Phenotypic Analysis of Extended-Spectrum Beta-Lactamase Production, Biofilm Capacity and Antimicrobial Resistance in *Escherichia coli* Isolates Obtained from Broilers

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ABSTRACT

Antimicrobial resistance has emerged as a significant issue in poultry farming, with extended-spectrum beta-lactamase (ESBL) production being one of the most critical mechanisms of resistance. The aim of this study was to phenotypically evaluate ESBL production, biofilm formation capacity and antimicrobial resistance profiles of *Escherichia coli* isolates causing colibacillosis in broilers. The study material consists of 180 *E. coli* isolates obtained from the livers of broilers diagnosed with colibacillosis. ESBL production was preliminarily screened using CHROMagar™ ESBL (CHROMagar, Paris, France) medium and confirmed through the double-disk synergy test (DDST). Biofilm formation capacity was determined via the microplate method, and antibiotic resistance profiles were assessed using the disk diffusion method. The relationship between biofilm formation capacity, multidrug resistance properties and ESBL production was analysed using the Chi-square test. Preliminary screening with CHROMagar™ ESBL medium revealed that 7.7% of isolates were ESBL-positive, and 78.6% of these isolates were confirmed to produce ESBL via the DDST. According to the findings, 6.1% of all isolates produced ESBL, 42.8% formed biofilms, and 53.3% exhibited multidrug resistance. Notably, resistance rates against commonly used antibiotics such as ampicillin, gentamicin, and tetracycline were significantly higher among ESBL-producing isolates. The study results demonstrated a statistically significant relationship ($p<0.05$) between ESBL production, biofilm formation capacity, and multidrug resistance rates. However, it was observed that 18.2% of ESBL-producing isolates did not form biofilms, and 9.1% did not exhibit multidrug resistance. Conversely, 40.2% of non-ESBL-producing isolates had the capacity to form biofilms, while 50.8% demonstrated multidrug resistance. The findings indicated that while a small proportion of *E. coli* isolates from broilers produce ESBL, a substantial portion possesses biofilm formation capacity and multidrug resistance traits. Additionally, it was observed that preliminary screening tests conducted with chromogenic agars, followed by confirmation via DDST, were beneficial for more accurately identifying ESBL production. Based on these results, it is recommended that future studies investigate the dissemination mechanisms of resistance genes and the clinical implications of biofilm formation in greater detail in order to contribute to the development of effective prevention and control strategies in this field.

Keywords: Broiler, *Escherichia coli*, Extended-Spectrum Beta-Lactamase, Biofilm.

INTRODUCTION

One of the leading mechanisms of antimicrobial resistance is the production of extended-spectrum beta-lactamase (ESBL) enzymes, which hydrolyse and inactivate beta-lactam antibiotics. These enzymes play a critical role in the emergence of resistant bacterial species, posing a significant threat to public health (1).

Different methods are used to detect ESBL-producing bacteria. CHROMagar™ ESBL has been shown to be a useful screening tool among these methods. However, it has been reported that false positive results may occur, which may negatively affect the specificity of the test (2). In order to eliminate these false positive results, the double disk synergy test (DDST) is one of the widely used confirmatory tests to phenotypically evaluate ESBL production capacities in *Escherichia coli* strains. DDST is used in microbiology laboratories due to its ease of application, high accuracy and low cost (3).

ESBL-producing *E. coli* isolates, which have been frequently reported in poultry are important for both animal and public health (4). The emergence of these pathogens has generally been associated with excessive antibiotic use or uncontrolled practices (5). Studies conducted in recent years have shown increased ESBL production in *E. coli* isolates isolated from chickens. For example, a study conducted in Türkiye in 2020 reported that 56.1% of *E. coli* isolates obtained from chicken meat produced ESBL and 94.3% showed multidrug resistance (6). Another study emphasized that similar resistance profiles are common and stated that plasmid-mediated gene transfer is one of the main reasons for these common resistance profiles (7).

Biofilm formation is one of the most important resistance mechanisms developed by bacteria against both their environment and antibiotics (8). Biofilm structures allow microbial cells to adhere to surfaces and organize in a protective matrix which reduce the effectiveness of antibiotics and help bacteria to gain resistance to environmental stress factors (9). Although some studies have reported a relationship between biofilm formation and certain antibiotic resistance profiles, it has been reported that strong biofilm-forming strains are more frequently detected among multidrug-resistant (MDR) and extensively drug-resistant (XDR) isolates (10). However, it has also been shown that biofilm production may not always be directly related to antibiotic resistance and that different biological mechanisms may play a role in this complex event (8). This study was aimed to phenotypically investigate ESBL production, biofilm formation and resistance to various antibiotics in *E. coli* isolates obtained from broilers. Obtaining information about these factors is important for both the livestock sector and public health in order to develop effective strategies to limit the spread of resistant pathogens.

MATERIALS AND METHODS

Ethical Approval This study was conducted with the ethical approval of Aydın Adnan Menderes University Local Animal Experiments Ethics Committee (ADU-HADYEK) under the approval dated 09.01.2025 and numbered 64583101/2025/015.

Material

The material for this study consisted of 180 *E. coli* isolates obtained from the livers of 1- to 5-day-old Ross 308 broiler chickens suspected of colibacillosis. These chickens were raised in 15 commercial broiler farms throughout 2022. Sampling was conducted randomly among animals exhibiting clinical disease symptoms, and liver tissue samples were collected under aseptic conditions. Clinical signs of infectious conditions, including pericarditis, peritonitis, perihepatitis, airsacculitis, and arthritis, were reported in the broilers from which the isolates were obtained.

Isolation and Identification

Liver tissue samples were cultured on selective and differential media, including MacConkey Agar (Merck 1.05465, Germany) and Eosin Methylene Blue (EMB) Agar (Merck 1.01347, Germany). Petri dishes were incubated aerobically at 37°C for 24 hours. At the end of the incubation period, colonies exhibiting lactose fermentation on MacConkey Agar and characteristic green metallic sheen on EMB Agar were considered presumptive *E. coli* isolates. Suspected colonies were examined using Gram staining, followed by identification through standard biochemical tests (e.g., oxidase, catalase, indole, methyl red, citrate, etc.) as described previously (11). For storage, the isolates were prepared in Brain Heart Infusion Broth containing 20% glycerol (Oxoid CM 1135, UK) and stored at -20°C.

Antibiotic Susceptibility Tests

The antimicrobial resistance of the isolates was examined using the standard disk diffusion method. The resistance of *E. coli* isolates was assessed against eight antibiotics representing eight antimicrobial classes. For this purpose, antibiotics (Oxoid, CM 1135, UK) were used: ampicillin (AMP, 10 **µg**), amoxicillin/clavulanic acid (AML, 20/10 **µg**), ertapenem (E, 10 **µg**), gentamicin (GM, 10 **µg**), tetracycline (T, 30 **µg**), ciprofloxacin (CIP, 5 **µg**), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 **µg**), and nitrofurantoin (NIT, 300 **µg**) (Table 1). The inhibition zone diameters were interpreted according to the Clinical and Laboratory Standards Institute (12) guidelines as susceptible (S), intermediate (I), or resistant (R). *E. coli* ATCC 25922 was used as the quality control strain for antibiotic susceptibility testing. Resistance to three or more antimicrobial classes was defined as multidrug resistance (MDR) (13).

Phenotypic Detection of ESBL Production

CHROMagar™ ESBL: CHROMagar™ ESBL (CHROMagar, Paris, France) was utilized for the phenotypic preliminary detection of ESBL-producing *E. coli* isolates. This medium is a selective and differential chromogenic culture medium specifically designed for the direct and qualitative detection of ESBL-producing Gram-negative pathogens. It is used to indicate the presence of ESBL-producing Gram-negative bacteria. On CHROMagar™ ESBL, ESBL-producing *E. coli* isolates form colonies ranging in colour from dark pink to red, whereas non-ESBL-producing isolates either forming colourless colonies or exhibit no growth (14).

- **Double Disk Synergy Test (DDST):** The ESBL production of *E. coli* isolates preliminarily identified on CHROMagar™ was confirmed using the DDST method. The isolated *E. coli* strains were adjusted to a turbidity standard of 0.5 McFarland in sterile physiological saline. Subsequently, they were inoculated onto Mueller-Hinton Agar (MHA) (Merck, Germany) plates using a three-way streaking technique with a sterile swab. For the test, an amoxicillin-clavulanic acid (AMC) disk was placed at the centre of the agar plate. Other antibiotic disks (ceftazidime [CAZ; 30 µg], ceftriaxone [CRO; 30 µg], cefotaxime [CTX; 30 µg], and aztreonam [ATM; 30 µg]) were positioned 20 mm centre-to-centre from the AMC disk. Petri dishes were incubated aerobically at 35°C for 18-24 hours. After incubation, synergy zones between the disks were evaluated. If the inhibition zones surrounding the third-generation cephalosporin disks (CTX, CAZ, CRO, ATM) extended toward the AMC disk (keyhole appearance or synergy effect), ESBL production was considered positive. Conversely, if no synergy was observed between the inhibition zones, ESBL production was deemed negative (15).

Microplate Method

For this test, 3-5 colonies of pure *E. coli* isolates were suspended in Müller Hinton Broth (MHB) containing 2% sucrose and incubated at 37°C under stationary conditions for 18 hours. Following incubation, the bacterial suspension was adjusted to a turbidity standard of 0.5 McFarland. Subsequently, 200 µl of the bacterial suspension was inoculated into each well, excluding the last wells designated as negative controls, which contained sterile MHB. The inoculated plates were incubated for 48 hours to allow biofilm formation. After the incubation period, the contents of the wells were emptied, and the remaining bacteria were washed with saline. The bacteria adhering to the wells were stained with 0.1% crystal violet solution at room temperature for 15 minutes. The dye was removed using a pipette, and the wells were rinsed with water. Each well was carefully filled with 150 µl of 95% ethanol using a pipette, and the wells were sealed to minimize evaporation. The results were evaluated using an ELISA reader (BioTek Instruments, Winooski, VT, USA) at a wavelength of 620 nm. The biofilm formation was assessed and categorized as weak, moderate, or strong, following previously reported methods (16).

Statistical Analysis

The statistical analysis of the data obtained in this study was performed using version 23.0 of the SPSS (Statistical Package for the Social Sciences) software (SPSS Inc., Chicago, IL, USA). The Pearson Chi-square (χ^2) Test, and Fisher's Exact Test, when necessary, were applied to compare frequency data. These analyses were conducted to evaluate the relationship between the biofilm formation capacities and multidrug resistance characteristics of ESBL-producing and non-ESBL-producing *E. coli* isolates. A 95% confidence interval was used for evaluating the results, and differences with $p<0.05$ were considered statistically significant.

RESULTS

Phenotypic Detection of ESBL Production

CHROMagar™ ESBL: For the phenotypic preliminary detection of ESBL-producing isolates, all isolates were cultured on CHROMagar™ ESBL medium. Among the 180 isolates, 7.7% (14/180) were identified as ESBL-positive. These 14 isolates, detected as ESBL-positive on CHROMagar™ ESBL, were further analysed using DDST.

DDST: Based on the results, 78.6% of these isolates (11/14; 6.1% of the total isolates, 11/180) were confirmed as ESBL producers using DDST. Later, the antibiotic resistance profiles and biofilm formation capacities of all isolates were comprehensively evaluated, followed by detailed analyses (Figure 1.).

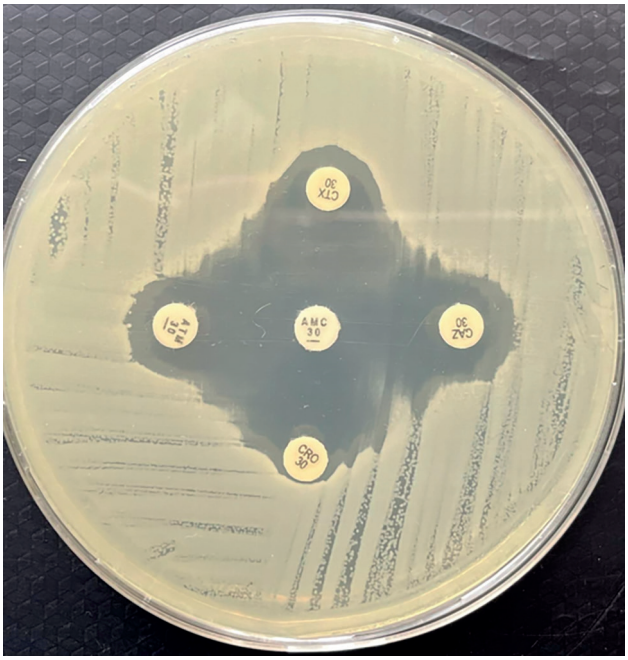

Figure 1: DDST test.

Antibiotic Resistance

High resistance rates to beta-lactam antibiotics were observed among ESBL-producing isolates, for example 90.9% for ampicillin and 63.6% for amoxicillin/clavulanic acid. Additionally, resistance rates for commonly used antibiotics gentamicin and tetracycline were identified as 72.7%. On the other hand, low resistance rates were recorded for antibiotics like nitrofurantoin and ertapenem, with both showing resistance rates of 9.1%.

In non-ESBL-producing isolates, overall antibiotic resistance rates were lower; however, resistance levels were observed for ampicillin (69.2%) and tetracycline (69.8%). When considering all isolates collectively, the highest resistance rates were identified for ampicillin (70.6%) and tetracycline (70.0%), while the lowest resistance rates were recorded for nitrofurantoin (5.0%) and ertapenem (3.3%) (Table 1, Figure 2).

Multidrug Resistance: Among ESBL-producing isolates, 90.9% (10/11) exhibited MDR characteristics, whereas 50.9% (86/169) of non-ESBL-producing isolates showed the same trait. Overall, the MDR rate across all isolates was determined to be 53.3% (96/180) (Table 2).

Table 1: Antibiotics used evaluation criteria and antimicrobial resistance profiles of isolates.

Antimicrobial Family	Antibiotic (Abbreviation) (Disk Content, µg)	Evaluation		Number of resistant isolates (%)					
		≥S	≤R	ESBL (+) Isolate		ESBL (-) Isolate		All Isolates	
				(N=11)	(%)	(N=169)	(%)	(N=180)	(%)
Penicillin	Ampicillin (AMP) (10)	17	13	10	(90.9)	117	(69.2)	127	(70.6)
Beta lactam	Amoxicillin Clavulonate (AMC) (20/10)	18	13	7	(63.6)	61	(36.1)	68	(37.8)
Carbapenem	Ertapenem (E) (10)	22	18	1	(9.1)	5	(2.9)	6	(3.3)
Aminoglycoside	Gentamicin (GM) (10)	15	12	8	(72.7)	51	(30.2)	59	(32.8)
Tetracycline	Tetracycline (T) (30)	15	11	8	(72.7)	118	(69.8)	126	(70.0)
Quinolone	Ciprofloxacin (CIP) (5)	31	20	7	(63.6)	55	(32.5)	62	(34.4)
Folate	Trimethoprim Sulfamethoxazole (TS) (1.25/23.75)	16	10	4	(36.4)	29	(17.2)	33	(18.3)
Nitrofuran	Nitrofurantoin (NIT) (300)	17	14	1	(9.1)	8	(4.7)	9	(5.0)

N: Isolate number

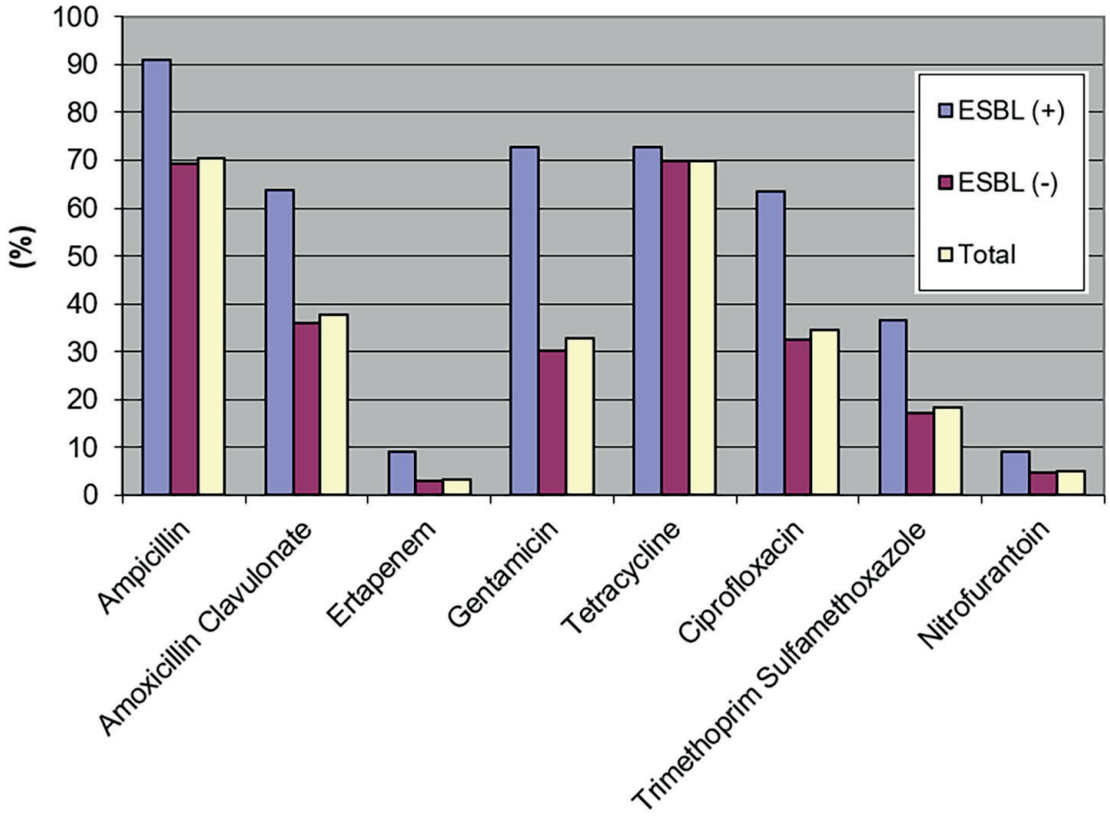

Figure 2: Antibiotic resistance status of ESBL-producing, non-ESBL-producing and all isolates.

Biofilm Formation Capacity

In this study, the biofilm formation capacities of ESBL-producing, non-ESBL-producing, and all *E. coli* isolates were assessed. The analysis revealed that 72.7% (8/11) of the ESBL-producing isolates exhibited weak biofilm formation, 9.1% (1/11) showed moderate biofilm formation, and 18.2% (2/11) did not form biofilms.

In contrast, 59.8% (101/169) of the non-ESBL-producing isolates did not form biofilms, while 24.8% (42/169) exhibited weak biofilm formation, and 15.4% (26/169) showed moderate biofilm formation.

When all isolates were evaluated together, 57.2% (103/180) were found to lack biofilm formation, whereas 42.8% (77/180) formed biofilms. Among those forming bio-

Table 2: MDR status of isolates.

Number of Resistant Antimicrobial Families	ESBL (+) Isolates		ESBL (-) Isolates		All isolates	
	(N=11)	(%)	(N=169)	(%)	(N=180)	(%)
0	0	(0.0)	4	(2.4)	4	(2.22)
1	0	(0.0)	28	(16.6)	28	(15.56)
2	1	(9.1)	51	(30.2)	52	(28.89)
3	1	(9.1)	49	(28.99)	50	(27.78)
4	5	(45.4)	29	(17.16)	34	(18.89)
5	3	(27.3)	8	(4.73)	11	(6.211)
6	1	(9.1)	0	(0.00)	1	(0.56)

ESBL+: Number of bacteria producing extended-spectrum beta-lactamase
ESBL-: Number of bacteria not producing extended-spectrum beta-lactamase
NMDR: Non-multiple antibiotic resistant

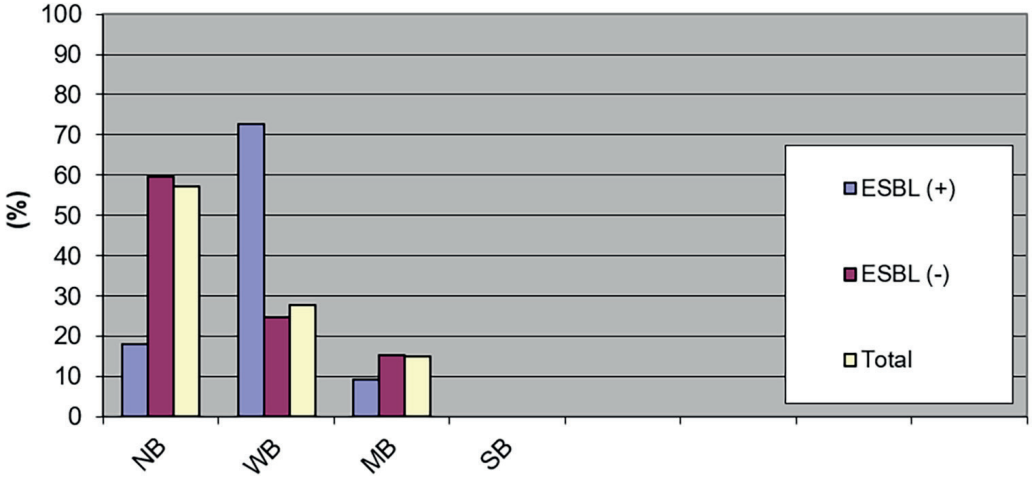

Figure 3: Biofilm formation status of isolates.

films, 27.8% (50/180) exhibited weak biofilm formation, and 15.0% (27/180) demonstrated moderate biofilm formation (Table 3, Figure 3).

Statistical Analysis

In this study, a statistically significant relationship was found between the biofilm formation capacity and MDR status of ESBL-producing and non-ESBL-producing *E. coli* isolates. According to the data, biofilm formation rates (P=0.01, Chi-square=7.2) and MDR rates (P=0.01, Chi-square=6.6) were significantly higher in ESBL-producing isolates (Table 4.)

DISCUSSION

The intensive use of antibiotics in the poultry sector accelerates the selection of resistant bacteria, causing significant damage to environmental ecosystems and posing serious

Table 3: Biofilm formation status of ESBL-producing and non-ESBL-producing isolates.

Biofilm formation status	ESBL (+) Isolate		ESBL (-) Isolate		All isolates	
	(N=11)	(%)	(N=169)	(%)	(N=180)	(%)
NB	2	(18.2)	101	(59.8)	103	(57.2)
WB	8	(72.7)	42	(24.8)	50	(27. 8)
MB	1	(9.1)	26	(15.4)	27	(15.0)
SB	0	(0.0)	(0.00)		(0.0)	

NB: Number of non-biofilm forming isolates; WB: Number of weak biofilm forming isolates
MB: Number of moderate biofilm forming isolates; SB: Number of strong biofilm forming isolates.

Table 4: Relationship between biofilm-forming capacities and MDR status of ESBL-producing and non-ESBL-producing *E. coli* isolates.

Number	ESBL (+) Isolate		ESBL (-) Isolate		P	χ^2
	(N=11)	(%)	(N=169)	(%)		
B+	9	(81.8)	68	(40.2)	0.01	7,2**
B-	2	(18.2)	101	(59.8)		
MDR+	10	(90.9)	86	(50.8)	0,01	6,6**
MDR-	1	(9.1)	83	(49.2)		

B+: Number of bacteria forming biofilm; B-: Number of bacteria not forming biofilm.
MDR+: Number of bacteria resistant to multiple antibiotics.
MDR-: Number of bacteria not resistant to multiple antibiotics.

threats to public health (17). In particular, infections like colibacillosis not only lead to substantial economic losses in broiler production but also facilitated the spread of resistant *E. coli* strains, resulting in secondary public health impacts (18). Key factors in the dissemination of these resistant bacteria include the food chain, manure applications, and contaminated water sources (17). Therefore, the phenotypic examination of ESBL production and antimicrobial resistance mechanisms in *E. coli* isolates from broilers is crucial for both infection control and understanding resistance mechanisms.

Of the 180 isolates, 7.7% (14 isolates) were identified as ESBL-positive using CHROMagar™ ESBL as a screening medium. As a confirmatory test, 6.1% (11 isolates) of all isolates were confirmed as ESBL producers by DDST. CHROMagar™ ESBL is reported as a preferred method with its high sensitivity and specificity in the rapid and cost-effective screening of ESBL-producing bacteria. However, literature has shown that this medium may produce false-positive results, especially in the presence of bacteria producing AmpC type beta-lactamase (2). Therefore, after the preliminary screening test, isolates should be tested with a confirmatory test using the DDST test and the results should be confirmed. DDST is recommended as a reliable method especially in the detection of bacteria resistant to third-generation cephalosporins (19). However, DDST has the disadvantage that the presence of other beta-lactamase enzymes can mask ESBL production and, in some cases, potentially give false-negative results. Therefore, using both methods together can minimize false-positive and false-negative results in detecting ESBL production (2). One advantage of CHROMagar™ ESBL is its ability to easily assess polymicrobial cultures. However, it has been reported that the false-positive rate may increase due to overlap in culture morphology or cross-enzyme effects. By using clavulanic acid as an inhibitor, DDST is known to increase the detection of ESBL production, specifically addressing such issues (2).

In our study, the resistance profiles of *E. coli* isolates obtained from broilers to antibiotics were investigated. High resistance rates were found in ESBL-producing isolates to antibiotics commonly used in the treatment of poultry diseases, such as ampicillin, gentamicin and tetracycline. These findings suggest selective pressure mechanisms driven by excessive and uncontrolled use of antibiotics. Interestingly, low resistance rates were found for antibiotics such as nitrofurantoin and ertapenem, which are not commonly used in broiler farming. This result may be related to environmental factors such as cross-resistance mechanisms or horizontal gene transfer. It has been reported that resistance genes for nitrofurantoin and carbapenems (e.g. *bla*OXA-48, *bla*NDM-1, etc.) can be introduced into farm environments via water sources, feed and human waste and contribute to the spread of resistant microorganisms (2). Moreover, these resistance genes can be naturally present in the environment and can spread opportunistically, even at low levels, in farm environments. These findings indicate that further research is needed to better understand the potential sources and spread mechanisms of resistant bacteria and resistance genes in farm environments.

In our study, the relationship between biofilm formation capacity and MDR status of ESBL-producing and non-ESBL-producing *E. coli* isolates was investigated. The findings showed that 18.2% of ESBL-positive isolates did not form biofilm, while 81.8% exhibited weak or moderate biofilm formation capacity. This suggests that the relationship between ESBL production and biofilm formation is complex and is potentially mediated by independent genetic and environmental mechanisms (19, 20). For example, in ESBL-positive isolates that do not form biofilms, it is thought that environmental factors or genetic mechanisms may have inhibitory effects on biofilm formation (2). Similarly, it was found that 9.1% of ESBL-producing isolates did not exhibit MDR properties, while 40.2% of ESBL-negative isolates were able to form biofilms and 50.8% exhibited MDR properties. Our results revealed that ESBL production is not always directly linked to MDR or biofilm formation. This may be explained by differences in the interactions between bacterial resistance genes and differences in horizontal gene transfer mechanisms (21). These findings indicate the need for more detailed molecular studies of the relationships between ESBL production, biofilm formation, and MDR. Elucidating the genetic mechanisms underlying these processes may benefit the development of strategies to combat antimicrobial resistance more effectively.

To the best of our knowledge, this article is the first comprehensive study examining the phenotypic ESBL production, biofilm capacity and antibiotic resistance profiles altogether of *E. coli* isolates in broilers in Türkiye. These results shows that detailed molecular studies are needed

on biofilm formation and antibiotic resistance mechanisms of ESBL-producing *E. coli* isolates obtained from broilers. Preventive measures such as increasing biosecurity measures, encouraging alternative treatment approaches and implementing public health education programs are considered important in combating antimicrobial resistance.

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