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Original Article

Investigating the Presence of *bla*$_{CMY}$ and Extended Spectrum Beta-Lactamase Genes in *Klebsiella pneumoniae* Isolates Identified in the Clinical Samples of Patients in Dhi-Qar, Iraq

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Abstract

Background: *Klebsiella pneumoniae* is an opportunistic pathogen that can cause nosocomial infections due to its high virulence factors and multiple antimicrobial resistance (AMR) mechanisms. This bacterium can produce various beta-lactamases, including extended-spectrum beta-lactamase (ESBL) and AmpC. This study aimed to investigate the presence of plasmid AmpC and ESBL genes in *K. pneumoniae* isolates from the clinical samples of patients in Dhi-Qar, Iraq.

Methods: A total of 612 clinical samples were collected from different medical centers and laboratories in Dhi-Qar, Iraq, between April 2023 and February 2024. Then, the presence of *K. pneumoniae* in these samples was evaluated using conventional biochemical and microbiological methods. ESBL production was assessed phenotypically by the synergy double disk (SDD) method. The presence of the *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{CTX-M-3}$, *bla*$_{CMY}$, and *bla*$_{SHV}$ genes was analyzed using the polymerase chain reaction.

Results: Out of the 612 samples, 180 (29.4%) tested positive for *K. pneumoniae*. Of these, 40 isolates (22.2%) were positive in the SDD test and were considered ESBL producers. The *bla*$_{SHV}$, *bla*$_{CTX-M-3}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$ genes were detected in 8 (20%), 32 (80%), 21 (52.5%), and 22 (55%) isolates, respectively. The *bla*$_{CMY}$ gene was not found in any of the *K. pneumoniae* isolates.

Conclusion: Our study highlights high resistance against third-generation cephalosporins among *K. pneumoniae* isolates. The prevalence of ESBL genes, including *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$, among these isolates can cause serious challenges in the treatment of bacterial infections.

Keywords: *Klebsiella pneumoniae*, AmpC, Beta-Lactamase, Synergy double disk, Extended-spectrum beta-lactamase

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Introduction

Klebsiella pneumonia, a member of the *Enterobacteriaceae* family, is an opportunistic bacterium capable of causing different diseases such as urinary tract infections (UTIs), *pneumoniae*, septicemia, and soft tissue infections, particularly in hospitals (1). It accounts for 3%–8% of all nosocomial bacterial infections (2). *K. pneumoniae* expresses several virulence factors, including endotoxins, siderophores, adhesins, iron-scavenging mechanisms, and capsules (3). This is a serious threat to human health, making antimicrobial resistance (AMR), a major global infection resistant to third-generation cephalosporins (4). Health systems currently incur significant financial expenditures due to AMR (5). The prevalence and diversity of AMR genes make them one of the major challenges facing global healthcare systems (5).

Beta-lactam antibiotics are commonly consumed to treat diseases caused by *Enterobacteriaceae*. However, the loss of sensitivity to these antimicrobial agents in Gram-negative bacteria, particularly *Enterobacteriaceae*, is spreading rapidly worldwide. Resistance to beta-lactams is primarily attributed to the production of three types of beta-lactamases (BLs), namely, extended-spectrum beta-lactamase (ESBL), AmpC-type BLs (AmpC-BLs), and carbapenemases (6).

ESBLs are a complex, diverse, and quickly evolving category of enzymes that pose significant challenges in treating patients with both community- and hospital-acquired infections (7). Most ESBLs are plasmid-mediated. Members of the *Enterobacteriaceae* family can easily transfer these plasmids among themselves, leading to the accumulation of resistance genes and the production of strains with multidrug-resistant plasmids. Isolates that produce ESBLs are resistant to several antibiotic classes. Unfortunately, plasmids that generate ESBLs are relatively stable in the host bacterium (8).

Based on Ambler classification, ESBL enzymes are categorized into two A and D classes. ESBLs, or serine beta-lactamases, form the largest group of beta-lactamases (9). The most prevalent genes encoding enzymes in class A include bla_{TEM}, bla_{CTX-M}, and bla_{SHV} (10). Before the 2000s, TEM and SHV were the most widespread ESBLs, but later, CTX-M became the most frequent worldwide, leading to a gradual decline in TEM-type ESBLs. Most CTX-M enzymes are more effective on cefotaxime than ceftazidime (9).

AmpC BLs are able to hydrolyze broad-spectrum cephalosporins as well as penicillins. AmpC-BLs belong to Ambler Class C and can be encoded either by the chromosome or plasmids. Plasmid-encoded AmpCs (pAmpCs) are widely spread and encoded by different genes, among which bla_{CMY} is the most prevalent. The pAmpC genes can be disseminated through *K. pneumoniae* and *Escherichia coli*, the bacteria responsible for diverse hospital-acquired infections (11).

Our research attempted to evaluate the presence of two types of BLs, ESBL and pAmpC, in *K. pneumoniae* isolates from the clinical samples of patients in Dhi-Qar, Iraq.

Materials and Methods

Sample Collection and Identification

In total, 612 samples were gathered from individuals suffering from blood septicemia, respiratory tract infections, burns, wound infections, and UTIs. The samples were gathered from various hospitals in Dhi-Qar, Iraq, including Al-Hussein hospital, Al Nasiriya hospital, Al Haboubi hospital, Al-Musawi Children's hospital, Bint Al Huda hospital, and medical laboratories in the city of Nasiriyah from April 2023 to February 2024. The samples were cultivated on MacConkey agar, blood agar, and nutrient agar. All culture media were obtained from Merck Company (Germany). Morphological characteristics of colonies, such as shape, size, margins, and pigmentation, were investigated in this study. Grown colonies were identified using Gram staining and traditional biochemical and microbiological tests, including oxidase, catalase, IMViC, sulfur indole motility, and triple sugar iron agar.

Synergy Double Disk Method for the Detection of ESBL Isolates

To identify ESBL-producing isolates, ceftazidime- or cefotaxime-resistant isolates were evaluated using the SDD test, as recommended by CLSI 2023 (12). The ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), ceftazidime-clavulanic acid (TZL; 30-10 µg), and cefotaxime-clavulanic acid (CEC; 30-10 µg) disks were applied in this method. The test isolates were inoculated onto Muller-Hinton agar (Merck, Germany) plates, and the disks were placed 3.5 cm from each other. The diameter of the no-growth zones around the disks was measured after 16 hours of incubation. According to CLSI criteria, the isolates were recorded as ESBL producers if the diameter of the no-growth zone around ceftazidime-clavulanic acid or cefotaxime-clavulanic acid (combined disks) increased by ≥5 mm compared to ceftazidime or cefotaxime (individual disks).

DNA Extraction

A manual boiling process was applied to extract DNA from *K. pneumoniae* isolates (13). A single colony from MacConkey agar was picked with an inoculation loop and cultured into the nutrient broth, then incubated for 18 hours at 37 ˚C. After incubation, 200 µL of the cultured media was transferred to a 1.5 mL microtube and centrifuged for 5 minutes at 5000 rpm. Afterward, the supernatant was discarded, and 200 µL of water was added to the bacterial pellet. After pipetting, the suspensions were boiled for 10 minutes and then centrifuged for 5 minutes at 10 000 rpm. The supernatant was collected as the template for amplification and stored at -20 ˚C.

Detection of Extended-Spectrum Beta-Lactamase Genes and bla_{CMY}

The test isolates were evaluated for the presence of four ESBL genes, including bla_{TEM}, $bla_{CTX-M-3}$, bla_{SHV}, and bla_{CTX-M}, along with the bla_{CMY} gene related to the pAmpC-BL type. The primer sequences are provided in Table 1. The polymerase chain reaction conditions were applied as previously described (14-18).

Statistical Analysis

The χ^2 or Fisher's exact test was applied to analyze descriptive data, and a P value of ≤0.05 was considered statistically significant.

Results

Out of 612 different samples, 180 (29.4%) were positive for *K. pneumonia*, including urine, burn, sputum, wound, blood, and pulmonary fluid samples. Details are included in Table 2. Of the positive samples, 48.3% (n = 87) and 51.7% (n = 93) were from females and males, respectively ($P > 0.05$).

Phenotypic Detection of Extended-Spectrum Beta-Lactamase-Producing Isolates

AMR was observed against cefotaxime, ceftazidime, or both in 139 (77.2%), 117 (65%), and 95 (58%) of *K. pneumoniae* isolates, respectively. After subjecting these isolates to the SDD test, 40 (22.2%) were detected as ESBL producers.

Detection of Extended-Spectrum Beta-Lactamase Genes and bla_{CMY}

In total, eight (20%), 32 (80%), 22 (55%), and 21 (52.5%) isolates harbored bla_{SHV}, $bla_{CTX-M-3}$, bla_{CTX-M}, and bla_{TEM}. The bla_{CMY} was found in none of the *K. pneumoniae* isolates (Figure 1). Four (10%) isolates did not exhibit any of the tested genes. Some isolates harbored multiple ESBL genes, with eight (20%) containing two ESBL genes and five (12.5%) consisting of three ESBL genes. The frequency of ESBL gene profiles is provided in Table 3.

Table 1. The Primer Sequences for the Detection of ESBL Genes and bla_{CMY}

Gene	Sequences	Annealing Temperature (°C)	Product Size	Reference
bla_{TEM}	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC	56	1080	(14)
bla_{CTX-M}	F: CGATGTGCAGTACCAGTAA R: TTAGTGACCAGAATCAGCGG	60	585	(15)
$bla_{CTX-M-3}$	F: CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	60	550	(16)
bla_{CMY}	F: ATGATGAAAAAATCGTTATGCT R: TTATTGCAGCTTTTCAAGAATGCG	60	1140	(17)
bla_{SHV}	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	50	885	(18)

Note. ESBL: Extended-spectrum beta-lactamase.

Table 2. The Number of *Klebsiella pneumonia* Isolates From Different Clinical Samples

Sample	Urine		Burn		Wound		Sputum		Blood		Pulmonary Fluids	
No. (%)	59 (32.8)		8 (4.4)		22 (12.2)		68 (37.8)		13 (7.2)		10 (5.6)	
Genus	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
N (%)	26 (44.1)	33 (55.9)	5 (62.5)	3 (37.5)	10 (45.4)	12 (54.6)	40 (58.8)	28 (41.2)	9 (69.2)	4 (30.8)	3 (30)	7 (70)

Note. M: Male; F: Female; N: Number.

Table 3. The ESBL Gene Profiles Found Among *Klebsiella pneumoniae* Isolates

Genes	bla_{SHV} N (%)	$bla_{CTX-M-3}$ N (%)	bla_{CTX-M} N (%)	bla_{TEM} N (%)	bla_{CMY} N (%)	$bla_{CTX-M-3}$- bla_{TEM} N (%)	$bla_{CTX-M-3}$- bla_{SHV} N (%)	$bla_{CTX-M-3}$ or bla_{CTX-M}-bla_{TEM} N (%)	$bla_{CTX-M-3}$-bla_{SHV}-bla_{TEM} N (%)	No Gene N (%)
Sum	8 (20)	32 (80)	22 (55)	21 (52.5)	0 (0)	4 (10)	1 (2.5)	7 (17.5)	5 (12.5)	4 (10)

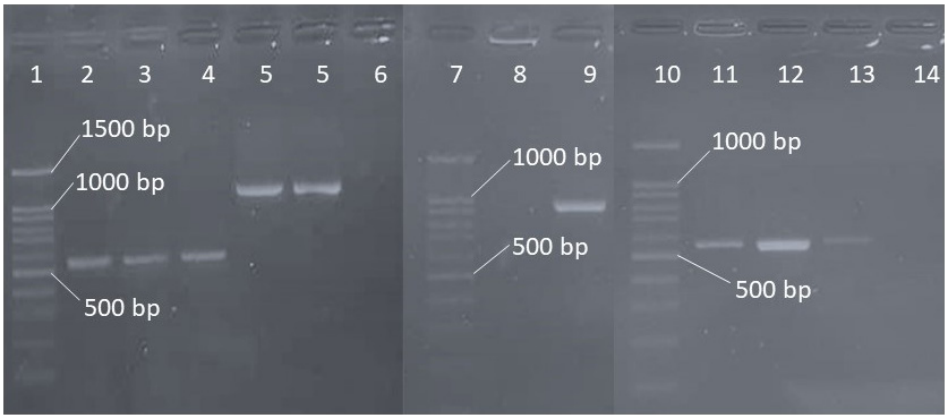

Figure 1. PCR Results of ESBL Genes. *Note*. PCR: Polymerase chain reaction; ESBL: Extended-spectrum beta-lactamase. Wells 1, 8, and 11: Ladder (100 bp); Wells 2, 3, and 4: $bla_{CTX-M-3}$; Wells 5 and 6: bla_{TEM}; Well 10: bla_{SHV}; Wells 12, 13, and 14: bla_{CTX-M}; Wells 7, 9, and 15: Negative isolates

Discussion

Enterobacteriaceae, especially *K. pneumonia*, cause nosocomial infections. Recently, AMR, due to the presence of BL, has increased worldwide, leading to resistance against a wide variety of antimicrobial agents (19). The prevalence of beta-lactamase producers and related genes varies greatly across different regions and from one year to another. For the accurate detection of ESBL-producing microorganisms, genotypic tests are required in addition to phenotypic tests (20).

The results of our study revealed that $bla_{CTX-M-3}$ and bla_{CTX-M} were the most frequent ESBL genes among *K. pneumoniae* isolates, respectively. Multiple studies have reported that CTX-M–type ESBLs are probably the most common ESBL type worldwide (7). CTX-M has different types, among which CTX-M-3 BL is the most common ESBL expressed by *K. pneumoniae*, *E. coli*, and different serotypes of non-typhoid *Salmonella (21-23)*. The universal primers of bla_{CTX-M} and specific primers were used for $bla_{CTX-M-3}$ because, in some cases, universal primers cannot detect all $bla_{CTX-M-3}$ genes. Only one isolate was negative for $bla_{CTX-M-3}$ but positive for bla_{CTX-M}, demonstrating that approximately 95% of bla_{CTX-M}-positive isolates harbored $bla_{CTX-M-3}$. These isolates may carry other types of bla_{CTX-M} simultaneously.

The frequency rate of bla_{CTX-M} varies in different geographic areas of Iraq. Studies conducted in Baghdad (24), Najaf (25), and Erbil (26) reported frequencies of 90%, 88.2%, and 41.1% for bla_{CTX-M}, respectively. Raouf et al found a prevalence of 47.4% for bla_{CTX-M} among *K. pneumoniae* isolates of patients with community-acquired pneumonia (27). In our study, bla_{TEM} was observed in 52.5%

of ESBL-producing isolates. In some studies performed in Iraq, this gene was the most prevalent ESBL gene (24, 26). The frequency of bla_{TEM} in Baghdad (24), Erbil (26), and Sulaimani (20) was 95%, 64.7%, and 53.7%, respectively. bla_{SHV}, another important ESBL gene, was observed in 20% of ESBL-producing isolates. Different frequencies ranging from 15.8% (27) to 92.85% (20) have been reported in other studies. The results of our investigation differed from those of some other studies, suggesting that different geographical regions may have various prevalence rates and types of ESBL genes. In addition, the sample size, type of sample, year of sampling, and method used for gene detection may have affected the study results.

The findings related to the prevalence of ESBL genes outside of Iraq also vary widely. In a study conducted by Saisi et al, the most common genes responsible for producing ESBLs were CTX-M (100%), TEM (97%), and SHV (94%) in Kenya, respectively (28). CTX-M was found to be the predominant gene according to studies from South America, the UK, Spain, the USA, and numerous regions of the Indian subcontinent (29). The bla_{TEM} gene was confirmed to be more prevalent than SHV in a Chinese investigation (30).

The presence of more than one BL gene within each isolate occurred in 13 ESBL-producing isolates. Notably, most isolates had a double or triple combination of ESBL genes, which is probably due to their transport by a common plasmid. Such plasmids can usually carry other resistance genes, leading to resistance against other antimicrobial categories. In Tunisia, Alibi et al found a triple combination of SHV/TEM/CTX-M (31). In Tanzania, Mshana et al reported a combination of CTX-M/TEM in 11.96% and SHV/CTX-M in 10.87% of the isolates (32).

Based on the results, the bla_{CMY} gene was not observed among cefotaxime- or ceftazidime-resistant isolates. There have been limited studies on the worldwide distribution of pAmpC-BLs, including bla_{CMY}, compared to the more frequently reported carbapenemase- and ESBL-producing bacteria (33-35). In general, the lowest frequency was found in Europe, including 0.06% in Denmark (36), 2.6% in Holland (37), and 11.9% in Germany (38), followed by America, with rates ranging from 1.3% in 2016 (37) to 3.42% in 2019 (39).

However, the prevalence of this type of BL is higher in the Middle East and Asia than in the rest of the world, especially in Iran (20.50% in 2020) (40), China (31.5% in 2015) (41), and Nepal (40.26% in 2020) (42). It was impossible to find information on the frequency of bla_{CMY} among *K. pneumoniae* strains isolated from patients in Iraq.

Among identified ESBL-producing *K. pneumoniae* isolates, 10% were negative for the investigated genes. ESBL production in these isolates may be attributed to other ESBL genes, such as bla_{OXA}, or other beta-lactamase groups, including metallo-beta-lactamase, carbapenemase, or other serine beta-lactamases.

Conclusion

Klebsiella pneumoniae isolates detected in our study showed a high level of resistance against third-generation cephalosporins. ESBL genes, including bla_{TEM}, bla_{CTX-M}, and bla_{SHV}, were frequent among these isolates, which can lead to serious challenges in the treatment of bacterial infections. The threat posed by this group of AMR bacteria to public health should not be underestimated. Extensive studies and the development of alternative solutions are needed to address this issue.

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Authors' Contribution

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Writing–review & editing: Seyedeh Elham Rezatofighi and Hossein Motamedi.

Competing Interests

The author declared no conflict of interests.

Ethical Approval

The ethical permission of the present study was taken from the Ethics Committee of Shahid Chamran University of Ahvaz according to the Declaration of Helsinki (IR.SCU.REC.1403.073).

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