


Original Article

Phenotypic and Molecular Characterization of the *bla*TEM Gene in Extended-Spectrum Beta-Lactamase-Producing *Klebsiella pneumoniae*

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Abstract**Introduction**

There has been a notable rise in antibiotic resistance among enterobacteria. This issue is primarily attributed to the emergence of extended-spectrum beta-lactamases (ESBLs), which present a significant concern for public health worldwide. This study investigates the prevalence of ESBL production, antibiotic resistance profiles, and molecular identification of the *bla*TEM gene in *Klebsiella pneumoniae* isolates.

Methods

The samples were randomly collected from several medical facilities in Erbil city. The VITEK 2 system was used for bacterial identification, antibiotic susceptibility, and ESBL production testing. The Double Disc Synergy Test (DDST) confirmed ESBL production. Polymerase chain reaction was conducted on all DNA samples, and the amplified DNA was analyzed using agarose gel electrophoresis to detect the *bla*TEM gene.

Results

A total of 43 samples were collected, of which the majority were urine (56%), followed by sputum (28%), blood (9%), and wound (7%). *Klebsiella pneumoniae* isolates exhibited the highest prevalence of resistance against ceftazidime (72%), ceftriaxone (70%), ciprofloxacin (63%), amoxicillin-clavulanic acid (60%), amikacin (58%), cefotaxime/tazobactam (56%), and gentamicin (53%). The DDST results indicated positive ESBL production in 15 isolates (35%), as evidenced by an increase or distortion in the inhibition zone toward the amoxicillin-clavulanate disc. Of the 43 isolates, 34 (79%) carried the *bla*TEM gene.

Conclusion

The study area shows a significant level of antibiotic resistance in ESBL-producing *Klebsiella pneumoniae* isolates, which, if not adequately addressed, could soon lead to severe health and therapeutic consequences.

1. Introduction

Antibiotic resistance is a dynamic and ongoing challenge. Over the past three decades, there has been a notable rise in antibiotic resistance among enterobacteria, particularly concerning third-generation cephalosporins. This issue is primarily attributed to the emergence of extended-spectrum beta-lactamases (ESBLs). ESBLs are a group of beta-lactamase enzymes produced by gram-negative bacteria that can hydrolyze and inactivate many antibiotics, including aztreonam, penicillin, and cephalosporins. These enzymes, such as TEM, SHV, and CTX-M, and their variants, allow enterobacteria to resist β -lactam antibiotics. The encroachment of these multi-drug-resistant pathogens into community settings raises significant alarm. The swift spread of the genes that encode these ESBLs, primarily via plasmids, has facilitated their rapid proliferation, leading to an alarming increase in the global prevalence of ESBL-producing bacteria. This situation presents a significant concern for public health worldwide [1,2]. *Klebsiella pneumoniae* is an opportunistic pathogen responsible for nosocomial and community-acquired infections [3]. This bacterium is usually found as an intestinal microflora. It can cause severe diseases like ventilator-associated pneumonia, catheter-related urinary tract infections, meningitis, bacteremia, septicemia, infection of surgical and non-surgical wounds, diarrhea, prosthetic valve endocarditis, peritonitis, and osteomyelitis [1]. The first strains of *Klebsiella pneumoniae* with ESBL (KP-ESBL) were identified in Europe in 1982, marking the emergence of resistance to ceftazidime and aztreonam due to plasmid-mediated beta-lactamase enzymes. These resistance traits rapidly disseminated to other gram-negative bacteria, including *Escherichia coli*. Since the discovery of these enzymes, their prevalence has continued to rise, with over 200 distinct ESBL enzymes identified today. The impact of ESBL-producing strains is particularly pronounced in intensive care units, which pose a high risk for epidemic outbreaks. *K. pneumoniae* and *E. coli* are the two most frequently encountered bacterial species associated with ESBL production. Implementing proactive monitoring of ESBL-producing pathogens in high-risk populations is crucial through suitable antimicrobial strategies. This necessity arises from the tendency of these pathogens to display multidrug resistance, which complicates treatment options and increases the potential for severe infections [4]. This study investigates the prevalence of ESBL production, antibiotic resistance profiles, and molecular identification of the *bla*TEM gene in *Klebsiella pneumoniae* isolates in Erbil City.

2. Methods

2.1. Study design and sample collection

This retrospective cross-sectional study was conducted between September 2021 and January 2022. The ethics board of the College of Health Sciences at Hewler Medical University approved the study. Patient consent was verbally obtained from the patients to collect their samples and be published in this study. The samples were randomly collected from several medical facilities in Erbil city, such as Nanakali Hospital, Hawler Teaching Hospital, and private laboratories. The collected samples included urine, sputum, or wound infection.

2.2. Isolation, identification, and susceptibility test of *Klebsiella pneumoniae*

The samples were cultured on MacConkey agar and incubated at 37°C for 24 hours. *Klebsiella pneumoniae* colonies were isolated based on their large, pink-to-red mucoid appearance. The VITEK 2 system (bioMérieux, France) was used for bacterial identification and antibiotic susceptibility testing. A sterile swab or stick was used to transfer an adequate number of colonies from the cultured sample into 3.0 ml of sterile saline (0.45% to 0.50% NaCl, pH 4.5 to 7) in a 12 × 75 mm polystyrene test tube. The suspension's turbidity was then adjusted to match the 0.5 McFarland standard for antimicrobial susceptibility testing and measured using a DensiChek™ turbidimeter.

2.3. Phenotypic Detection and Confirmation of ESBLs

ESBL production was detected among the isolates using the VITEK 2 system. The testing panel included six wells containing 10 µg/ml ceftriaxone, 0.5 µg/ml cefotaxime, or 0.5 µg/ml ceftazidime, alone or in combination with clavulanic acid (10 µg/ml, 4 µg/ml, and 4 µg/ml, respectively). Bacterial growth in each well was quantitatively assessed using an optical scanner. A proportional reduction in growth in the wells containing cephalosporin combined with clavulanic acid, compared to those containing cephalosporin alone, was considered indicative of ESBL production. The Double Disc Synergy Test (DDST) confirmed ESBL production. Each *Klebsiella pneumoniae* isolate was inoculated on Müller-Hinton agar plates for susceptibility testing. Discs containing 30 µg of cefotaxime and ceftazidime were placed on either side of a disc with co-amoxiclav (20/10 µg), positioned 20 mm apart (center to center). ESBL production was indicated when the inhibition zone of either cephalosporin was expanded by clavulanic acid, often resulting in a distinctive "champagne-cork" or "keyhole" shape zone [1].

2.4. Molecular detection of *bla*TEM gene

DNA extraction of *Klebsiella pneumoniae* was carried out using the simple boiling method. Isolated colonies from overnight cultures were placed in a test tube containing 1 ml of distilled water and heated in a water bath for 10 minutes. The sample was centrifuged at 1,000 rpm for 5 minutes to complete the extraction process. Polymerase chain reaction (PCR) was conducted on all DNA samples of *Klebsiella pneumoniae*. The concentration of extracted double-stranded DNA was combined with a PCR master mix (Promega, USA), which included Taq DNA polymerase, deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), buffers, and salts. Specific forward (5'-GAG TAT CAA CAT TTC CGT GTC-3') and reverse (5'-TAA TCA GTG GGC ACC TTC TC-3') primers were added to amplify the *bla*TEM target gene. The PCR mixture was then incubated in a thermocycler, undergoing 32 cycles of alternating temperatures: a denaturation step at 94°C for one minute to separate the double-stranded DNA into single strands, an annealing step at 57°C for one minute to allow the primers to bind to the target DNA, and an extension step at 72°C for 10 minutes for the Taq DNA polymerase to synthesize new DNA strands. This process generated billions of copies of the target DNA sequence within a few hours. Subsequently, the amplified DNA was separated and analyzed using agarose gel electrophoresis.

3. Results

A total of 43 samples were collected; 22 (51%) were obtained from females and 21 (49%) from males. The majority of the samples were urine (56%), followed by sputum (28%), blood (9%), and wound (7%). *Klebsiella pneumoniae* isolates exhibited the highest prevalence of resistance against ceftazidime (72%), ceftriaxone (70%), ciprofloxacin (63%), amoxicillin-clavulanic acid (60%), amikacin (58%), cefotaxime/tazobactam (56%), and gentamicin (53%). In contrast, the lowest prevalence of resistance was observed with imipenem (26%) and meropenem (28%) (Table 1). The DDST results indicated positive ESBL production in 15 isolates (35%), as evidenced by an increase or distortion in the inhibition zone toward the amoxicillin-clavulanate disc. The remaining samples (65%) tested negative for ESBL production (Figure 1). Of the 43 isolates, 34 (79%) carried the *bla*_{TEM} gene (Figure 2). Overall, 11 isolates (26%) tested positive in the DDST and PCR, while five isolates (12%) were negative. Additionally, four isolates (9%) were positive in DDST but negative in PCR, whereas 23 isolates (53%) were negative in DDST but positive in PCR (Table 2).

4. Discussion

KP-ESBL has emerged as a significant concern due to its virulence factors and role as a leading cause of infectious diseases. It is categorized within the multidrug-resistant group of bacteria. *Klebsiella pneumoniae* exhibits antibiotic resistance through several mechanisms, including the enzymatic degradation or inactivation of antibiotic compounds, alterations in membrane permeability, and modifications of antibiotic target sites via bacterial protein mutations. It has been reported that *K. pneumoniae* acquires numerous ESBL enzymes, which serve as its primary defense mechanism against antibiotics [5]. The prompt and precise identification of ESBL-positive Enterobacteriaceae strains is critical for guiding appropriate patient management and developing and enforcing targeted infection control protocols [6]. Over the past decade, numerous studies have highlighted the prevalence of ESBL-mediated resistance in infectious bacteria, with a higher prevalence in *Escherichia coli* and *Klebsiella pneumoniae* [2,5,6-9]. In the study by Benbrahim et al., 15 out of 40 (37.5%) *Klebsiella*

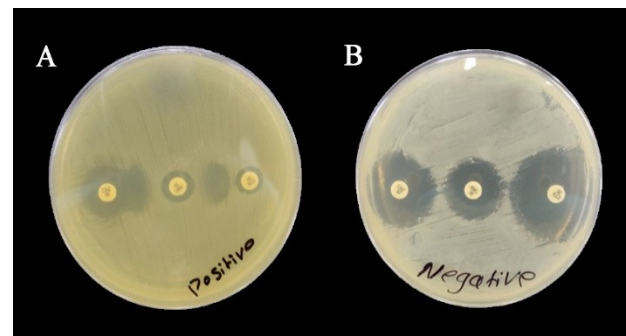


Figure 1. (A) shows the positive result of the Double-disk synergy test, and (B) is the negative result of the Double-disk synergy test.

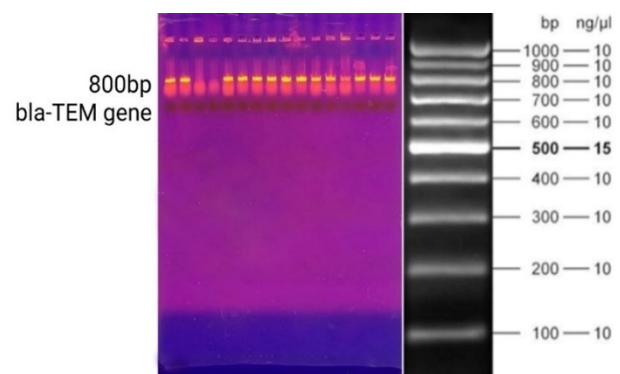


Figure 2. Amplified *bla*_{TEM} gene on 1% agarose gel electrophoresis.

pneumoniae isolates were identified as ESBL-producing strains, a figure close to the 41.1% reported by Pirzaman et al. [10]. Another study reported a prevalence of 31.8% [2]. Higher rates were observed in Asian countries, reaching up to 75% [1]. In this study, the prevalence of isolates with positive ESBL enzymes was 35%, close to previously reported [1,2,10].

Infections caused by ESBL-producing bacteria can affect individuals of all ages, though their distribution may be influenced by patients' immunological status and the prevalence of antibiotic misuse. Benbrahim et al. found that KP-ESBLs were detected across all age groups, with the highest incidence (20%) observed in individuals aged 21-30 [1].

Table 1. Antibiotic resistance pattern of *K. pneumoniae* isolates.

Antibiotic	No. of resistant isolates	Frequency (%)	No. of sensitive isolates	Frequency (%)
Ceftazidime	31	72%	12	28%
Ceftriaxone	30	70%	13	30%
Ciprofloxacin	27	63%	16	37%
Amoxicillin clavulanic acid	26	60%	17	40%
Amikacin	25	58%	18	42%
Cefotaxime	24	56%	19	44%
Tazobactam	24	56%	19	44%
Gentamicin	23	53%	20	47%
Meropenem	12	28%	31	72%
Imipenem	11	26%	32	74%

Table 2. Outcomes of double-disk synergy test (phenotypic detection) and polymerase chain reaction results (genotypic detection of *bla-TEM* gene) among *K. pneumoniae* isolates.

Isolated bacteria	DDST results	PCR results	Number	Frequency (%)
	(+)	(+)	11	26%
(-)	(-)	5	12%	
(+)	(-)	4	9%	
(-)	(+)	23	53%	

Gravey et al. reported a KP-ESBL prevalence of 4.1% in individuals aged 18–64 and 4.2% in those over 65 [11]. Due to the retrospective nature of the present study, the age preference among samples was unknown. In the present study, females comprised 51% of the sample, while males accounted for 49%, a distribution that closely aligns with the findings reported by Marra et al. [12]. However, a study by Ali and Ismael observed a higher proportion of female samples (72.72%) than male samples (22.73%) among 84 *Klebsiella pneumoniae* isolates [13]. Conversely, a study by Nirwati et al. found a higher frequency of male samples (64.07%) than female samples (35.93%) in 167 *K. pneumoniae* isolates [14]. In the study by Benbrahim et al., KP-ESBL strains were most frequently isolated from urine samples, which the authors suggested is likely due to urine being one of the most commonly collected specimens for clinical investigation [1]. Similarly, most of the samples (56%) in the present study were urine.

Antimicrobial resistance in pathogenic bacteria presents a global challenge, contributing to high mortality and morbidity rates. Infections caused by multidrug-resistant strains are often difficult or impossible to treat with conventional antimicrobials. The widespread, usually unnecessary use of antibiotics stems from the failure of many healthcare centers to promptly diagnose causative microorganisms and determine their antimicrobial susceptibility in patients with bacteremia and other serious infections [15]. In this study, *Klebsiella pneumoniae* strains exhibited the highest resistance rates against ceftazidime (72%), ceftriaxone (70%), ciprofloxacin (63%), and amoxicillin-clavulanic acid (60%). The resistance rate for ceftazidime in this study was slightly similar to that reported in another study conducted in Erbil, which documented a resistance rate of 62.5% [16]. Notably, our study found significantly higher resistance rates for ciprofloxacin (63% vs. 4.2%), gentamicin (53% vs. 4.2%), and amikacin (58% vs. 4.2%). Conversely, the resistance rate for amoxicillin-clavulanic acid was lower in our study than in the previous one (60% vs. 81.25%) [16]. Another study conducted in Iran reported even higher resistance rates for amoxicillin-clavulanic acid (100%), ceftazidime (84%), tazobactam (80%), ciprofloxacin (80%), and amikacin (60%) [17]. In contrast to our findings, sensitivity rates of 90% and 100% by *K. pneumoniae* isolates have been reported for amoxicillin-clavulanic acid and imipenem, respectively [1,18]. Muggeo et al. and Rasamiravaka et al. found a sensitivity rate of 83% to amikacin, while it was 42% in the present study. In the study by Bora et al., imipenem and meropenem were identified as one of the most effective antimicrobial agents against ESBL-producing isolates, comparable to the findings in this study [7].

The prevalence of the *blaTEM* gene in *Klebsiella pneumoniae* isolates varies across studies. Alibi et al. reported that *blaTEM*

gene was present in 56.8% of their isolates [19]. In contrast, Paterson et al. found a prevalence of 87% [20]. Guesseend et al. reported that 63.4% of their isolates in Abidjan, Côte d'Ivoire, carried the *blaTEM* gene [21]. The prevalence rates of 31.57% and 55% for the *blaTEM* gene in *Klebsiella pneumoniae* have also been reported [2,5]. Based on genotypic detection, in this study, 79% of the isolates carried the *blaTEM* gene, which may indicate uncontrolled antibiotic misuse in our locality. In line with this finding, Bora et al. found that genes responsible for ESBL were positive in 79.45% of the *Klebsiella pneumoniae* isolates. However, in the phenotypic confirmatory test for ESBL production, 53.42% of the isolates tested positive, suggesting that the phenotypic test results may be subject to false positives or negatives. Several factors can contribute to this issue, including the production of multiple β -lactamase types by a single bacterial isolate, the co-production of ESBLs and constitutive AmpC β -lactamases, variations in substrate affinities, and the inoculum effect [7]. Accordingly, in the current study, phenotypic detection revealed that only 15 isolates (34.9%) were positive for ESBL production, while genotypic detection identified 34 isolates (79%) as positive for the *blaTEM* gene. Therefore, PCR using oligonucleotide primers specific to ESBL genes is considered the most straightforward and reliable method for detecting the presence of ESBLs [7].

This study has several limitations that may impact the generalizability of its findings, including small sample size, sampling restricted to a specific geographic area of the city, and the exclusive focus on the *blaTEM* gene. Future research with larger sample sizes and broader geographic coverage, extending beyond the city to the entire country, is needed to provide deeper insights into this issue.

5. Conclusion

The study area shows a significant level of antibiotic resistance in KP-ESBL strains, which, if not adequately addressed, could soon lead to severe health and therapeutic consequences. Molecular methods, which detect the genes responsible for resistance, may offer greater sensitivity in identifying antibiotic resistance.

Declarations

Conflicts of interest: The author(s) have no conflicts of interest to disclose.

Ethical approval: The ethics board of the College of Health Sciences at Hewler Medical University approved the study.

Patient consent (participation and publication): Written informed consent was obtained from patients for publication.

Source of Funding: Medical Research Center of Hawler Medical University.

Role of Funder: The funder remained independent, refraining from involvement in data collection, analysis, or result formulation, ensuring unbiased research free from external influence.

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Use of AI: AI was not used in the drafting of the manuscript, the production of graphical elements, or the collection and analysis of data.

Data availability statement: Not applicable.

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