 with seven plasmids in third, and DSK 7 and 31 strains with six plasmids in the fourth place. The strains with the largest plasmid were DSK 34, 45 and 46; although the strain with the smallest plasmid was found to be DSK 35 (Table 4). As a result of the plasmid profiling carried out according to the sizes of the plasmids they contain, 22 different plasmid profiles were revealed within the strains (Table 4). Strain numbered DSK 35 showed the highest number of profiles (7). DSK 2 with eight plasmids showed five profiles; DSK 28 with five plasmids and DSK 31 with six plasmids showed four plasmid profiles. It was observed that DSK strains 7, 12, 21, 40, 41 and 42 displayed three plasmid profiles.

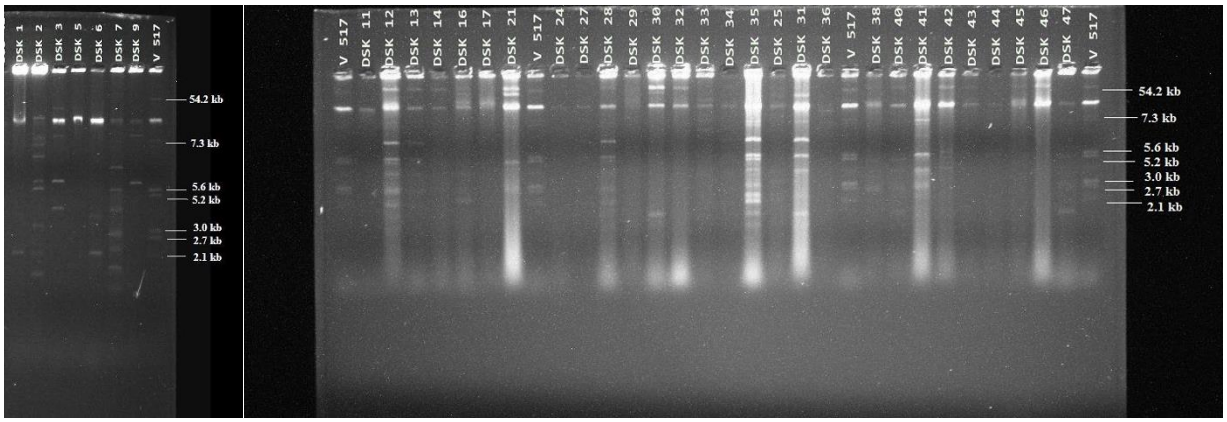

Figure 2a-2b Image of plasmids extracted from *E. coli* strains isolated from clinical samples on agarose gel. *E. coli* V517 plasmid DNA fragments were used as a size standard.

Table 4 Plasmid sizes and profiles of *E. coli* isolates

Strains	Number of plasmids	Size (kb)	Profile No
DSK1	1	2.3 kb	1
DSK2	8	14.5, 12.2, 9.3, 5.9, 5.2, 3.6, 2.3, 1.9 kb	1, 2, 3, 4, 5
DSK3	4	40.4, 15.2, 5.9, 4.0 kb	2, 6
DSK6	2	3.6, 2.3 kb	1, 4
DSK7	6	7.5, 5.2, 3.9, 2.9, 2.4, 2.0 kb	3, 7, 8
DSK9	2	15.1, 5.7 kb	
DSK12	7	59.8, 6.9, 4.6, 4.2, 3.5, 2.7, 2.1 kb	9, 10, 11
DSK13	4	59.8, 34.7, 6.7, 4.6 kb	9, 10
DSK14	1	59.8 kb	9
DSK16	4	63.3, 42.7, 26.6, 2.3 kb	1, 12
DSK21	4	59.8, 42.7, 4.4, 2.7 kb	9, 11, 12
DSK25	1	40.1 kb	13
DSK28	5	67.0, 7.0, 4.9, 2.7, 2.2 kb	11, 14, 15, 16
DSK30	3	67.0, 47.0, 1.9 kb	5, 14
DSK31	6	40.1, 12.9, 7.3, 5.1, 3.8, 1.9 kb	5, 13, 17, 18
DSK32	1	67.0 kb	14
DSK33	2	30.2, 9.6 kb	
DSK34	2	77.1, 7.4 kb	19, 20
DSK35	11	12.9, 11.2, 7.4, 5.1, 4.6, 3.8, 2.9, 2.5, 2.2, 1.9, 1.6 kb	5, 7, 10, 16, 17, 18, 20
DSK38	1	2.7 kb	11
DSK40	4	67.0, 4.6, 3.7, 2.0 kb	8, 10, 14
DSK41	5	13.3, 8.3, 5.2, 3.8, 2.8 kb	3, 18, 21
DSK42	4	72.5, 5.5, 4.9, 4.0 kb	6, 15, 22
DSK43	1	72.5 kb	22
DSK45	1	77.1 kb	19
DSK46	1	77.1 kb	19
DSK47	3	5.0, 2.8, 1.9 kb	5, 21

Profile no 1: 2.3 kb; **Profile no 2:** 5.9 kb; **Profile no 3**: 5.2 kb; **Profile no 4**: 3.6 kb; **Profile no 5**: 1.9 kb; **Profile no 6:** 4.0 kb; **Profile no 7:** 2.9 kb; **Profile no 8:** 2.0; **Profile no 9**: 59.8 kb; **Profile no 10**: 4.6 kb; **Profile no 11:** 2.7 kb; **Profile no 12:** 42.7 kb; **Profile no 13:** 40.1 kb; **Profile no 14:** 67.0 kb; **Profile no 15:** 4.9 kb; **Profile no 16:** 2.2 kb; **Profile no 17:** 12.9, 5.1 kb; **Profile no 18:** 3.8 kb; **Profile no 19:** 77.1 kb; **Profile no 20:** 7.4 kb; **Profile no 21:** 2.8 kb; **Profile no 22:** 72.5 kb.

DISCUSSION

Bacteria are commonly found in the body, in food, and in the environment, and are important to living organisms because of the delicate balance between symbiotic and parasitic relationships they establish. Among some potentially pathogenic microorganisms known in the environment, coliform bacteria and especially *E. coli* have been extensively studied by researchers in determining water and food quality **(Manning, 2010)**. *E. coli* strains were not considered an important pathogen until they were recognized as a source of epidemics in some countries in the 1980s **(Wells *et al.*, 1983)**. The Centers for Disease Control (CDC) in the USA reported 73,000 cases and 61 deaths from toxinogenic strains of *E. coli* during a year **(Rangel *et al.*, 2005)**. The frequency of reports of this pathogen in various foods and drinking waters indicates the need for rapid, accurate and cost-effective diagnostic systems to reduce exposure to *E. coli* infection. This bacterium can be isolated from many different sources in nature, and their isolation and identification are carried out by various classical methods. However, even if the bacteria is diagnosed, whether these microorganisms are genotypically similar to the pathogen strains can only be determined by molecular methods **(Foley *et al.*, 2009)**. Many methods have been developed to control the spread of beneficial and pathogen microorganisms, allowing their identification. PFGE is widely used for genotypic characterization of microorganisms. It is preferred in the analysis of most bacterial pathogens due to its high discrimination and reproducibility. Although it is a very important tool in epidemic research, it is not only in this field; it is also an important method to determine the clonal relationships and genetic diversity of microorganisms in the environment **(Goering, 2010)**. In our study; it is aimed to reveal the similarities and differences of clinically originated *E. coli* strains with PFGE method and to reach detailed information about clone sources in this direction. The phylogenetic dendrogram of strains were established according to PFGE profiles obtained after restriction with XbaI. With a degree of similarity of 80%, the results from PFGE separated the E. coli strains into 39 different groups representing four subtypes. . According to the data obtained, high genetic diversity was detected among the isolated *E. coli* strains (Figure 1).

Similarly, **Edberg *et al.* (1994)** investigated the clonal relationship of a total of 56 *Enterobacter cloacae* strains, 23 from the water distribution system in New Haven, 5 from the spring waters and 28 clinical isolated from various units of the city center hospitals. The PFGE method was applied to determine whether all of these strains were similar to each other, whether the strains obtained from the water distribution service were the same as the strains isolated from the spring waters, and whether the isolated strains from the water distribution service were similar to the clinical strains. As a result of the study, they reported that the strains isolated from the water distribution network were the same, however, these strains were completely different from the strains isolated from the hospital and the strains obtained from the spring water. Researchers have stated that there is heterogeneity between strains obtained from different sources in the same region. In our study, the homogeneity and heterogeneity of *E. coli* strains were investigated. As a result, high level diversity was observed among clinical isolates.

Parveen *et al.* (2001) tried to reveal the source of fecal contamination by various methods. A total of 104 strains of *E. coli* of human origin (53) and non-human (51) were isolated from estuary waters. Genetic relationships were investigated by PFGE method. As a result of these phenotypic and genotypic analyzes, no relatedness was determined between both human and non-human origin strains. PFGE profiles of clinical strains showed less genetic variation than profiles of strains of non-human origin. In another study, **Rios *et al.* (1999)** defined the clonal relationships of enterohemorrhagic *E. coli* strains isolated from clinical isolates (hemolytic uremic syndrome cases), animal sources and food from in Chile by colony blot hybridization and PFGE methods. Among the 39 *E. coli* O157:H7 strains analyzed, 37 different profiles were revealed. PFGE profiles were widely distributed among isolates obtained from hemolytic uremic syndrome cases and asymptomatic individuals; It has been reported that genetic relatedness is observed between hemolytic uremic syndrome and O157 strains isolated from pigs. Based on this result, some different EHEC clones have spread in Chile; It has been determined that pigs are an important animal source in human infections. In our study, no research or serotyping was performed for a specific serotype. The clonal similarity rate was found to be low among the strains isolated from different clinical cases. Subtypes generally occurred among strains isolated from urinary tract infection (Figure 1).

Bae *et al.* (2014) reported that 41 ESBL producing *E. coli* isolates were subjected to three different molecular typing methods such as repetitive extragenic palindromic PCR (REP-PCR), Multilocus Sequence Typing (MLST) and PFGE. Researchers emphasized that PFGE showed higher discriminative power than rep-PCR in *E. coli* isolates. The study conducted by **Ludden *et al.* (2014)** manifested that 94 ESBL producing *E. coli* strains isolated from different sources (nursing homes, residents, hospitals) revealed 65 different pulsotypes. Similarly, **Toka Ozer *et al.* (2018)** stated that four different pulsotypes were defined in45 ESBL producing *E. coli* strains. So C group pulsotype showed six different sub-types. 120 *E. coli* strains isolated from urinary tract infections subjected to ESBL producing test, antibiotic resistance and PFGE typing. Results showed that four different resistance patterns and three PFGE groups were observed among strains **(Tutun *et al.*, 2019)**. In our study, the PFGE method showed a high degree of differentiation within the strains and revealed 39 different pulsotypes. Our results obtained coincide with the results defined by other studies showing a high degree of heterogeneity in PFGE method.

Antibiotic resistance in Gram-negative pathogens is a global problem that contributes to higher healthcare costs and mortality rates, treatment failures, and a longer duration of clinical disease. Because many bacterial infections are treated with antibiotics, many bacteria have developed ways to resist the killing mechanism of antibiotics. For *E. coli*, antibiotic resistance has become more common due to the overuse and misuse of antibiotics. Moreover, *E. coli* can serve as reservoirs of resistance genes that are efficiently exchanged not only with each other but also with other enteric pathogens of humans and animals **(van den Bogaard *et al.*, 2001)**. Worldwide, the prevalence of Enterobacteriaceae, which produce broad-spectrum β-lactamase or carbapenemase enzymes, continues to increase at alarming rates **(Zowawi *et al.*, 2015)**. Routine monitoring of antibiotic resistance in *E. coli* provides data for antibiotic therapy and resistance control, as the prevalence of resistance in commensal *E. coli* is a useful indicator of antibiotic resistance in bacterial isolates from humans and the environment **(OBrien, 1997)**. In this study, *E. coli* strains isolated from clinical sources were found to be highly resistant to the antibiotics tested in this study, as expected. The highest resistance was observed against Ampicillin 100% (47 strains), followed by ticarcillin/clavulanic acid and cephalothin with a rate of 89.36 % (42 strains each), cefuroxime at a rate of 87.24% (41 strains), and tetracycline and cefotaxime 80.85% (38 strains) (Table 2). The MAR index values of 43 strains were found to be greater than 0.2, therefore it was discussed that they originated from the regions where these antibiotics to which they are resistant are used intensively. This situation may be an indicator of uncontrolled and unconscious antibiotic use. The majority of clinical strains have been isolated from various samples such as urinary tract infections, vaginal smear, throat infection, blood. Among the strains, 36 different resistance profiles were defined (Table 3). With the exception of one strain (DSK 36), multiple antibiotic resistances were observed in the remaining strains. The resistance rates obtained were compared to similar studies.

In a study conducted by **Bijapur *et al.* (2015)**, ampicillin resistance rates in *E. coli* strains were determined as 91.66%; cefuroxime 82.29%; cefotaxime 79.16%; 75% of ciprofloxacin. However, they did not detect any resistance to imipenem and meropenem. **Kudinha *et al.* (2013)** revealed that 47.63% of 953 *E. coli* isolates were resistant to tetracycline and 33.47% to cephalothin. In addition, the researchers determined that 6.4% of the total isolates were ESBL-producing strains. In another study, **Rehab *et al.* (2019)** determined phenotypically ESBL positivity in 113 of 168 *E. coli* and K. pneumoniae strains, and they found that 95.58% of these strains also genotypically carried blaTEM, blaSHV and blaCTX-M genes. The antibiotic resistance rates of the strains were mostly shown against third generation cephalosporins; they also emphasized that the highest sensitivity was shown against meropenem and imipenem. In our study all *E. coli* strains were susceptible against meropenem (100%) and the resistance rates of cephalothin and cefuroxime were defined as 89% and 87%, respectively. The results of the researchers found to be consistent with the resistance rates detected in our study. **van Driel *et al.* (2019)** stated that *E. coli* strains were responsible for 83% of the strains isolated from urinary tract infections, and compared their antibiotic susceptibility status, which was performed every 5 years between 2004-2009-2014, with their current studies. In terms of antibiotic susceptibility, the strains were stable over time except for ciprofloxacin (96% in 2004, 97% in 2009 and 94% in 2014; $P < 0.05$); co-amoxiclav sensitivity increased by 88%, 87% and 92% in 2004, 2009 and 2014, respectively; They reported that the prevalence of ESBL-producing *E. coli* increased from 0.1% in 2004 to 2.2% in 2014.

In a study on ESBL-producing bacteria isolated from pregnant women in Beirut, Lebanon, **Gaddar *et al.* (2020)** found that most of the 59 *E. coli* strains were highly sensitive to meropenem and imipenem, with a sensitivity of 93.2%. The most resistance was determined against aztreonam, cefepime and sulfomethoxazole. **Ugwu *et al.* (2020)** reported that the dominant ESBL gene in 58 isolates was blaTEM and the most resistant antibiotic was cotrimaxazole in 100 uropathogenic *E. coli* strains, also they stated that the antibiotics to which they were most sensitive were aztreonam and ceftazidime. The rate of ESBL-producing strains were defined as 51.06% in our study and this result was higher than the results of other researchers.

According to the 2018 report of surveillance studies conducted in Europe; In the European countries, the highest resistance against third generation cephalosporin was 38.7% in Bulgaria; 37.1% in Cyprus; 30.1% in Slovakia; 28.7% in Italy and 20.2% in Romania. In the ranking of the lowest resistance European countries were determined as, 6.8% in Norway; 7.3% in Netherlands; 7.6% in Finland; 7.7% in Denmark; 8.1% in Iceland; 8.3% in Switzerland; 9% in Belgium; 9.6% in France; 11% in United Kingdom; 12.2% in Germany **(ECDC, 2019)**. In our current study, resistance to third generation cephalosporins was found to be 38% (ceftazidime) and 80% (cefotaxime). In accordance with these resistance rates, we concluded that Turkey is at the forefront of resistance with rates of 38% and 80%.

The highest resistances to fluoroquinolone group antibiotics in European countries were reported as follows: 42.4% in Cyprus; 42.1% in Slovakia; 41.9% in Malta; 41.8% in Bulgaria; in Italy 41.7%. The countries with the least resistance are Finland with a rate of 11.4%; Norway with a rate of 12.9%; Denmark with a rate of 13.3%; Netherlands with a rate of 14.9% and France with a rate of 16.3% **(ECDC, 2019)**. In our study, the resistance determined against ciprofloxacin was 57.45%. This situation causes us to rank high in this table. Turkey ranks first with

a rate of 52% in the 2013 report **(Allocati *et al.*, 2013)**. The resistance observed in aminoglycoside groups was the highest in Bulgaria with a rate of 28.4%; Slovakia with a rate of 21.6%. Also the lowest rates were found in Finland, Norway and Denmark (4.3%, 5.7%, 5.7%, respectively). In our study, *E. coli* strains were resistant to amikacin (2.12%); kanamycin (4.25%) and gentamicin with a rate of 55.32% (Table 2). When compared in terms of resistance to aminoglycosides, Turkey ranks first with a rate of 35% in the 2013 report **(Allocati *et al.*, 2013)**.

It is seen that ESBL production rates have increased before 2010 and in the last 10 years after 2010 in the world. Accordingly, the ESBL rates determined in *E. coli* strains isolated from UTI infections were increased from 4.6% to 6.6% in the United Kingdom; from 1.1% to 3.3% in France; in Spain from 2.4-18.2% to 8.9-23.69%. These ratios were increased in Italy from 3.5% to 6.7%, in Turkey from 8-13.1% to 24% in Mediterranean countries. ESBL producing *E. coli* rates increased from 27.1% to 33.2% in South Asian countries, from 4.8-7.5% to 7.6-10.7% in Far East Asia. It rose from 1.7% to 7.1-2.5% in Latin American countries and from 7.4% to 1.8-8% in the USA and Canada **(Lee *et al.*, 2018)**. ESBL-producing Enterobacteriaceae members were studied in Turkey in the 2007-2020 period. ESBL positivity rates were reported as follows: 11% by **Azap *et al.* (2010)**; 20.2% by **Yilmaz *et al.* (2009)**; 43% by **(Kizilca *et al.*, 2012)**; 23.5% by **Azap *et al.* (2013)**, 55% by **Aladag *et al.* (2013)**; 20.75% by **Khorshed and Arslan (2015)**; 24% by **Yılmaz *et al.* (2016)**; 13.40% by **Uysal *et al.* (2018)**; 11.8% by **Bozkır *et al.* (2020)**. In our current study, an ESBL positivity rate of 51.06% was observed, which was higher than the results of other researchers. The fact is that ESBL-producing *E. coli* strains are increasing worldwide.

The result of plasmid profiling assay revealed that 57.44% of the clinical isolates carried plasmids. Forty-six (97.8%) of the strains showed multiple antibiotic resistance, and 57 plasmid bands differing from each other were observed between the strains (Table 4). Although a large number of multi-antibiotic resistant strains were detected; It was observed that some of the strains (DSK 34, 35, 40) that showed this situation carried plasmid, while some (DSK 10, 11, 19, 20, 24, 27) did not carry plasmids. In fact, it has been defined that some of the strains (DSK 31 and 15) showing the same resistance profile have plasmid and some do not have plasmid (Table 4). This situation revealed the view that the plasmids were randomly distributed among the strains and that the resistance that occurred was not related to the number and size of plasmids. Multi-antibiotic resistance detected in strains is highly likely to be a chromosomal resistance.

Karbasizaed *et al.* (2003) determined resistance to heavy metals and antibiotics and revealed plasmid profiles in *E. coli* strains isolated from nosocomial infections and healthy human feces. Plasmids were determined in 25 strains; 13 of these were found to be transmissible R plasmids. It has been reported that 8 nosocomial strains contain conjugative plasmid encoding resistance to heavy metals and antibiotics. However, they found that there was no consistent relationship between antibiotic resistance and plasmid profiles. In our study; it has been demonstrated that there is no consistent relationship between resistance and plasmid profiles of clinically originated strains. The findings of the researchers and our results support each other.

Wan *et al.* (2003) investigated multiple antibiotic resistance rates and plasmid profiles in enteropathogenic *E. coli* (EPEC) strains that cause diarrhea in infants. They detected one or more plasmids in the range of 1.0-30.9 mDa (mega dalton) in 22 of the strains. Since the conjugation experiment showing the transfer of the antibiotic resistance phenotype was not performed, a definite relationship between the antibiotic resistance profile and the plasmids cannot be mentioned; In this case, they stated that there is no direct relation between the plasmid and the antibiotic resistance profile. They also emphasized that multi-antibiotic resistance can occur without plasmids or transposons. The presence of strains resistant to 10, 11, 12 antibiotics in our study, although they do not carry plasmids, supports this idea and it is supported by the results of **Teophilo *et al.* (2002)**, **Aladağ *et al.* (2009)**, **Ozbakir *et al.* (2010)** and **Shahriar and Khair (2011)** as well. **Çelebi *et al.* (2007)** determined the resistance and plasmid profile in *E. coli* causing urinary tract infections; so they clarified that some of the strains showing multi-antibiotic resistance did not contain plasmids, and that the multi-antibiotic resistance and plasmid profiles were not related to each other in some of the isolated strains. This idea is consistent with the results obtained from our study.

CONCLUSION

Humans, animals and plants are always in a close relationship with microorganisms that are common in nature. This relationship may rarely occur in the form of disease. Due to the recent increase in world population, pollution of some water and food sources directly affects this situation. The increasing resistance to antibiotics worldwide and the transmission of this resistance to other microorganisms by pathogenic bacteria, which are at the heart of resistance, also puts humans in a difficult situation in the fight against diseases. Strategies to be developed against these strains are only possible with the detection of various virulence factors and antibiotic resistance levels of these bacteria. In other words, to fight against the enemy without knowing its identity; can cause loss of time, money, labor and life. So characterization of bacteria that causing various diseases is important at this point.

Today, molecular typing methods are widely used for the rapid detection of such harmful bacterial agents and to determine their genetic relationships. When evaluating the knowledge gained in our study, the PFGE method proved to be a very useful method for determining the clonal relatedness of clinical *E. coli* strains and showed high discriminatory power between the isolates.

It is well known that resistance increases as a result of the indiscriminate and unnecessary use of antibiotics. If strain virulence factors are added to these resistance factors, it is not possible to control and combat the situation. Monitoring of antimicrobial resistance levels is demonstrated through continuous surveillance studies. Such studies should be conducted and followed up on a regular basis.

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