Molecular Detection of ESBL and Integron Genes in Multidrug-Resistant *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Mastitic Cow Milk

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ABSTRACT

Antibiotic resistance represents an escalating global concern affecting both human and veterinary medicine. The dissemination of plasmid-mediated extended-spectrum β-lactamase (ESBL) genes among bacterial populations poses a serious threat to public health and food safety. This study investigated the prevalence, antimicrobial resistance profiles, and genetic characteristics of ESBL-producing Gram-negative bacteria isolated from bovine mastitic milk. A total of 312 milk samples were collected from dairy farms, yielding 21 *Escherichia coli* and 7 *Klebsiella pneumoniae* isolates confirmed as ESBL producers through chromogenic media and phenotypic confirmation by the combined disk test. Antimicrobial susceptibility testing was performed using the disk diffusion method with twelve antibiotics from eight classes. Molecular identification of *E. coli* was conducted by uspA and trpBA gene amplification, while 16S rRNA gene sequencing was used for *K. pneumoniae*. PCR was applied to detect *blaCTX-M*, *blaTEM*, *blaSHV*, *int1*, and *int2* genes. Multidrug resistance (MDR) was identified in 86% of isolates, with complete resistance to cefotaxime and ceftriaxone. Gene prevalence rates were 93% (*blaCTX-M*), 67% (*blaTEM*), 67% (*blaSHV*), 64% (*int1*), and 4% (*int2*). Chi-square analysis revealed significant associations between MDR and the presence of *blaTEM*, *blaSHV*, and *int1* ($p<0.05$). These findings underscore the public health risks posed by MDR bacteria in dairy herds and highlight the need for integrated surveillance and prudent antimicrobial stewardship.

Keywords: Bovine mastitis; Drug Resistance; Beta-Lactamases; *Escherichia coli*; *Klebsiella pneumoniae*

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are enzymes produced by Gram-negative bacteria such as *Escherichia coli* and *Klebsiella* spp., enabling them to hydrolyze β-lactam antibiotics and contribute significantly to antimicrobial resistance (1).

The first ESBL-producing *Klebsiella pneumoniae* strain was reported in 1983 in Germany (2), and since then, ESBL-producing bacteria have spread widely, including into livestock populations (3).

Dairy cattle represent a critical node in the One Health interface, where mastitis-associated pathogens may harbor and disseminate resistance genes through milk and environmental contamination (4). Chromogenic media like CHROMagar™ ESBL allow rapid detection (5), while the Combined Disk Test (CDT) and PCR targeting *blaCTX-M*,

blaTEM, and *blaSHV* enable confirmatory and genotypic detection (6,7).

In Türkiye, ESBL-producing *E. coli* have also been reported in healthy cattle, indicating a potential gastrointestinal reservoir (8), and recent evidence confirms their presence in mastitic milk (9). Integrons, particularly *int1* and *int2*, are often plasmid-borne and mediate horizontal gene transfer (10), with *int1* frequently associated with *blaCTX-M* genes (11).We hypothesize that Gram-negative mastitis agents may harbor ESBL genes and integrons, and that these elements contribute to multidrug resistance. This study investigates ESBL-producing *E. coli* and *K. pneumoniae* from mastitic milk, characterizes their resistance profiles, and assesses the distribution of *blaCTX-M*, *blaTEM*, *blaSHV*, *int1*, and *int2*.

MATERIAL AND METHODS

Ethics Approval

This is an observational study. The Animal Experiments Local Ethics Committee (ADU-HADYEK) of Aydın Adnan Menderes University confirmed that no ethical approval was required, as procedures such as diagnostic sampling and milking are exempt according to Article 8k/2 of the ADU-HADYEK Directive.

Sample Collection

Between June 2023 and June 2024, 312 bovine milk samples were collected from 27 dairy farms in Aydın Province, Türkiye. Subclinical mastitis was diagnosed using the California Mastitis Test (CMT; Bavivet CMT Liquid, Kruuse®) (12). From each positive cow, 5–10 mL of milk was aseptically collected from the most affected quarter into sterile tubes. All animals were antibiotic-free for at least three weeks prior to sampling. Samples were transported under cold chain conditions for immediate processing.

Isolation of ESBL-Producing Gram-Negative Bacteria

Milk samples were directly inoculated onto CHROMagar™ ESBL (Paris, France) and incubated at 37°C for 24–48 hours. Presumptive colonies (*Escherichia coli*: dark pink; *Klebsiella* spp.: metallic blue; *Acinetobacter* spp.: cream; *Pseudomonas* spp.: translucent) (13) were subcultured onto EMB and MacConkey agar (Oxoid, UK) for purification and preliminary biochemical identification (12), followed by molecular confirmation.

Phenotypic Confirmation of ESBL Production

Phenotypic detection was performed using the Combined Disk Test (CDT), employing cefotaxime, ceftazidime, and cefepime discs, both alone and combined with clavulanic acid. A ≥5 mm increase in inhibition zone in the presence of clavulanic acid indicated ESBL production. Double Disk Synergy Test (DDST) was used as confirmatory testing (6). *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 served as positive and negative controls, respectively.

Antimicrobial Susceptibility

The Kirby-Bauer disk diffusion method was applied using twelve antibiotics across eight classes: amoxicillin-clavulanic acid, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, aztreonam, imipenem, ciprofloxacin, levofloxacin, sulfamethoxazole-trimethoprim, chloramphenicol, and gentamicin. Interpretations followed Clinical and Laboratory Standards Institute (14) guidelines. Multidrug resistance (MDR) was defined as resistance to ≥3 antibiotic classes (15). *Escherichia coli* ATCC 25922 was used for quality control.

DNA Extraction

Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) according to manufacturer instructions, based on the alkaline lysis protocol (16,17). Chromosomal DNA was isolated via a modified cetyltrimethylammonium bromide (CTAB) method incorporating sonication to enhance cell lysis (18). DNA quality and concentration were assessed by spectrophotometry (MaestroNano MN-913, Maestrogen, Malaysia) and 1% agarose gel electrophoresis.

Polymerase Chain Reaction (PCR)

PCR was performed in 20 µL reactions using FIREPol® 5× Master Mix (Solis BioDyne, Estonia) containing 12.5 mM $MgCl_2$. The protocol included initial denaturation (95°C, 5 min), followed by 30 cycles of denaturation (95°C, 30 secs), annealing (primer-specific), extension (72°C, 30 secs), and a final extension (72°C, 10 min). Amplified products were resolved on 2% agarose gels stained with

Table1. The primers used in the study

Primer	Sequence (5'-3')	Amplicon Size (bp)	Tm	Reference
16S rRNA	AGAGTTTGATCCTGGCTCAG GACGGGCGGTGTGTACAA	1371	58	19
*trpB*AF *trpB*AR	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	489	57	20
*usp*AF uspAR	CCGATACGCTGCCAATCAG ACGCAGACCGTAGGCCAGAT	884	59	21
*CTX-M*F *CTX-M*R	CGCTGTTGTTAGGAAGTGTG GGCTGGGTGAAGTAAGTGAC	569	57	22
*SHV*F *SHV*R	CGCCTGTGTATTATCTCCCT CGAGTAGTCCACCAGATCCT	293	57	
*TEM*F *TEM*R	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATCA	1080	49	23
*int1*F *int1*R	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280	57	24
*int2*F *int2*R	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	51	25

SafeView (ABM, Canada) and visualized using a Vilber Lourmat imaging system. A single band at the expected size (Table 1) was considered evidence of the presence of the target gene.

Molecular Identification

Species confirmation of *E. coli* was achieved by PCR amplification of *uspA* (21) and *trpBA* (20) genes. Positive and negative controls included *E. coli* ATCC 35150 and *Salmonella* Typhimurium ATCC 14028, respectively. *Klebsiella pneumoniae* isolates were confirmed by 16S rRNA gene sequencing (19,26), with BLAST analysis against GenBank reference sequences.

Detection of ESBL and Integron Genes

The presence of *blaCTX-M*, *blaTEM*, *blaSHV*, *int1*, and *int2* genes was determined by PCR using previously validated primers (22,23,24,25). Amplicons were identified by expected fragment size following electrophoresis.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics (v25). Associations between multidrug resistance (MDR) and the presence of resistance genes (*blaCTX-M*, *blaTEM*, *blaSHV*) and integrons (*int1*, *int2*) were evaluated using the Chi-square (χ^2) test. For significant associations, odds ratios (OR) with 95% confidence intervals were calculated to assess the strength of association. A p-value <0.05 was considered statistically significant.

RESULTS

Isolation of ESBL-Producing Gram-Negative Bacteria

Gram-negative bacteria were isolated from 27% (85/312) of the examined milk samples. Among these, *E. coli* accounted for 31 isolates (36%), *Klebsiella* spp. for 35 isolates (41%), *Pseudomonas* spp. for 6 isolates (7%), and *Acinetobacter* spp. for 13 isolates (15%) (Fig. 1). Presumptive identification was performed using conventional biochemical tests. Both *E. coli* and *Klebsiella* spp. were catalase-positive, lactose-fermenting, and negative for oxidase and hydrogen sulfide production. *E. coli* isolates demonstrated motility, indole positivity, and were negative for urease and citrate utilization, whereas *Klebsiella* spp. isolates were non-motile, indole-negative, and positive for urease and citrate tests.

Phenotypic Confirmation of ESBL Production

Phenotypic screening by the Combined Disk Test (CDT) confirmed extended-spectrum β-lactamase production in 21 of 31 *E. coli* isolates (68%) and 7 of 35 *Klebsiella* spp. isolates (20%), yielding a total ESBL-positive rate of 33% (28/85). All CDT-positive isolates were further evaluated by the Double Disk Synergy Test (DDST), which fully corroborated the CDT findings (Fig. 2).

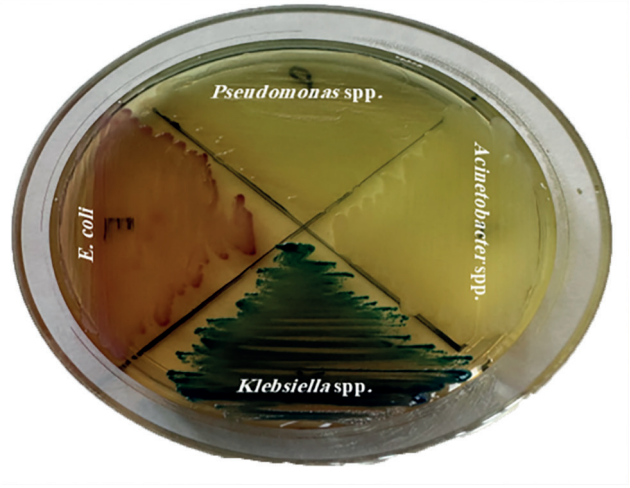

Figure 1: Chromogenic differentiation of ESBL-producing Gram-negative bacteria on CHROMagar™ ESBL.Representative plate image showing distinct colony pigmentation of *E. coli* (pink-red), *Klebsiella* spp. (metallic green), *Pseudomonas* spp. (pale yellow), and *Acinetobacter spp.* (colorless to light yellow) cultured on CHROMagar™ ESBL.

Antimicrobial Resistance

Among *Escherichia coli* isolates, complete resistance (100%) was observed to both cefotaxime and ceftriaxone. Similarly, all *Klebsiella* spp. isolates exhibited 100% resistance to cefotaxime, ceftriaxone, and ceftazidime. When considering all ESBL-producing isolates collectively, the highest resistance rates remained for cefotaxime and ceftriaxone (100%), while cefoxitin demonstrated the lowest resistance rate (4%) (Table 2, Fig. 3).

Multidrug Resistance (MDR)

Multidrug resistance was identified in 81% (17/21) of *E. coli* isolates and in 100% (7/7) of *Klebsiella pneumoniae* isolates. Overall, a high prevalence of MDR was evident among the extended-spectrum β-lactamase producers (Table 3, Fig. 4, Fig. 5).

Molecular Identification

All extended-spectrum β-lactamase-producing *E. coli* isolates (n=21) were confirmed at the species level by polymerase chain reaction targeting the *uspA* and *trpBA* gene regions (Fig. 6A). The seven *Klebsiella* spp. İsolates were identified to the species level as *K. pneumoniae* by sequencing of the 16S rRNA gene, with BLAST analysis revealing a 99.7% sequence identity to reference strains in the GenBank database (Accession numbers: PV925344–PV925350) (Fig. 6B).

Distribution of ESBL Genes

Analysis of the extended-spectrum β-lactamase gene profiles in *E. coli* isolates demonstrated the presence of *blaCTX-M* in 95%, *blaTEM* in 71%, and *blaSHV* in 62% of strains. Among *K. pneumoniae* isolates, *blaCTX-M* and *blaSHV* were each detected in 86%, while *blaTEM* was identified in 57% of isolates. When all ESBL-producing isolates were considered collectively, the overall gene prevalence was 93% for *blaCTX-M*, 67% for *blaTEM*, and 67% for *blaSHV* (Table 4, Fig. 7, Fig. 8).

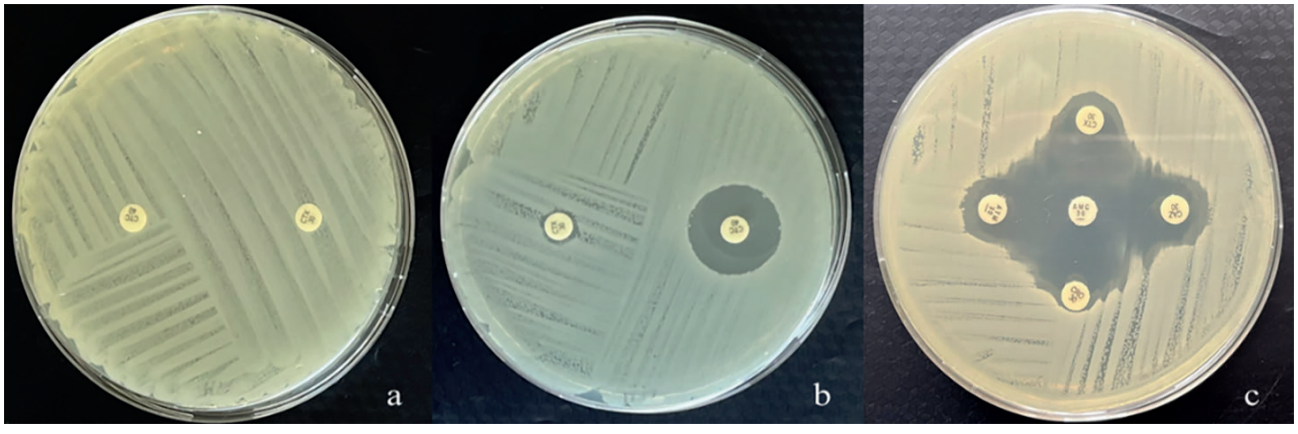

Figure 2. Phenotypic confirmation of ESBL production using the combined disk test (CDT). (a) Negative result with no enhancement in inhibition zone between cephalosporin and cephalosporin/clavulanic acid. (b) Positive result showing ≥5 mm increase in zone diameter around CTX/CLA versus CTX alone. (c) Strong synergy with CTX/CLA and CAZ/CLA in a confirmed ESBL producer. All CDT-positive strains (n = 28) were confirmed by the double disk synergy test (DDST).

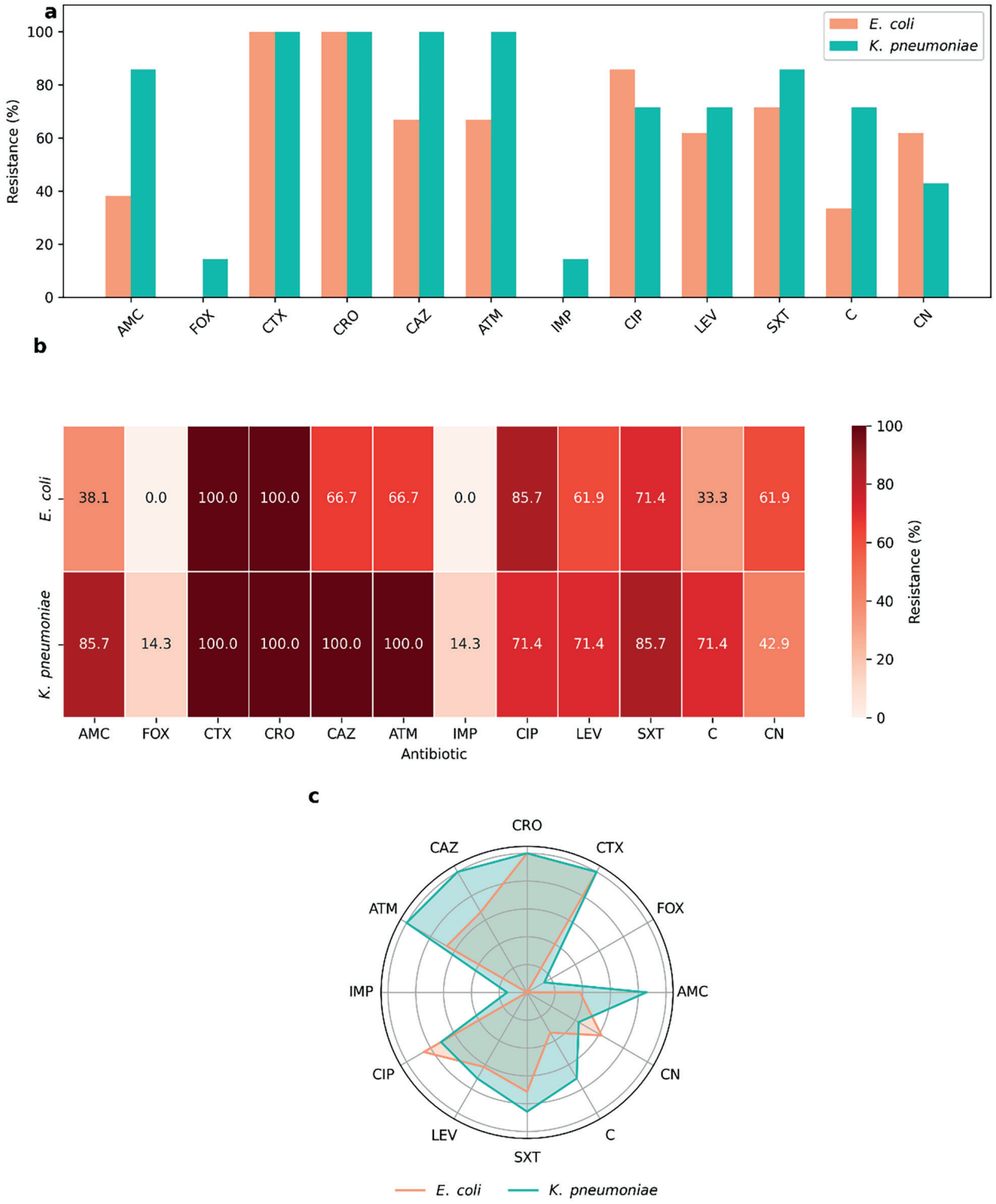

Figure 3: Antimicrobial resistance profiles of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates. (**a**) Bar chart showing resistance percentages across 12 antibiotics for both species. (**b**) Heatmap illustrating the intensity of resistance per antibiotic–species pair; darker shades indicate higher resistance rates. (**c**) Radar plot summarising the geometric distribution of resistance across all tested antimicrobials.

Table 2. Resistance status of ESBL producing isolates to antibiotics.

Name of Antibiotic	Zone diameter (mm)		Resistant Isolate count (n/N, %)		
(Disk content (µg))	≥S	≤R	*E. coli* (N=21)	*K. pneumoniae* (N=7)	Total (N=28)
AMC (30)	18	13	8 (38)	6 (86)	14 (50)
FOX (30)	18	14	0 (0)	1 (14)	1 (4)
CTX (30)	26	22	21 (100)	7 (100)	28 (100)
CRO (30)	23	19	21 (100)	7 (100)	28 (100)
CAZ (30)	21	17	14 (67)	7 (100)	21 (75)
ATM (30)	21	17	14 (67)	7 (100)	21 (75)
IMP (10)	23	19	0 (0)	1 (14)	1 (4)
CIP (5)	26	21	18 (86)	5 (71)	23 (82)
LEV (5)	21	16	13 (62)	5 (71)	18 (64)
STX (25)	16	10	15 (71)	6 (86)	19 (68)
C (30)	18	12	7 (33)	5 (71)	20 (71)
CN (10)	18	14	13 (62)	3 (34)	10 (36)

Table 3. MDR isolates percentages

Antimicrobial Class Count	*E. coli* (N=21)(%)	MDR (N=21) (%)	*K. pneumoniae* (N=7) (%)	MDR (N=7) (%)	Total (N=28) (%)	MDR (N=28) (%)
0	0 (0)	4 (19)	0 (0)	0 (0)	0 (0)	4 (14)
1	0 (0)		0 (0)		0 (0)	
2	4 (19)		0 (0)		4 (14)	
3	5 (24)	17 (81)	1 (14)	7 (100)	6 (21)	25 (86)
4	4 (19)		1 (14)		5 (18)	
5	3 (14)		0 (0)		3 (11)	
6	3 (14)		2 (29)		5 (18)	
7	2 (10)		3 (43)		5 (18)	
8	0 (0)		0 (0)		0 (0)	

Table 4. ESBL genes distribution within the isolates

	E. coli (N=21) (%)	*K. pneumoniae* (N=7) (%)	Total (N=28) (%)
CTX-M	20 (95)	6 (86)	26 (93)
TEM	15 (71)	4 (57)	19 (67)
SHV	13 (62)	6 (86)	19 (67)

Distribution of Integron Genes

Among *E. coli* isolates, the *int1* gene was detected in 76% (16/21), while *int2* was identified in only one isolate (5%). In *K. pneumoniae* isolates, *int1* was present in 29% (2/7), with no detection of *int2*. Overall, across all ESBL-producing isolates, the prevalence of *int1* and *int2* genes was 64% and 4%, respectively (Table 5, Fig. 9, Fig. 10).

Statistical Analysis

Chi-square analysis revealed significant associations between multidrug resistance and the presence of *blaTEM* ($p=0.003$), *blaSHV* ($p<0.001$), and *int1* ($p=0.026$). The presence of *blaCTX-M* showed a positive but non-significant trend ($p=0.23$), while *int2* was rare and not associated with MDR.

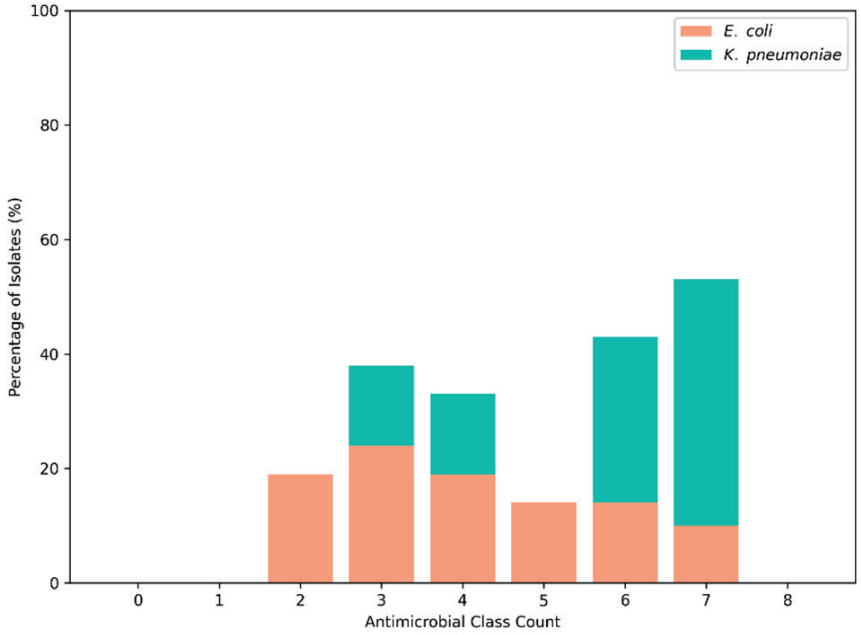

Figure 4. Distribution of antimicrobial resistance class counts among ESBL-producing *E. coli* and *K. pneumoniae*. Stacked bar chart showing percentage of isolates resistant to 0–8 antibiotic classes. *E. coli* clustered in class counts 2–5, while *K. pneumoniae* predominated in higher classes, especially class 7, indicating broader resistance.

DISCUSSION

Bovine mastitis remains a major threat to both animal health and public safety due to its economic impact and zoonotic potential (27). The emergence of extended-spectrum β-lactamase-producing Gram-negative bacteria, particularly *E. coli* and *K. pneumoniae*, further complicates treatment outcomes by limiting therapeutic options and increasing resistance dissemination risks (28). In this study, CHROMagar™ ESBL demonstrated high selectivity for extended-spectrum β-lactamase producers, though phenotypic confirmation revealed that only 33% of chromogenic isolates were ESBL-positive, suggesting possible co-occurrence of other β-lactamases such as AmpC or metallo-β-lactamases (5,13,29). Resistance profiling revealed complete resistance to third-generation cephalosporins, consistent with the hydrolytic capacity of extended-spectrum β-lactamases. Notably, susceptibility to carbapenems and cefoxitin remained high, although the limited availability of veterinary formulations restricts the clinical utility of cefoxitin (30,31). High fluoroquinolone resistance rates likely reflect the presence of plasmid-mediated quinolone resistance determinants co-localized

Table 5. Integron genes distribution within the isolates

	E. coli (N=21) (%)	*K. pneumoniae* (N=7) (%)	Total (N=28) (%)
int1	16 (76)	2 (29)	18 (64)
int2	1 (5)	0 (0)	1 (4)

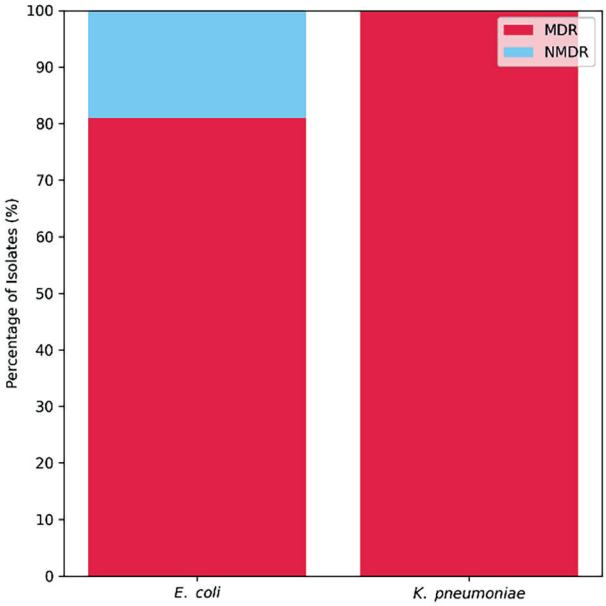

Figure 5. Proportion of multidrug-resistant (MDR) and non-multidrug-resistant (NMDR) isolates among ESBL-producing *E. coli* and *K. pneumoniae*.

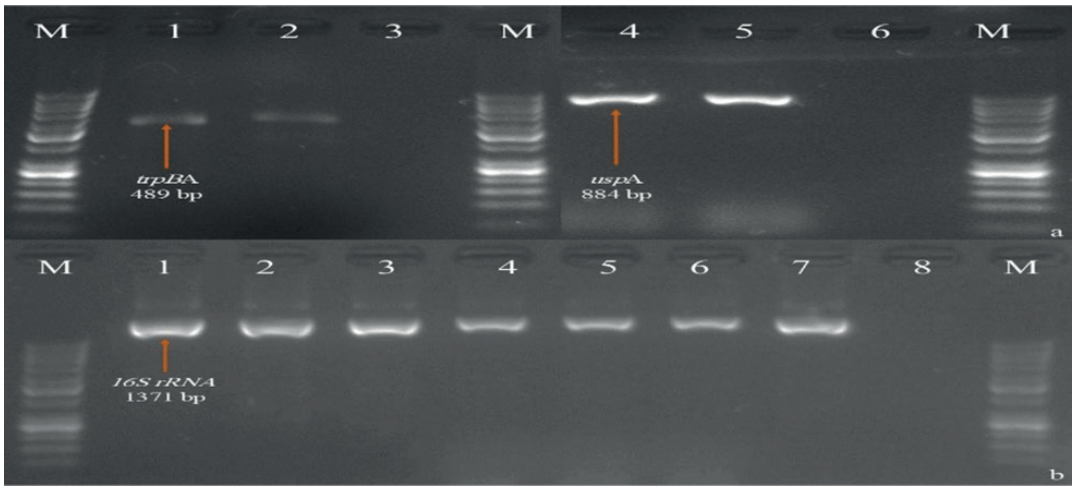

Figure 6. Molecular identification of *E. coli* and *K. pneumoniae* isolates.
(**a**) Multiplex PCR targeting the *trpBA* (489 bp) and *uspA* (884 bp) genes used for confirmation of *E. coli* isolates.
(**b**) PCR amplification of the 16S rRNA gene (1371 bp) from *K. pneumoniae* isolates. Lane M: molecular size marker (50 bp DNA ladder); lanes 1–6: clinical *E. coli* and *K. pneumoniae* isolates ; lane 7: Positive control; lane 8: Negative control (master mix without DNA).

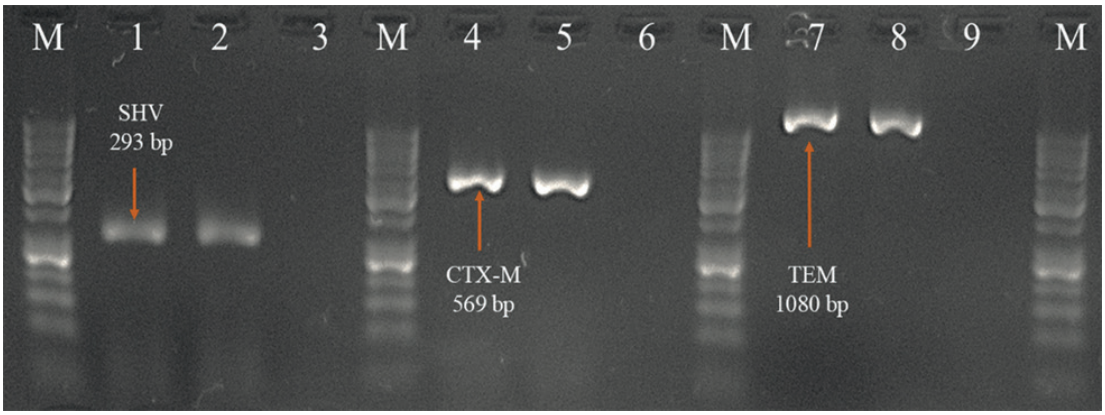

Figure 7. Detection of ESBL genes by PCR.
Amplification of *blaSHV* (293 bp, lane 1: positive control; lane 2: test isolate), *blaCTX-M* (569 bp, lane 4: Positive control; lane 5: test isolate), and *blaTEM* (1080 bp, lane 7: Positive control; lane 8: test isolate). Lanes 3, 6, and 9: Negative controls; M: 50 bp molecular size marker.

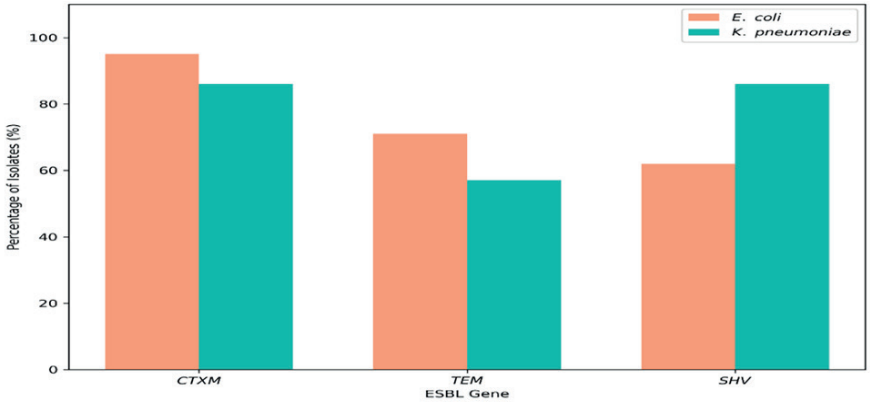

Fig. 8. Prevalence of ESBL genes among *E. coli* and *K. pneumoniae*.
Bar chart showing PCR-based detection rates of *blaCTX-M*, *blaTEM*, and *blaSHV*. *blaCTX-M* was most frequent overall, while *blaSHV* predominated in *K. pneumoniae*.

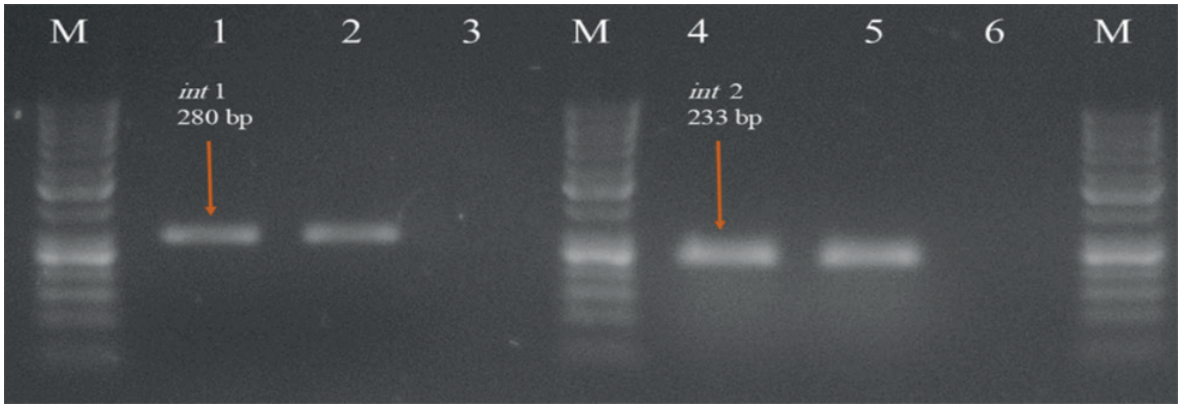

Figure 9. PCR detection of class 1 and class 2 integrons.
Amplification of *int 1* (280 bp; lane 1: Positive control, lane 2: test isolate) and *int 2* (233 bp; lane 4: Positive control, lane 5: test isolate). Lanes 3 and 6: no-template controls; M: 100 bp molecular size marker.

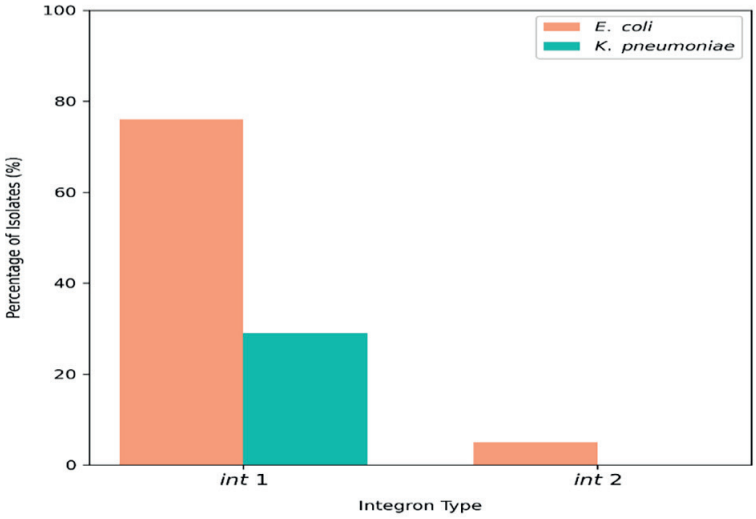

Figure 10. Distribution of integron genes among ESBL-producing isolates.

with extended-spectrum β-lactamase genes (32). Multidrug resistance was widespread, affecting 81% of *E. coli* and 100% of *K. pneumoniae* isolates, aligning with global reports of increasing resistance burdens in dairy herds (33,34). Molecular analysis confirmed the predominance of *blaCTX-M*, further emphasizing its leading role in bovine mastitis-associated resistance (33,35). The frequent coexistence of *blaTEM* and *blaSHV* genes underscores the contribution of plasmid-mediated horizontal gene transfer to multidrug resistance development. Chi-square analysis confirmed significant associations between MDR and the presence of *blaTEM* ($p = 0.003$), *blaSHV* ($p < 0.001$), and *int1* ($p = 0.026$), while *blaCTX-M* showed a positive but non-significant trend ($p = 0.23$).

These results statistically support the role of these genetic determinants in resistance burden and highlight their epidemiological significance in mastitis surveillance. Integron analysis revealed a high prevalence of *int1* among *E. coli* isolates, and its co-occurrence with *blaCTX-M* in multiple strains suggesting a strong potential for co-selection and mobile genetic element-mediated dissemination (36,37). Regional variation in integron carriage likely reflects differences in antimicrobial use policies, environmental reservoirs, and horizontal gene transfer dynamics.

CONCLUSION

This study revealed a high prevalence of extended-spectrum β-lactamase (*blaCTX-M, blaTEM, blaSHV*) and class 1 integron (*int1*) genes among *E. coli* and *K. pneumoniae* isolates from bovine mastitic milk, with significant associations between *blaTEM, blaSHV*, and *int1* and multidrug resistance phenotypes. The persistence of resistance despite these associations indicates that additional mechanisms such as porin modifications, efflux pump overexpression, or other mobile genetic elements also contribute to the resistance burden. These findings highlight the multifactorial nature of antimicrobial resistance evolution in dairy-associated *Enterobacteriaceae* and emphasize the need for integrated, cross-sectoral surveillance and prudent antimicrobial stewardship within the One Health framework.

STATEMENTS AND DECLARATİONS

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Farouk Hassan Farouk, Mehmet Kenan Türkyılmaz and Süheyla Türkyılmaz. The first draft of the manuscript was written by Farouk Hassan Farouk, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The 16S rRNA gene sequences of the *Klebsiella pneumoniae* isolates have been deposited in GenBank under accession numbers PV925344–PV925350.

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