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Original article

PREVALENCE AND MOLECULAR CHARACTERIZATION OF *BLA*^{TEM}, *BLA*^{SHV} AND *BLA*$^{CTX-M}$ GENES IN ESBL-PRODUCING *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATED FROM INTENSIVE CARE UNIT PATIENTS

Alla Shamsaldin Abdullah [1, *], and Pishtiwan Ahmad Hamad [1]

[1] Department of Biology, College of Education, Salahaddin University- Erbil, Erbil, Kurdistan Region, Iraq.

*Corresponding Author Email: alla.abdullah@su.edu.krd (Tel: +9647504626670)

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ABSTRACT

The rise and spread of antimicrobial resistance (AMR) not only significantly hinder the effective treatment of infectious diseases, but also lead to prolonged illness, treatment failures, and increased mortality. In intensive care units (ICUs), extended-spectrum β-lactamase (ESBL)-producing bacteria, such as *Escherichia coli (E. coli)* and *Klebsiella pneumoniae* (*K. pneumoniae*), are among the most concerning multidrug-resistant organisms (MDROs. This research was conducted to find out the causative agents of ICU infections, identify their profiles of antimicrobial resistance, and assess associated virulence factors. Discrepancies were found in bacterial identification by molecular analysis using *16S rRNA* sequencing compared to the Vitek2 system. More than half (50%) carried all three ESBL genes, while the prevalence of bla^{SHV} + bla^{CTX-M} carriers were the lowest. Isolates of *E. coli* from the study were not found to be salmon single ESBL genes (bla^{SHV}, bla^{CTX}, or bla^{TEM}), only 5.26% of *K. pneumoniae* isolates carried bla^{TEM} among them. Most of the strains had moderate to high biofilm-forming ability, which is the key to their MDR. In other words, ICU patients are vulnerable to colonization and infection with MDR pathogens, and still, *E. coli* and *K. pneumoniae* are the major threats that are linked to the resistance mechanisms and virulence factors of these pathogens.

KEYWORDS: ESBL, *E. coli*, *K. pneumonia*, 16S rRNA, ICU

1. INTRODUCTION

Infection management is increasingly challenged by antimicrobial resistance (AMR), which leads to treatment failures, prolonged illness, and higher mortality rates. Its economic impact is particularly severe in low- and middle-income countries, where healthcare resources are often limited (Murray *et al.,* 2022, Taher and Othman, 2024). Limiting treatment options for Enterobacteriaceae resistant to third-generation cephalosporins and carbapenems has been recognized as essential for public health (Oliveira *et al.* 2015, Ali, 2025). Assessing the adaptive patterns and genomes of pathogenic bacteria in specific geographic regions is crucial for monitoring and controlling drug resistance (Hami & Khalid, 2023; Issa, 2024). In vitro analyses showed that Carbapenems were the most effective antibacterial agents, while Amikacin and Ciprofloxacin demonstrated minimal activity. Rupp and Fey (2003) found that TEM-type extended-spectrum β-lactamases (ESBLs) occurred more frequently than other ESBL variants. Enterobacteriaceae continue to pose treatment challenges as they steadily develop antibiotic resistance. ESBLs, in particular, drive one of the most widespread resistance mechanisms in Gram-negative bacteria (Ojdana *et al.,* 2014). Inhibition of ESBLs enzymes by Clavulanic acid allows certain bacteria resistant to Aztreonam, Ceftazidime, Cefotaxime, Oxyimino-β-lactams, cephalosporins, and penicillins. ESBL Enzymes are broadly classified into three primary families: TEM, SHV, and CTX-M (Castanheira *et al.,* 2021). Among all, CTX-M catalysts have grown into quite common, surpassing both SHV and TEM in frequency, and are rapidly expanding across diverse clinically relevant bacterial species and geographic regions (Husna *et al.,* 2023). Additionally, ESBL-producing strains commonly demonstrate co-resistance to distinct sorts of antimicrobials, as aminoglycosides, fluoroquinolones, and sulfonamides, thereby complicating therapeutic strategies (Rupp and Fey, 2003). Although several ESBL types exist, including OXA and AmpC, most variants belong to the SHV, TEM, and CTX-M families, which are frequently identified in *K. pneumoniae* and *E. coli* (Ghenea *et al.,* 2022). This study aimed to investigate the prevalence of extended-spectrum β-lactamases (ESBLs) and detect the presence of the *bla*^{SHV}, *bla*$^{CTX-M}$, and *bla*^{TEM} genes among bacterial isolates recovered from clinical specimens in Erbil. By evaluating the antimicrobial resistance (AMR) profiles and multidrug susceptibility of infectious agents, particularly those isolated from intensive care unit (ICU) patients, the study sought to generate insights that supported precise treatment decisions, minimized therapeutic failures, and curbed the spread of resistant pathogens. Ultimately, these findings aimed to strengthen evidence-based infection control strategies and improve patient outcomes in healthcare settings.

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2. MATERIALS AND METHODS

Bacterial isolates:

Between September 2024 and February 2025, a total of 35 consecutive, non-duplicate isolates of *Escherichia coli* (n = 16) and *Klebsiella pneumoniae* (n = 19) were obtained from various clinical specimens (blood, urine, sputum and wound) at an intensive care unit (ICU) facility in Erbil, Iraq. For initial isolation and identification, standard microbiological techniques through culture on MacConkey agar and conventional biochemical tests, including catalase, citrate utilisation, indole, oxidase, and methyl red assays, were carried out. For confirmation, the VITEK 2 Compact system (bioMérieux, France) was employed to re-identify E. coli and K. pneumoniae isolates (BioMerieux, France).

Antimicrobial susceptibility testing:

Antimicrobial susceptibility of the isolates was assessed on Mueller–Hinton agar (MHA) plates using the Kirby–Bauer disc diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The study employed commonly prescribed antibiotics, including ampicillin (10 µg), amikacin (30 µg), cefotaxime (30 µg), cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), and meropenem. Results were interpreted using CLSI breakpoints to classify isolates as susceptible, intermediate, or resistant (Balouiri *et al.,* 2016)..

Amplification of *16S rRNA* gene for sequencing the isolated bacteria:

Universal primers 8F (forward) AGAGTTTGATCCTGGCTCAG and 1492R (reverse) GGTTACCTTGTTACGACTT with the amplicon size 1500bp were used to amplify the target DNA and the 1391R (reverse) primer GACGGGCGGTGTGTRCA was used to sequence the 16S rRNA gene. Amplification of the 16S rRNA gene was performed according to (Turner *et al.,* (1999). All PCR reactions were performed by using 2 µl DNA template (density of 10 ng/µl), 1.5 µl of each primers, 25µl of the Master Mix consisting of 3 mM MgCl2, 0.2% Tween 20, 20 mM Tris-HCl pH 8.5, (NH4)2SO4, 0.4 mM for each dNTP, and 0.2 units/µl Ampliqon Taq DNA polymerase, and 20 µl water nuclease free in a final volume of 50 µl. The conditions of PCR included primary denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 45 sec, at 55 °C for 45 sec, and at 72 °C for 90 sec, and a last extension at 72°C for 6 (Sambrook and Russell 2001).

Sequencing of *16S rRNA* gene:

The PCR products were purified and sequenced using the automated sequencer ABI 3100 with Big Dye Terminator Kit v. 3.1 at MACROGEN in Seoul, Korea. The primers 1391R (5'-GACGGGCGGTGTGTRCA -3') were used for sequencing (Turner *et al.,* 1999).

Detection of ESBL genotypes by multiplex PCR amplification:

The PCR assay was employed to detect the bla*SHV*, bla^{CTX-M}, and bla^{TEM} genes in isolates that tested positive during the initial enzyme screening, following the method described by Monstein *et al.* (2007) with minor modifications. Genomic DNA was extracted from 35 freshly cultured bacterial isolates using the Presto™ Mini gDNA Bacterial Kit. PCR products were then separated by agarose gel electrophoresis to confirm the presence of the target genes, as detailed in Table 1..

Table 1: Primers used for ESBL genotypes Multiplex PCR amplification.

Target Genes	Primers	Seq. (5`-3`)	Amplicon	Ref.
Bla^{TEM}	Forward	TC GCC G CAT ACACTAT TCTCAGAAT GA	445bp	Ghenea *et al.* 2022
	Reverse	ACGC TCAC CGGCT CC AGATT TAT		
Bla^{SHV}	Forward	ATGC GTTA TAT TCGCC TGTG	747bp	
	Reverse	TGCTT TGTTA TTCG GGC CAA		
Bla^{CTX-M}	Forward	ATGT GCA GYAC CAGTA ARGT KAT GGC	593bp	
	Reverse	TG GGTRAART ARGTSA CCAG AAYCAG C GG		

Biofilm Formation:

The biofilm-forming ability of Gram-negative isolates was quantitatively assessed using pre-sterilized 96-well polystyrene microtiter plates. After incubation, the adherent biofilm was stained, and absorbance was measured at 630 nm using an ELISA reader. The optical density (OD) values reflected the thickness of the biofilm produced by each strain. Biofilm production was classified according to the criteria of Stepanović *et al.* (2007) criteria, with the optical density cut-off (ODc) defined as the mean OD of the negative control plus three standard deviations (SD).

3. RESULTS

A total of 112 clinical specimens, including urine (22), blood (15), wound (15), and sputum (60), were collected and analyzed for bacterial culture. Among these, 35 samples showed positive bacterial growth, with *K. pneumoniae* as the predominant isolate (19 isolates, 54.3%), followed by *E. coli* (16 isolates, 45.7%). *E. coli* was most frequently recovered from urine samples (43.75% of E. coli isolates), reflecting its strong association with urinary tract infections, whereas *K. pneumoniae* was primarily isolated from sputum samples (52.6% of Klebsiella isolates), indicating its significant role in respiratory infections. The highest positive culture rate was observed in urine samples (50%), followed by wound (40%) and sputum (26.7%), with blood samples showing the lowest recovery rate (13.3%). These findings highlighted the clinical significance of *K. pneumoniae* in respiratory infections and *E. coli* in urinary tract infections, emphasizing the need for targeted antimicrobial therapy based on infection site-specific prevalence.

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Table 2: Prevalence of studied bacteria.

Isolates	No.	Sputum (60)	Wound (15)	Blood (15)	Urine (22)
E. coli	16	6	3	-	7
K. pneumoniae	19	10	3	2	4
Total	35	16(56.66%)	6(73.33%)	2(20%)	11(72.27%)

Molecular identification using *16S rRNA* gene sequencing

Molecular methods were employed to confirm the results obtained through conventional identification techniques and the VITEK 2 Compact system. The 16S rRNA gene (~1500 bp) was amplified and sequenced for 35 isolates, comprising 16 *E. coli* and 19 *K. pneumoniae*. Sequencing results revealed discrepancies in identification: 13 isolates were confirmed as *E. coli*, 21 as *K. pneumoniae*, and one remained unidentified. To ensure accurate species-level identification (Figure 1), PCR-amplified 16S rRNA products from all 35 isolates were sequenced (Table 3).

Table 3: Conventional and automated Recognition of isolates.

Bacteria	Clinical Identification	
	Conventional & Automatic	16S rRNA
Escherichia coli	16	13
Klebsiella pneumoniae	19	21
Total	35	34

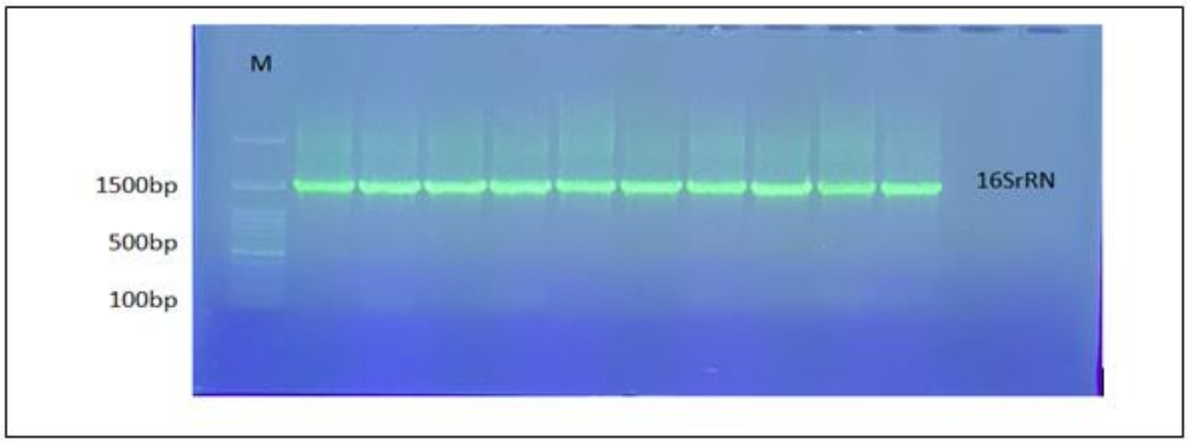

Figure 1: Agarose gel electrophoresis image showing the amplification of 16S rRNA, showing amplicons of 1500 bp. Lane M: DNA marker with 100 bp, with lanes (1-10) showing positive bands produced.

A slightly different resistance pattern was observed for *K. pneumoniae*. More than 84% of isolates were resistant to ampicillin, and approximately 74% were resistant to cefotaxime, cefepime, and gentamicin. Notably, considerable resistance was also observed to ciprofloxacin and meropenem. Encouragingly, amikacin resistance remained relatively low, with only about 10.5% of *K. pneumoniae* isolates exhibiting resistance (Table 4).

Table 4: Number and percentage of antimicrobial resistance of the isolated bacteria.

Antibiotic	Symbol	*E. coli* (n=16)	*K. pneumoniae* (n=19)	Resistant strains
Ampicillin	AMP	12 (75%)	16 (84.2%)	28
Amikacin	AK	2 (12.5%)	2 (10.5%)	4
Cefotaxime	CTX	11 (68.7%)	14 (73.6%)	25
Cefepime	CEF	12 (75%)	14 (73.6%)	26
Ceftazidime	CAZ	10 (62.5%)	12 (63.1%)	22

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Ciprofloxacin	CIP	8 (50%)	13 (68.4%)	21
Gentamicin	GEN	12 (75%)	14 (73.6%)	26
Imipenem	IPM	1 (6.25%)	9 (47.3%)	10
Meropenem	MEM	2 (12.5%)	13 (68.4%)	15
TMP-SMX	TMP-SMZ	6 (37.5%)	9 (47.3%)	15

The findings of this investigation revealed that, among the 35 isolates of *E. coli* and *K. pneumoniae*, the latter was the predominant species, accounting for 19 isolates (54.29%), while *E. coli* comprised 16 isolates (45.71%). Consequently, we focused on studying ESBL production in both species.

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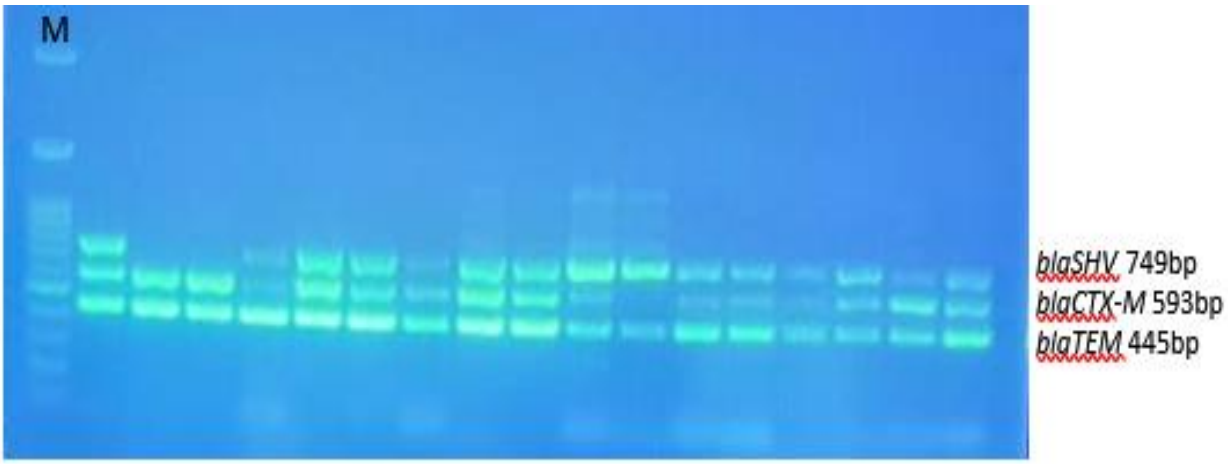

Figure. 2: Agarose gel electrophoresis image showing the amplification of *bla*^{SHV} 749bp, *bla*$^{CTX-M}$ 593bp and *bla*^{TEM} 445bp in isolates. Lane M: ladder (100 bp).

Table 5: Distribution of ESBL types among isolates.

ESBL genes Positive by PCR	*E. coli* No. (%)	*K. pneumonia* No. (%)
Existence of ≥ 2 ESBL genotypes		
bla^{TEM}+ *bla*^{SHV}+*bla*$^{CTX-M}$	8(50%)	9(47.36%)
bla^{TEM}+*bla*^{SHV}	4(25%)	2(10.52%)
bla^{TEM}+ *bla*$^{CTX-M}$	3(18.75%)	6(31.57%)
bla^{SHV}+*bla*$^{CTX-M}$	1(6.25%)	1(5.26%)
Existence of single ESBL genotype		
bla^{TEM}	0	1(5.26)
Bla^{SHV}	0	0
bla^{CTX-M}	0	0

According to current results, 100% of *Klebsiella pneumoniae* isolates produce biofilms. The strains were split into three groups: high, moderate, and weak producers. Regarding to the biofilm formation in this study 12 (63.15%) were biofilm strong producers, 5 (26.32%) were biofilm moderate producers, and 2 (10.53%) were biofilm weak producers (see table 6).

Table 6: Number and percentage of isolated bacteria for the occurrence of biofilm via using microtiter.

Isolated bacteria	No.	Strongly	Moderately	weakly	none	Total of biofilm producer
E. coli	16	10(62.5%)	4(25%)	2(12.5%)	0	16(100%)
K. pneumoniae	19	12(63.15)	5(26.31%)	2(10.52)	0	19(100%)
Total	35	22(62.85%)	9(25.71%)	4(11.42%)	0	35(100%)

4. DISCUSSION

Extended-spectrum β-lactamases (ESBLs) are enzymes produced by certain Gram-negative bacteria, notably *E. coli* and *K. pneumoniae*, conferring resistance to a broad range of β-lactam antibiotics, including third-generation cephalosporins and monobactams (Husan *et al.,* 2023). Among ESBLs, CTX-M enzymes, particularly CTX-M-15, have emerged as the predominant type globally, commonly found in both *E. coli* and *K. pneumoniae*. SHV-type ESBLs are traditionally associated with *K. pneumoniae* (e.g., SHV-12), while TEM-type ESBLs,

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though once widespread, have declined in prevalence (Castanheira *et al.,* 2021). OXA-type β-lactamases, primarily linked to penicillin resistance, are less commonly classified as ESBLs (Bush & Bradford, 2016). These resistance genes are often plasmid-mediated, enabling rapid horizontal transfer and contributing to multidrug resistance and therapeutic failures (Michaelis and Grohman, 2023). Detection through PCR remains crucial for effective infection control and antibiotic stewardship (Bonnet, 2004; Ibrahim, 2023).

In the present study, 35 Gram-negative isolates were identified, comprising two species from two genera: *K. pneumoniae* (19 isolates, 29.69%) and *E. coli* (16 isolates, 25%). These results align with prior research reporting *K. pneumoniae* and *E. coli* as leading opportunistic pathogens in both hospital- and community-acquired infections (Al-Qaysi *et al.,* 2024). Previous studies have shown *E. coli* as the most common cause of urinary tract infections, particularly in female patients, while *K. pneumoniae* is frequently implicated in pneumonia and wound infections (Babypadmini & Appalaraju, 2004; Gupta *et al.,* 2011). The predominance of *K. pneumoniae* in sputum samples observed here is consistent with its capacity to colonize the respiratory tract and cause severe pulmonary infections, particularly in immunocompromised individuals (Liu *et al.,* 2023). Conversely, the high prevalence of *E. coli* in urine samples reaffirms its established role in urinary tract infections. These findings underscore the need for ongoing surveillance of pathogen distribution to guide empirical treatment and strengthen infection control measures (Alameer *et al.,* 2025).

Additionally, *16S rRNA* sequencing was employed for precise identification of bacterial isolates, demonstrating high accuracy in differentiating challenging-to-identify organisms. This approach is particularly valuable for detecting biochemically fastidious or poorly characterized species. For instance, members of the genus *Pantoea* remain difficult to classify using conventional methods (Delétoile *et al.,* 2009; Brady, 2013). Bacterial species in research and clinical diagnostics are commonly identified using conserved housekeeping genes, particularly the 16S rRNA gene (Clarridge, 2004; Petti *et al.,* 2005). Numerous studies have confirmed the reliability of 16S rRNA sequencing in medical microbiology. Drancourt *et al.* (2000) demonstrated its superiority over phenotypic methods in 177 bacterial isolates, including 81 clinical strains. Bosshard *et al.* (2003) validated its effectiveness for aerobic Gram-negative bacilli, while Spilker *et al.* (2004) found frequent misidentification of Pseudomonas spp. by phenotypic tests compared to *16S rRNA* sequencing.

Intensive care unit (ICU) patients are highly vulnerable to colonization and infection by multidrug-resistant organisms (MDROs). Carbapenem-resistant Enterobacteriaceae (CRE), particularly *E. coli* and *K. pneumoniae*, have emerged as major threats due to limited therapeutic options and high mortality rates. The data found that resistance among *E. coli* isolates was especially pronounced, with 75% resistant to ampicillin, cefepime, and gentamicin, and high resistance was also observed for cefotaxime (69%) and ceftazidime (63%). In contrast, imipenem (6.25%) and amikacin (12.5%) retained activity against most *E. coli* isolates, indicating their continued utility as treatment options. These findings highlight the urgent need for vigilant antimicrobial stewardship and the development of new strategies to combat rising resistance in critical care settings.

Molecular characterization of ESBL genotypes revealed that most *Klebsiella pneumoniae* and Escherichia coli isolates carried at least one of the ESBL genes screened in this study, with all phenotypically ESBL-positive isolates (100%) testing positive for ESBL genes. Multiplex PCR analysis demonstrated that 3.13% of isolates harbored the bla^{TEM} gene (445 bp) (Table 7). The majority of isolates (51.56%) carried all three genes, while 21.87% possessed bla^{TEM} and bla^{CTX-M}. Additionally, 15.62% contained bla^{TEM} alone, and 7.81% carried bla^{SHV} together with bla^{CTX-M} These findings align with previous reports (Ibrahim & Youssef, 2015; Liao *et al.,* 2017), confirming the widespread presence of ESBL genes and the predominance of multi-gene carriage among clinical isolates.

The identification of β-lactamase-producing strains is essential for understanding the epidemiology of antibiotic resistance. CTX-M-type extended-spectrum β-lactamases (ESBLs) have become the most widespread globally, surpassing SHV and TEM variants in prevalence (Jorgensen *et al.,* 2010). In this study, bla^{TEM} was the dominant genotype, detected in *E. coli* and *K. pneumoniae* isolates, consistent with reports by Kaur *et al.* (2013) and Chowdhury *et al.* (2016), who also documented high ESBL production rates among these pathogens. Geographic variation likely explains differences across studies, as ESBL prevalence can fluctuate widely, ranging from 5–52% in Western countries to 10–46.5% in Asian regions (Babypadmini and Appalaraju, 2004). The high burden of ESBL production poses a serious threat to β-lactam therapy, particularly since many strains may be misclassified as susceptible due to limitations of phenotypic detection methods (MacKenzie *et al.,* 2002).

Molecular analysis revealed that bla^{SHV} was more frequently detected in *K. pneumoniae* than in *E. coli*, whereas bla^{CTX-M} was also present in both species. All ESBL-producing isolates harbored at least one ESBL gene, with bla^{TEM} emerging as the predominant genotype in both species. Co-existence of multiple ESBL genes was common, with *E. coli* frequently carrying both bla^{CTX-M} and bla^{TEM}, while *K. pneumoniae* more often harbored all three (bla^{SHV}, bla^{TEM}, and bla^{CTX-M}), reflecting considerable genetic diversity among ESBL determinants.

Biofilm formation, another key virulence factor, was observed in all *E. coli* isolates, with 62.5% classified as strong, 25% as moderate, and 12.5% as weak producers. These findings contrast with reports from Risal *et al.* (2018), who observed fewer strong biofilm producers, and Hussein *et al.* (2018), who found a majority of *K. pneumoniae* isolates to be weak producers. Biofilm-associated resistance mechanisms—including delayed antibiotic penetration, altered bacterial metabolism, and matrix-mediated protection—contribute to the persistence of infections and therapeutic failures (Karigoudar *et al.,* 2019; Gurung *et al.,* 2013). Early detection of biofilm production may therefore improve treatment outcomes by preventing chronic infections and immune-mediated complications (Deka, 2014).

The study has several limitations. The study is limited by a small sample size, short duration, and focus on only two bacterial species. It lacks patient-level clinical data, broader resistance gene screening (e.g., carbapenemases), and statistical correlation between resistance genes, biofilm formation, and clinical outcomes, which may affect the generalizability and depth of the findings. However, this study demonstrates several key strengths, including its focus on ICU patients—a high-risk group for multidrug-resistant infections—and the use of molecular techniques (*16S rRNA* sequencing and multiplex PCR) to accurately identify ESBL-producing *E. coli* and *K. pneumoniae* and characterize resistance genes (bla^{TEM}, bla^{SHV},

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bla^{CTX-M}). It also integrates antimicrobial susceptibility testing and biofilm formation analysis, providing a comprehensive view of resistance and virulence.

CONCLUSION

This study highlighted discrepancies between bacterial identification using the VITEK 2 system and molecular analysis through *16S rRNA* sequencing. Nearly half of the isolates carried all three ESBL genes, with the lowest prevalence observed for the bla^{SHV} + bla^{CTX-M} combination. No *E. coli* isolates harbored single ESBL genes (*bla^{SHV}*, *bla^{CTX}*, or *bla^{TEM}*), and only 5.26% of *K. pneumoniae* isolates carried *bla^{TEM}* alone. Most isolates were strong or moderate biofilm producers, a factor contributing to their multidrug resistance. Patients admitted to intensive care units remain at high risk of colonization and infection by multidrug-resistant organisms (MDROs). In particular, carbapenem-resistant Enterobacteriaceae (CRE), such as *E. coli* and *K. pneumoniae*, represent a growing threat due to limited therapeutic options and associated high mortality rates.

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Conflict of interest:

None.

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Author Contribution:

The authors contributed equally to the conception, implementation, analysis, and writing of this manuscript.

Ethical Approval:

This study received full approval from the HRECs at Salahaddin University-Erbil.
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