Adıyaman University Journal of Science
https://dergipark.org.tr/en/pub/adyujsci
DergiPark AKADEMİK
ISSN 2147-1630
e-ISSN 2146-586X

Multiplex PCR-based Molecular Characterization of Fecal-Derived Antibiotic Drug Resistant *Escherichia coli* Isolates

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Received: 01.04.2026 **Accepted:** 08.06.2026 **Published:** 30.06.2026

Abstract

Antimicrobial resistance (AMR) in *Escherichia coli* poses a serious public health threat, particularly when resistant strains spread through contaminated food and water. This study characterized fecal-derived multidrug-resistant (MDR) *E. coli* isolates using multiplex polymerase chain reaction (PCR) to detect extended-spectrum β-lactamase (ESBL) gene variants (bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$), sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*), and integron-associated integrase genes (*int1*, *int2*, and *int3*). We analyzed 62 *uspA*-positive *E. coli* isolates. Results showed that 82.26% of isolates carried none of the targeted *bla* genes. The $bla_{CTX\text{-}M}$ and bla_{OXA} genes were each present in 9.68% and 9.68% of isolates, respectively. Co-occurrence of *bla* and *sul* genes was observed in 8.06% of isolates. Co-occurrence of *bla* and *int* genes occurred in 14.52%. Only 8.06% of isolates carried at least one gene from all three categories, indicating potential MDR genotypes. These findings highlight the importance of molecular

* Corresponding Author DOI: 10.37094/adyujsci.1919076

surveillance in tracking resistance genes outside clinical settings and support the need for integrated One Health strategies to combat AMR.

Keywords: *Escherichia coli*; Multiplex PCR; ESBL; Sulfonamide resistance; Integrons, fecal isolates.

Dışkı Kaynaklı Antibiyotik Dirençli *Escherichia coli* İzolatlarının Çoklu PCR Temelli Moleküler Karakterizasyonu

Öz

Escherichia coli'de antimikrobiyal direnç (AMD), özellikle dirençli suşların kontamine yiyecek ve su yoluyla yayılması durumunda ciddi bir halk sağlığı tehdidi oluşturmaktadır. Bu çalışmada, fekal kaynaklı ilaç dirençli *E. coli* izolatları, genişlemiş spektrumlu β-laktamaz (ESBL) gen varyantlarını (bla_{TEM}, bla_{OXA}, bla_{SHV} ve bla_{CTX-M}), sülfonamid direnç genlerini (*sul1*, *sul2* ve *sul3*) ve integron ilişkili integraz genlerini (*int1*, *int2* ve *int3*) tespit etmek amacıyla multipleks polimeraz zincir reaksiyonu (PZR) kullanılarak karakterize edilmiştir. Altmış iki *uspA*-pozitif *E. coli* izolatı analiz edilmiştir. Sonuçlar, izolatların %82,26'sının hedef alınan *bla* genlerinin hiçbirini taşımadığını göstermiştir. bla_{CTX-M} ve bla_{OXA} genleri her iki izolat için de %9,68 bulunmuştur. *bla* ve *sul* genlerinin birlikte bulunma oranı izolatların %8,06'sında görülmüştür. *bla* ve *int* genlerinin birlikte bulunma oranı ise %14,52 olarak gerçekleşmiştir. İzolatların yalnızca %8,06'sı her üç kategoriden de en az bir gen taşımakta olup bu durum potansiyel çoklu ilaç direnci genotiplerine işaret etmektedir. Bu bulgular, klinik ortamlar dışında direnç genlerinin izlenmesinde moleküler sürveyansın önemini vurgulamaktadır. Ayrıca, AMD ile mücadele için bütünleşik "Tek Sağlık" stratejilerine duyulan ihtiyacı desteklemektedir.

Anahtar Kelimeler: *Escherichia coli*; Multipleks PZR; GSBL; Sülfonamid direnci; İntegronlar; Fekal izolatlar.

1. Introduction

Escherichia coli, a Gram-negative bacterium of the family Enterobacteriaceae, is a ubiquitous component of the intestinal microbiota in humans and other warm-blooded animals. While most strains are harmless commensals, certain pathogenic variants, including both intestinal and extraintestinal pathotypes, can cause a range of diseases from self-limiting diarrhea to life-threatening systemic infections such as bacteremia, urinary tract infections, and neonatal meningitis [1,2]. The clinical and public health burden posed by *E. coli* has been significantly

worsened by the global rise of antimicrobial resistance (AMR), particularly the emergence and dissemination of multidrug-resistant (MDR) strains.

A critical driver of MDR in *E. coli* is the production of extended-spectrum β-lactamases (ESBLs), enzymes that hydrolyze and confer resistance to a broad range of β-lactam antibiotics, including penicillins, cephalosporins, and aztreonam. The most prevalent ESBL genes are variants of bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$, with $bla_{CTX\text{-}M}$ enzymes now dominating globally in both clinical and community settings [3,4]. Additionally, plasmid-mediated resistance to sulfonamides, historically among the first synthetic antimicrobials, is frequently conferred by *sul1*, *sul2*, and *sul3* genes, which encode dihydropteroate synthase variants with reduced drug affinity [5]. The genetic mobility and persistence of these resistance determinants are often facilitated by mobile genetic elements, particularly integrons. Integrons are genetic platforms that can capture, integrate, and express resistance gene cassettes via a site-specific recombination mechanism. Class 1 integrons that carrying *int1*, are strongly associated with MDR profiles in clinical *Enterobacteriaceae*, serving as key vectors for the accumulation and dissemination of resistance genes, including those encoding ESBLs and sulfonamide resistance [6,7].

Fecal carriage of MDR *E. coli*, including ESBL producers, represents a significant reservoir for community transmission and a potential source of infections. Humans can acquire these strains through the consumption of contaminated food or water, direct contact with animals, or environmental exposure. Once established in the gut, resistant *E. coli* can persist, facilitate further horizontal gene transfer, and serve as a silent reservoir for future infections or transmission to others [8,9]. Therefore, surveillance of resistance genes in fecal-derived isolates is crucial for understanding the epidemiology of AMR outside hospital settings and for informing public health interventions.

Conventional phenotypic methods for antimicrobial susceptibility testing, though essential, are time-consuming and provide limited information on the underlying genetic mechanisms. Molecular techniques, particularly polymerase chain reaction (PCR), offer a rapid, specific, and sensitive alternative for detecting resistance genes directly from bacterial DNA. Multiplex PCR, which enables the simultaneous amplification of multiple target genes in a single reaction, is especially valuable for high-throughput screening and comprehensive resistance profiling [10,11].

This study aimed to conduct a detailed molecular characterization of fecal-derived *E. coli* isolates from a clinical collection. Using a multiplex PCR approach, we targeted a panel of clinically relevant genes, including four ESBL variants (bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$),

three sulfonamide resistance determinants (*sul1*, *sul2*, and *sul3*), and three integron-associated integrase genes (*int1*, *int2*, and *int3*). The objectives were to determine the prevalence and distribution of these resistance markers, analyze their co-occurrence patterns to assess potential MDR profiles, and contribute to the epidemiological understanding of resistance gene carriage in community-associated *E. coli* strains in the study region.

2. Materials and Methods

2.1. Bacterial Isolates

A total of 62 *E. coli* isolates, previously isolated from human stool samples, were obtained from the laboratory collection. The original isolates were received as fully anonymized and de-identified cultures with no link to patient identifiers. All isolates were confirmed as *E. coli* by amplification of the *uspA* gene (884 bp) using specific primers (Table 1) [12].

Table 1: Primer sequences used for the detection of antibiotic resistance and virulence genes in *E. coli* isolates by PCR in this study.

Gene	Primer Sequences (5'→3')	Amplicon length (bp)	Reference
	Molecular Verification of *E. coli* (Species-specific)		
uspA	F: CCGATACGCTGCCAATCAGT	884	[12]
	R: ACGCAGACCGTAGGCCAGAT		
	ESBL Gene Variants		
bla$_{CTX-M}$	F: CGCTTTGCGATGTGCAG	551	[13]
	R: ACCGCGATATCGTTGGT		
bla$_{OXA}$	F: TCAACTTTCAAGATCGCA	610	
	R: GTGTGTTTAGAATGGTGA		
bla$_{SHV}$	F: TTATTTCCCTGTTAGCCACC	727	
	R: GATTTGCTGATTTCGCTCCGG		
bla$_{TEM}$	F: ATAAAATTCTTGAAGACGAAA	1080	
	R: GACAGTTACCAATGCTTAATC		
	Sulfonamide Resistance Genes		
sul1	F: CGGCGTGGGCTACCTGAACG	433	[14]
	R: GCCGATCGCGTGAAGTTCCG		
sul2	F: GCGCTCAAGGCAGATGGCATT	293	
	R: GCGTTTGATACCGGCACCCGT		
sul3	F: GAGCAAGATTTTTGGAATCG	750	[15]
	R: CTAACCTAGGGCTTTGGATAT		
	Integron Genes		

int1	F: CCCGAGGCATAGACTGTA R: CAGTGGACATAAGCCTGTTC	160	[16]
int2	F: CACGGATATGCGACAAAAAG R: GATGACAACGAGTGACGAAA	788	
int3	F: GCCTCCGGCAGCGACTTTCAG R: ACGGATCTGCCAAACCTGACT	979	

2.2. DNA Extraction

DNA was extracted by boiling method as described by Arslan and Uçan [17]. Briefly, bacterial suspensions were heated at 95°C for 5 min, centrifuged, and supernatants were stored at −20°C for PCR analysis.

2.3. Multiplex PCR Assays

Three separate multiplex PCR assays were optimized for the detection of:

a. ESBL genes: bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$

b. Sulfonamide resistance genes: *sul1*, *sul2*, and *sul3*

c. Integron genes: *int1, int2,* and *int3*

Primer sequences and amplicon sizes are listed in Table 1. In the current study, all used PCR conditions followed as suggested by the protocols of the cited research [12-16]. Briefly, to identify ESBL gene variants, PCR conditions were as follows: 95°C for 13 minutes; 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. For detection of sulfonamide resistance genes, PCR conditions were: 94°C for 5 minutes; 36 cycles of 94°C for 15 seconds, 67°C for 30 seconds, 72°C for 60 seconds; and 72°C for 7 minutes. For determination of integron class-specific genes, PCR conditions were: 94°C for 10 minutes; 32 cycles of 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds; and a final extension at 72°C for 10 minutes.

2.4. Gel Electrophoresis

PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light. A 100 bp DNA ladder was used as a molecular size marker.

3. Results and Discussion

3.1. Molecular Confirmation of E. coli Isolates

All 62 presumed *E. coli* isolates included in this study underwent molecular confirmation by PCR targeting the species-specific *uspA* (universal stress protein A) gene. Amplification of the expected 884-bp fragment was successfully observed in all 62 isolates, confirming their identity as *E. coli* with 100% positivity (Fig. 1). This step ensured the genetic integrity of the sample set prior to resistance gene profiling.

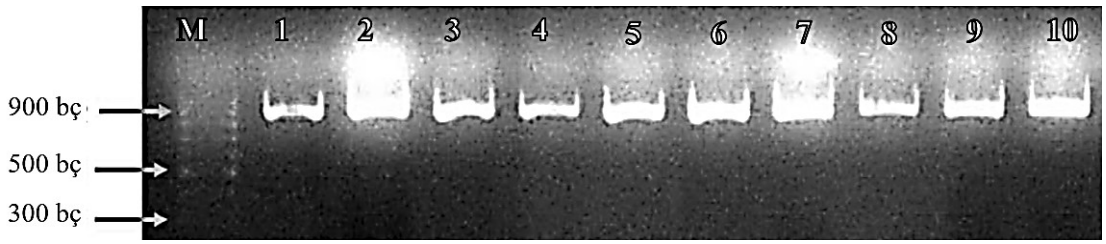

Figure 1: *E. coli* confirmation, gel images of the amplicons belonging to the *uspA* gene (884 bp, samples 1–10, M: Marker)

3.2. Prevalence and Distribution of ESBL Gene Variants

Multiplex PCR analysis for four major ESBL gene families revealed a notably low overall prevalence in this collection of fecal-derived isolates. As detailed in Table 2, the majority of isolates (51/62, 82.25%) did not harbor any of the targeted *bla* genes (bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$).

Table 2: Distribution of resistance genes by genetic profile among fecal E. coli isolates (n=62).

Profile	Gene Combination	Isolate Count (n)	Isolate Numbers
A	No gene detected	15	15, 16, 29, 30, 32, 34, 35, 36, 47, 48, 50, 51, 52, 53, 55
B	*sul1* only	2	31, 43
C	*sul2* only	1	44
D	*int1* only	16	1, 4, 5, 11, 14, 19, 21, 22, 23, 24, 25, 57, 58, 59, 61, 62
E	*int2* only	11	17, 18, 20, 26, 27, 37, 38, 39, 40, 41, 42
F	bla_{OXA} only	2	33, 49
G	$bla_{CTX\text{-}M}$ + *int1* + *int2*	1	2
H	$bla_{CTX\text{-}M}$ + *sul1* + *sul2* + *int1*	2	3, 9
I	$bla_{CTX\text{-}M}$ + *sul1* + *int1*	2	7, 8
J	$bla_{CTX\text{-}M}$ + bla_{OXA} + *sul1* + *int1* + *int2*	1	13
K	bla_{OXA} + *int1*	3	54, 56, 60
L	*sul1* + *int1*	1	12
M	*sul1* + *int2*	1	28
N	*sul2* + *int1*	2	6, 10
O	*int1* + *int2*	2	45, 46

Among the *bla*-positive isolates, bla_{CTX-M} was the most frequently detected variant, identified in 6 isolates (9.68% of the total). This was closely followed by bla_{OXA}, found in 6 isolates (9.68%). A critical observation was the absence of the bla_{TEM} and bla_{SHV} genes in any isolate. Furthermore, no isolate was found to carry more than a single *bla* gene; the simultaneous presence of bla_{CTX-M} and bla_{OXA} was detected in only one isolate (1.61%), and no other combinations were observed. The electrophoretic profile of *bla* gene amplification is presented in Fig. 2, clearly showing the specific bands corresponding to bla_{CTX-M} in positive samples.

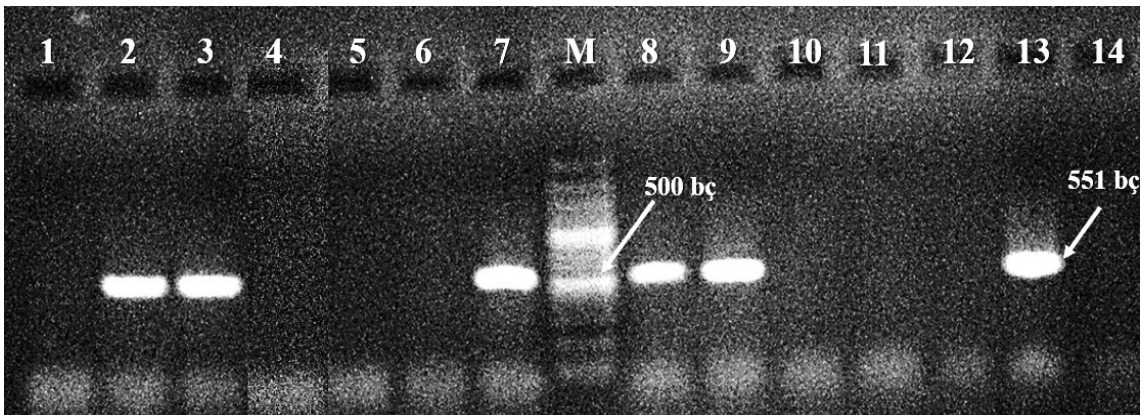

Figure 2: *bla* genes: agarose gel electrophoresis image of products after multiplex PCR. Samples 1–15, M: 100 bp DNA marker (bla_{CTX-M} = 551 bp)

3.3. Profile of Sulfonamide Resistance Genes

Screening for sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) demonstrated a higher detection frequency than ESBL genes. In total, 12 isolates (19.35%) carried at least one *sul* gene (Table 2). The *sul1* gene was the most common variant, present in 9 isolates (14.52%). The *sul2* gene was detected in 6 isolates (9.68%). Notably, the *sul3* gene was not identified in any isolate. Regarding gene combinations, 12 isolates (19.35%) carried a single *sul* gene (either *sul1* or *sul2*) while 2 isolates (3.23%) harbored both *sul1* and *sul2* simultaneously.

3.4. Detection and Frequency of Integron-Associated Genes

Integron screening produced the highest positivity rate among the three gene categories tested. At least one integron integrase gene (*int1*, *int2*, or *int3*) was detected in 42 isolates (67.74%). The class 1 integron gene (*int1*) was by far the most prevalent, found in 30 isolates (48.39%). The class 2 integron gene (*int2*) was present in 16 isolates (25.81%). As with *sul3*, the class 3 integron gene (*int3*) was absent from all tested isolates (Table 2).

Analysis of co-occurrence among the integron genes showed that 3 isolates (4.84%) carried both *int1* and *int2* simultaneously. No isolate harbored all three integron genes. The PCR results

for integron genes are visualized in Figs. 3(A) and (B), showing distinct bands for *int1* (160 bp) and *int2* (788 bp).

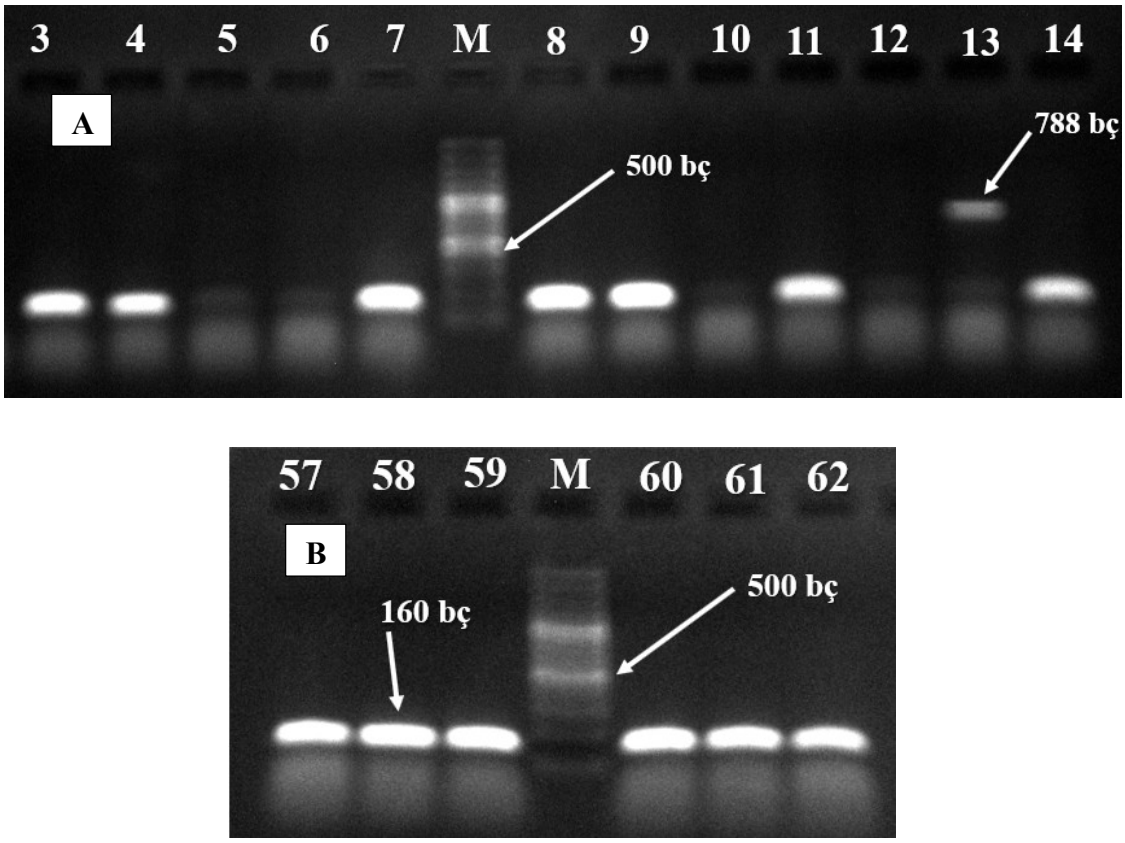

Figure 3: Gel images of PCR products for integron gene detection. A) Lanes 3–14 show amplified DNA from bacterial samples. B) Lanes 57–62 show amplified DNA from additional bacterial samples. A 100 bp DNA ladder (lane M) is included for size comparison. Target bands: *int1* gene at 160 bp, *int2* gene at 788 bp

3.5. Co-occurrence Analysis of Resistance Genes Across Categories

A comprehensive analysis was conducted to investigate the simultaneous carriage of resistance genes from different functional classes (*bla*, *sul*, and *int*), which indicates potential multidrug-resistant (MDR) genotypes. The detailed combinations and frequencies are summarized in Table 3.

Table 3: Co-occurrence of *bla*, *sul*, and i*nt* resistance genes in *E. coli* isolates.

Gene Combinations	Number of Isolate (n)	Rate (%)	Explanations
bla* + *sul1	5	8.06	At least one *bla* gene together with *sul1*
bla* + *sul2	2	3.23	At least one *bla* gene together with *sul2*
bla* + *sul1* + *sul2	2	3.23	At least one *bla* gene together with *sul1* and *sul2*
bla* + *sul1* + *sul2* + *sul3	0	0.00	*sul3* gene not detected in any isolate
bla* + *int1	9	14.42	At least one *bla* gene together with only *int1*
bla* + *int2	2	3.23	At least one *bla* gene together with only *int2*
bla* + *int1* + *int2	2	3.23	At least one *bla* gene together with both *int1* and *int2*
bla* + *int1* + *int3	0	0.00	At least one *bla* gene together with both *int1* and *int3*
bla* + *int1* + *int2* + *int3	0	0.00	All three integron genes not observed together
sul* + *int1	9	14.52	At least one *sul* gene together with *int1*
sul* + *int2	4	6.45	At least one *sul* gene together with *int2*
sul* + *int1* + *int2	1	1.61	At least one *sul* gene together with both *int1* and *int2*
sul* + *int1* + *int3	0	0.00	Not Observed
bla* + *sul* + *int	5	8.06	At least one *bla*, one *sul*, and one *int* gene together

The co-occurrence of ESBL and sulfonamide resistance genes was observed in a subset of isolates. The combination of at least one *bla* gene with *sul1* was found in 5 isolates (8.06%), while a *bla* gene with *sul2* was present in 2 isolates (3.23%). Two isolates (3.23%) carried at least one *bla* gene together with both *sul1* and *sul2*.

The association between ESBL genes and integrons was also examined. The combination of a *bla* gene with the *int1* gene alone was detected in 9 isolates (14.52%). Similarly, a *bla* gene with *int2* alone was 2 isolate (3.23%), while 2 isolates (3.23%) carried a *bla* gene with both *int1* and *int2*.

The linkage between sulfonamide resistance and integrons was notably more common. At least one *sul* gene was found together with only *int1* in 9 isolates (14.52%). The combination of a *sul* gene with *int2* was less frequent (4 isolates, 6.45%), and only one isolate (1.61%) carried a *sul* gene with both *int1* and *int2*.

The most significant finding, indicating a complex MDR genetic background, was the simultaneous presence of at least one gene from all three categories: *bla*, *sul*, and *int*. This triad was identified in only 5 isolates, representing 8.06% of the total collection. These isolates constitute a high-risk subset, as they possess genetic determinants for resistance to critically important β-lactams and sulfonamides, together with the genetic machinery (integron) that facilitates the acquisition and expression of further resistance genes.

4. Discussion

This study provides a detailed molecular characterization of resistance genes in fecal-derived *Escherichia coli* isolates, focusing on the prevalence and co-occurrence of extended-spectrum β-lactamase (ESBL), sulfonamide resistance, and integron-associated genes. The findings contribute to understanding the reservoir of antimicrobial resistance (AMR) determinants in community-associated bacterial populations and their potential for dissemination.

The high proportion of isolates lacking any of the targeted *bla* genes (82.26%) suggests that ESBL production may not be highly prevalent among fecal *E. coli* in the sampled community. This contrasts with higher rates often reported in clinical isolates from healthcare settings, where antibiotic selection pressure is more intense [9,19]. The fecal origin of these isolates likely reflects a lower direct exposure to broad-spectrum antibiotics compared to hospitalized patients. However, individually, $bla_{CTX\text{-}M}$ and bla_{OXA} were each identified in 9.68% of the isolates. The presence of $bla_{CTX\text{-}M}$, in particular, aligns with its status as a globally dominant ESBL gene variant reported in both clinical and community studies [8,20]. The absence of bla_{TEM} and bla_{SHV}, which are also common in clinical isolates from Turkey and elsewhere, may indicate a different epidemiological pattern or selective advantage for $bla_{CTX\text{-}M}$ and bla_{OXA} in the community-gut environment. Notably, no isolate carried more than one *bla* gene, which contrasts with findings from some clinical and foodborne MDR strains where multiple β-lactamase genes co-exist, suggesting a more limited genetic repertoire in this fecal collection [21].

The profile of sulfonamide resistance genes revealed *sul1* as the most prevalent (14.52%), followed by *sul2* (8.06%), with *sul3* entirely absent. This distribution is consistent with reports from Turkey and other regions, where *sul1* and *sul2* are frequently detected in *E. coli* from clinical, environmental, and food sources, while *sul3* is less common [22-24]. The higher

frequency of *sul1* may be mechanistically linked to its common location within conserved regions of class 1 integrons, acting as a platform for capturing additional resistance gene cassettes. This association is supported by our finding that *sul1* often co-occurred with the class 1 integron gene *int1*. The use of sulfonamides in veterinary and agricultural settings, due to their low cost and broad-spectrum activity, likely drives the selection and persistence of these resistance genes, facilitating their entry into the human gut microbiota via the food chain [25,26].

Integron analysis showed a high detection rate, with 67.74% of isolates carrying at least one integron integrase gene. Class 1 integrons (*int1*) were by far the most abundant (48.39%), a finding consistent with numerous studies identifying class 1 integrons as the primary vehicle for resistance gene dissemination among Gram-negative bacteria [7,27]. The presence of class 2 integrons (*int2*) in 25.81% of isolates is notable, as they are typically less common. The simultaneous carriage of *int1* and *int2* in a subset of isolates (6.45%) suggests complex genetic backgrounds and potential for horizontal gene transfer between different mobile genetic elements. The complete absence of class 3 integrons (*int3*) aligns with their documented rarity in most geographical and clinical contexts [6]. The high prevalence of integrons, particularly class 1, underscores their critical role in assembling and spreading multidrug resistance cassettes, acting as a genetic library for resistance traits [28].

The co-occurrence analysis of resistance genes across different functional classes provides insight into the potential for multidrug resistance. Combinations of *bla* and *sul* genes or *bla* and *int* genes were observed in 14.42% and 20.97% of isolates, respectively. However, the simultaneous carriage of at least one gene from all three categories (*bla*, *sul*, and *int*) was limited to 8.06% (5 isolates). This indicates that while individual resistance determinants are circulating, the convergence of mechanisms conferring resistance to β-lactams and sulfonamides, coupled with an efficient gene-capturing integron platform, is not yet widespread in this specific fecal *E. coli* population. Nevertheless, these few isolates represent a high-risk genotype. They possess the genetic foundation for resistance to critically important antibiotic classes and the machinery (integron) to acquire further resistance genes, positioning them as potential precursors for pan-drug resistant clones [29].

The detection of clinically relevant resistance genes in fecal isolates has important public health implications. It confirms that asymptomatic individuals in the community can serve as reservoirs for antibiotic-resistant *E. coli*. These strains can persist in the gut and be shed into the environment, contaminating water sources and soil. They can also be transmitted through the food chain or direct contact, posing a risk for endogenous infections (e.g., urinary tract infections) or outbreaks [30,31]. The presence of $bla_{CTX\text{-}M}$ and *int1* in community isolates mirrors findings from

other Turkish studies on fecal carriage of ESBL producers, highlighting a national public health concern that extends beyond hospital wards [9]. These findings suggest a potential benefit of integrating molecular surveillance into AMR monitoring programs. Phenotypic susceptibility testing alone cannot reveal the genetic complexity and transmission potential of resistant bacteria. Techniques such as multiplex PCR, as employed here, offer a rapid and informative tool for tracking specific high-risk resistance genes and mobile elements in both clinical and community settings. Furthermore, combating AMR effectively requires a holistic "One Health" strategy. The interconnectedness of human, animal, and environmental health means that resistance genes selected in livestock through antibiotic use can appear in human commensals via food, and human waste can disseminate them into the environment [32,33]. Therefore, coordinated actions (including prudent antibiotic use in human medicine and agriculture, improved sanitation, wastewater treatment, and ongoing genomic surveillance) are essential to mitigate the spread of resistant bacteria like MDR *E. coli*.

5. Conclusion

This study demonstrates the utility of multiplex PCR for rapid resistance gene profiling in fecal-derived *E. coli*. Detecting ESBL, sulfonamide, and integron genes, even at relatively low frequencies, confirms the ongoing circulation of resistance determinants in the community. Continuous surveillance, rational antibiotic use, and targeted public health interventions are needed to mitigate MDR *E. coli* spread. Future studies should include larger sample sizes, temporal sampling, and genomic sequencing to better understand transmission dynamics and resistance mechanisms.

Acknowledgement

This manuscript is a part of Doğan SARIGÜL's thesis (Hatay Mustafa Kemal University / Institute of Natural Sciences / Department of Biology, BSc Thesis, 2025).

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