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Article

Distribution of *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ genes in Extended Spectrum β-Laktamase Producing *Klebsiella pneumoniae* from Clinical Isolates in Jakarta

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Abstract. *Klebsiella pneumoniae* is one of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) due to their high level of antibiotic resistance. Ceftriaxone is one of the cephalosporin antibiotic that functions inhibit bacterial cell wall synthesis and used for treating *K. pneumoniae* infections. Resistance to ceftriaxone in *K. pneumoniae* has been widely reported, with one contributing factor being the production of β-lactamase enzymes encoded by the genes *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$. This study characterized the presence of these genes in 12 clinical isolates of *K. pneumoniae* and analyzed their correlation with phenotypic resistance to ceftriaxone. All isolates characterized with antimicrobial susceptibility testing (AST) and disk diffusion methods to evaluate the phenotypic production of *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$. Molecular analysis using the polymerase chain reaction (PCR) method showed the genes *bla*$_{CTX-M}$ and *bla*$_{TEM}$ were detected in 11 isolates (91.67%), and *bla*$_{SHV}$ was found in 9 isolates (75%). The distribution pattern of the *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ resistance genes was present in 8 isolates (66.67%), with MIC values > 64 µg/mL. The presence of *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes together in *K. pneumoniae* isolates represents a potential risk for resistance to other β lactam antibiotics.

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1. Introduction

Klebsiella pneumoniae is one of the pathogens classified within the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which poses significant threats to human health [1]. In addition, *K. pneumoniae* is known to be a major cause of nosocomial infections in patients who are mechanically ventilated, have intravenous catheters, have wounds due to injury, or have been on prolonged courses of certain antibiotics, as well as community acquired infections [2].

The ability of *Klebsiella pneumoniae* to evade the immune system, combined with the presence of hypervirulent strains, significant challenges in managing infections caused by this pathogen [3]. The rise in infections may also be attributed to the emergence of *K. pneumoniae* strains resistant to multiple antibiotics, which increases the risk of infection spread and treatment failure [4]. High rates of multidrug resistance (MDR) in *K. pneumoniae* observed in Southeast Asia, with prevalence rates of 55% in both healthcare settings and community environments [5].

Ceftriaxone is a third generation cephalosporin antibiotic that is widely used to treat infections caused by *K. pneumoniae* and increased resistance to this antibiotic has been studied [6]. Study by Ahmad et al. (2022) reported that *Klebsiella pneumoniae* isolated from COVID-19 patients at RSUP Dr. Mohammad Husein exhibited resistance to ceftriaxone, with a resistance rate of 100% [7]. Another study by Elvionita et al. (2023) reported resistance to ceftriaxone reached 40.74% on pediatric patients with pneumonia at RSUP Dr. Sardjito Yogyakarta, with *K. pneumoniae* being a predominant bacterial pathogen in these infections [8].

Resistance to ceftriaxone in *Klebsiella pneumoniae* can be attributed to the production of beta-lactamase enzymes, particularly Extended-Spectrum β-Lactamases (ESBLs). The genes responsible for ESBL production are encoded by *bla* genes, which are the most important resistance genes within the Enterobacteriaceae family [9]. Consistent with the research of Mohammedkheir et al. (2024), who reported that $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} are among the *bla* genes commonly associated with resistance to β lactam antibiotics in *E. coli* and *Klebsiella* species [10].

Klebsiella pneumoniae is a member of Enterobacteriaceae that produces many ESBL genes, including $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} [11]. A study by Yamasaki et al. (2021) reported that *K.pneumoniae* isolates from urinary tract infections in Indonesia had the $bla_{\text{CTX-M-15}}$ gene, which was predominant among all resistant isolates, accounting for 89.4% of cases [12]. In line with the study conducted at the Clinical Microbiology Laboratory of the Faculty of Medicine, University of Indonesia (FKUI), data was obtained from January 2023 to December 2023 indicate that 32% of *K. pneumoniae* isolates showed resistance to ceftriaxone.

Based on data obtained, *Klebsiella pneumoniae* isolates resistant to ceftriaxone are frequently encountered. The specific genes responsible for this resistance have not been thoroughly investigated. The aim of this study is to identify the presence of the $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} genes in *K. pneumoniaea* and to see the correspondence between ceftriaxone resistance phenotypes by molecular characterization.

2. Experimental Section

2.1 Type of Research

This study is observational and the data obtained from the laboratory tests are analyzed descriptively to get a picture of the object under study so that it can be concluded that there is a match between the ceftriaxone resistant phenotypes and the molecular characteristics of the resistance encoding *bla* gene.

2.2 Culture of *K. pneumoniae*

This study used 43 *K. pneumoniae* isolates from January to December 2023. The isolates are part of the collection from the Clinical Microbiology Laboratory at the Faculty of Medicine, Universitas Indonesia and approved by the Ethics Committee of FKUI-RSCM.

Isolates were subcultured using the Koch dilution method on 5% blood agar media. Incubation was performed for 24 hours at a temperature of 35°C - 37°C. The growing colonies were subjected to Gram staining and microscopic observation to ensure the absence of contamination.

Antibiotic susceptibility tests (AST) were performed by disc diffusion (Kirby-Bauer) and with VITEK® 2 COMPACT system. Kirby-Bauer method was performed by attaching 30 µg ceftriaxone antibiotic disc paper on Mueller Hinton agar (MHA) containing *K. pneumoniae* culture with turbidity according to 0.5 McFarland standard. Incubation was carried out for 24 hours at a temperature of 35°C - 37°C. The zone of inhibition formed after incubation was measured and compared with the ceftriaxone standard in the Clinical & Laboratory Standards Institute (CLSI) 2020.

Antibiotic susceptibility testing (AST) with VITEK® 2 COMPACT system using a single colony culture in 0.45% NaCl solution. Inoculum turbidity was standardized using a DensiCheck range of 0.5 to 0.63 McFarland. Incubation was performed for 24 hours at a temperature of 35°C - 37°C. Selected *K. pneumoniae* isolates resistant to ceftriaxone based on Kirby-Bauer and AST methods are 12 consist of three tissue samples, one BAL (Bronchoalveolar Lavage), two urine samples, two pus samples, two sputum samples, and two feces samples. These isolates are used for extraction and PCR steps.

2.3 DNA Extraction and Amplification

DNA extraction from *K. pneumoniae* isolates was performed using the QIAamp® DNA Mini Kit. Target gene amplification was performed by polymerase chain reaction (PCR). A total of 1 µL of extracted DNA was used as template and the total reaction for PCR is 20 µL. The PCR reagents used are 10 µL of iMax II Intron, 1 µL each of forward and reverse primers, and 7 µL of nuclease-free water. Optimization of the three target genes began with a pre-denaturation step at 94°C for 4 minutes. Amplification proceeded with 40 cycles. Denaturation of the $bla_{\text{CTX-M}}$ gene at 94°C for 30 seconds, bla_{SHV} and bla_{TEM} genes at 94°C for 1 minute. Annealing of the $bla_{\text{CTX-M}}$ gene at 60°C for 30 seconds, bla_{SHV} and bla_{TEM} genes at 55°C for 1 minute. Extension of the $bla_{\text{CTX-M}}$ gene at 72°C for 40 seconds, bla_{SHV} and bla_{TEM} genes at 72°C for 1 minute. Final extension for all three genes was performed at 72°C for 5 minutes. The three primers used are presented in Table 1.

Tabel 1. Primer sequences used for PCR

Primer	Nucleotide Sequences (5'-3')	Amplicon Size (bp)	References
CTX-M-F	ATG TGC AGC ACC AGT AAA GTG ATG GC	593	[13]
CTX-M-R	TGG GTA AAG TAA GTG ACC AGA ATC AGC GG		
SHV-F	ATG CGT TAT ATT CGC CTG TG	868	[14]
SHV-R	GTT AGC GTT GCC AGT GCT C		
TEM-F	TCG GGG AAA TGT GCG	971	[15]
TEM-R	TGC TTA ATC AGT GAG GCA CC		

2.4 Electrophoresis

Gel electrophoresis was conducted by loading 5 µL of the PCR product onto a 1% agarose gel in 1x Tris-Acetate-EDTA (TAE) buffer. The 1% agarose gel was prepared by dissolving 1 gram of agarose in 100 mL of 1x TAE buffer and was stained with 3 µL of GreenSafe DNA dye. The DNA marker used ranged from 100 to 1400 base pairs (bp). Electrophoresis was performed at a voltage of 100 volts for 30 minutes. Visualization of the PCR products was carried out using UV light on a gel documentation system. PCR analysis is performed for detect the presence of $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} genes. Schematic of research show in Figure 1.

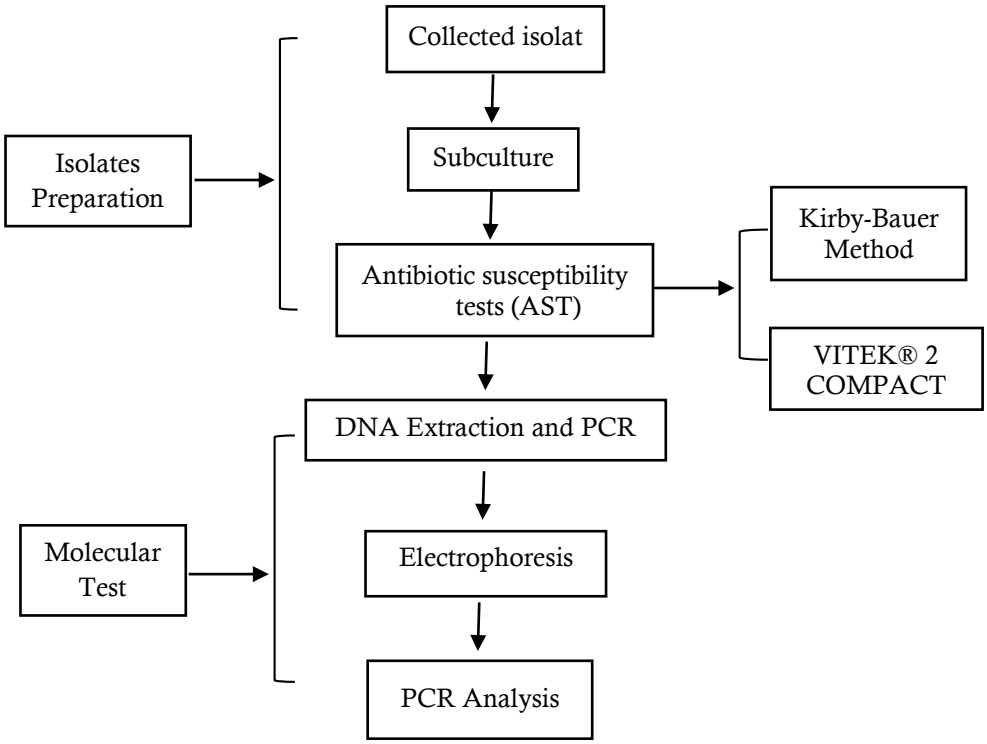
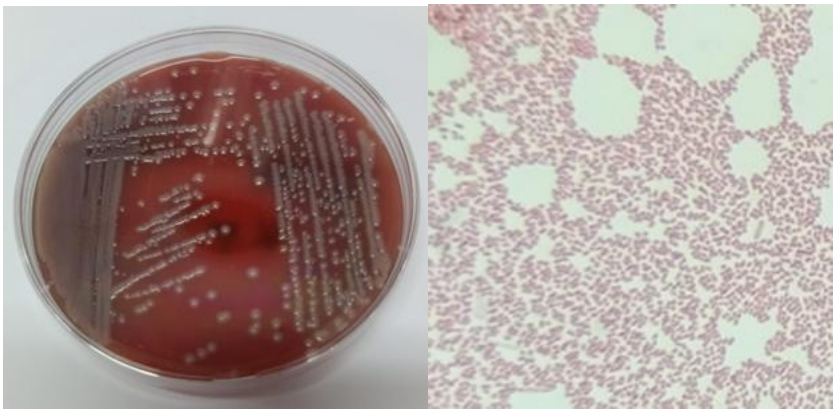


Figure 1. Schematic of research

3. Results and Discussion

A total of 43 *K. pneumoniae* cultures grown on 5% blood agar exhibited characteristic mucoid morphology, with a white to brownish color and non-hemolytic properties. Gram staining revealed *K. pneumoniae* morphology characterized pink with short rod-shaped Gram-negative bacteria. The absence of a hemolytic zone on blood agar may be attributed to the lack of hemolysins necessary to lyse red blood cells, distinguishing these bacteria from other Gram-negative organisms [16]. The mucoid surface of *K. pneumoniae* colonies indicates the presence of a capsular polysaccharide, which serves as a protective component of the bacterial outer structure and functions as a virulence factor. The cell wall of *K. pneumoniae* consists of a thin peptidoglycan layer, resulting in the observed pink coloration during Gram staining. This occurs because the bacteria are unable to retain the crystal violet stain and the lipid layer is decolorized by ethanol. The cells are subsequently stained by the counterstain safranin, which is insoluble in water [17].

Identification of *K. pneumoniae* was performed using an automated method by inserting a GN (Gram Negative) cassette into the VITEK system. Following the incubation period, the results indicated that all research isolates were free from contamination, with identification probabilities ranging from 98% to 99%. Identification with GN cards is based on biochemical methods and the use of substrates that utilize carbon sources, enzymatic activity and resistance. The tool analyzes the card using fluorescence kinetics, turbidity and colorimetric signals. The percentage of probability calculated by the tool refers to how accurate the observed biochemical reactions are compared to the typical reactions of each organism [18]. The bacterial cultures and the results of the microscopic observations are presented in Figure 2.

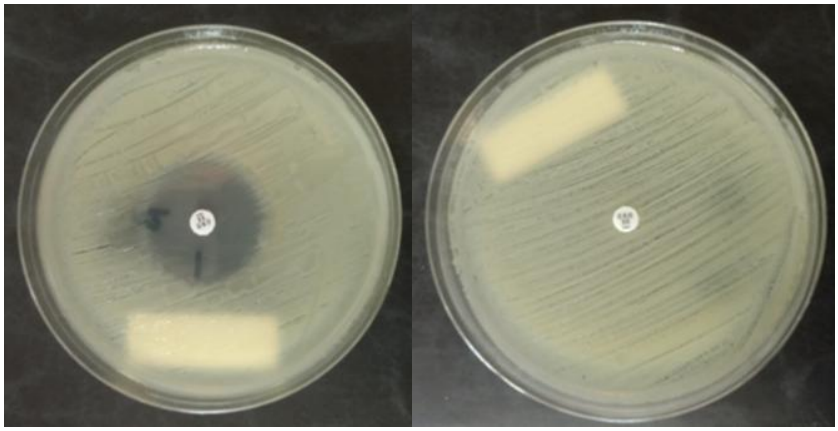


(A) (B)

Figure 2. Colonies of *K. pneumoniae* on 5% on blood agar and microscopic Gram staining at 10 x 100 magnification. (A) *K. pneumoniae* colonies, (B) results of Gram staining

Antibiotic susceptibility testing using the disk diffusion method (Kirby-Bauer) showed 12 isolates were resistant to ceftriaxone. No inhibition zones were formed for nine isolates Kp-5, Kp-21, Kp-28, Kp-40, Kp-56, Kp-57, Kp-60, Kp-68, and Kp-69. Additionally, three isolates Kp-3, Kp-7, and Kp-34 showed resistance with inhibition zone diameters of 8 mm, 9 mm, and 13 mm. According to CLSI (2020) guidelines for ceftriaxone at a concentration of 30 µg, the interpretation of inhibition zone diameters for Enterobacterales is classified as susceptible (S) if the diameter is ≥ 23 mm and resistant (R) if the diameter is ≤ 19 mm [19]. The disk diffusion method is relatively cost effective and easy to interpret. The inhibition zones formed around the antibiotic disks indicate whether the bacteria are susceptible, intermediate, or resistant to the tested antibiotics. However, a limitation of this method is that it cannot provide information on the effectiveness of the antibiotics. Additionally, the size of the inhibition zones is significantly influenced by the diffusion characteristics of the antibiotics in the agar medium and the bacterial inoculum size used [20].

Antimicrobial Susceptibility Testing (AST) for *Klebsiella pneumoniae* isolates showed twelve other isolates specifically Kp-3, Kp-5, Kp-7, Kp-21, Kp-28, Kp-34, Kp-40, Kp-56, Kp-57, Kp-60, Kp-68, and Kp-69 exhibited resistance to ceftriaxone, with MIC values ranging from 16 to ≥ 64 µg/mL. The MIC values obtained from the VITEK AST cassette were recorded between ≤ 1 µg/mL and ≥ 64 µg/mL. According to CLSI (2020) guidelines, ceftriaxone is classified as susceptible (S) for Enterobacterales, including *K. pneumoniae*, when the MIC is ≤ 2 µg/mL, and resistant (R) when the MIC is ≥ 4 µg/mL [19]. The Automated Susceptibility Testing (AST) method is employed to monitor emerging antimicrobial resistance patterns among pathogenic bacteria, enabling the Minimum Inhibitory Concentration (MIC) values obtained to be utilized in patient treatment protocols and infection control measures. However, the implementation of automated methods in AST must also take into account the costs and resources required for equipment, reagents, and trained personnel. These factors can pose significant barriers to the use of this testing method [21]. The representative susceptibility testing of the isolates under investigation on Mueller-Hinton agar (MHA) is shown in Figure 3.



(A) (B)

Notes: A. Isolate Kp-1 (sensitive), B. Isolate Kp-28 (resistant)

Figure 3. Sensitivity testing of *K. pneumoniae* on Mueller-Hinton agar (MHA) with ceftriaxone 30 µg

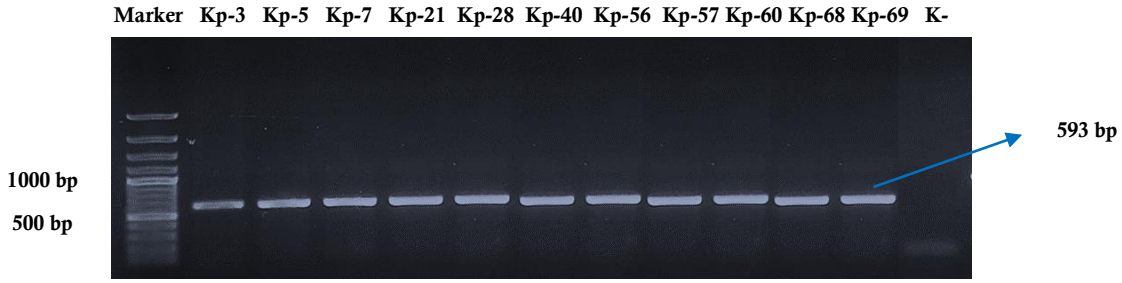
The results of the AST and antibiotic susceptibility testing using the disk diffusion method (Kirby-Bauer) are presented in Table 2.

Table 2. AST result and antibiotic susceptibility testing Kirby-Bauer method

Isolate Code	MIC Ceftriaxone µg/mL	Inhibition Zone Diameter (mm)	Interpretation
Kp-1	≤ 1	27	S
Kp-3	32	8	R
Kp-5	≥ 64	0	R
Kp-7	≥ 64	9	R
Kp-21	≥ 64	0	R
Kp-28	≥ 64	0	R
Kp-34	16	13	R
Kp-40	≥ 64	0	R
Kp-56	≥ 64	0	R
Kp-57	≥ 64	0	R
Kp-60	≥ 64	0	R
Kp-68	≥ 64	0	R
Kp-69	≥ 64	0	R
K. pneumoniae ATCC 13883	≤ 1	32	S

The amplification of the bla_{CTX-M} gene DNA was conducted using the polymerase chain reaction (PCR) method, employing primers based on previous research. The DNA band corresponding to the bla_{CTX-M} gene was detected in 11 out of 12 *Klebsiella pneumoniae* isolates (91.67%), specifically in isolates Kp-3, Kp-5, Kp-7, Kp-21, Kp-28, Kp-40, Kp-56, Kp-57, Kp-60, Kp-68, and Kp-69. The findings indicated that the gene bla_{CTX-M} was detected in 11 *K. pneumoniae* isolates, characterized by clearly visible DNA bands when visualized under UV light. The size of the PCR product for the gene bla_{CTX-M} was approximately 593 bp. According to data from GenBank (NCBI), the bla_{CTX-M} gene in *K. pneumoniae* has a length of 876 bp, indicating that the PCR product generated was shorter than the target gene. The presence of bla_{CTX-M} in this research demonstrates a match between ceftriaxone

resistance and genotype profile. Consistent with the research of Lubwama et al. (2024), *bla*$_{CTX-M}$ is one of the extended-spectrum beta-lactamase (ESBL) genes commonly identified in Enterobacteriaceae responsible for bacteremia in patients with hematologic cancers at UCI, Uganda [23]. Visualization of the *bla*$_{CTX-M}$ DNA band is shown in Figure 4.



Note: K- = negative control.

Figure 4. Detection of the *bla*$_{CTX-M}$ gene in *Klebsiella pneumoniae* isolates with a size of 593 bp

The DNA band corresponding to the *bla*$_{SHV}$ gene was clearly detected in 9 out of 12 *Klebsiella pneumoniae* isolates (75%), specifically in isolates Kp-5, Kp-7, Kp-21, Kp-28, Kp-34, Kp-56, Kp-57, Kp-68, and Kp-69, with the size of the amplified DNA band measuring approximately 868 bp. The size of the *bla*$_{SHV}$ gene according to GenBank is 861 bp, thus the PCR product produced was slightly longer than the target gene. The presence of *bla*$_{SHV}$ in this research will show the match between the resistance to fenotif ceftriaxone and the genotype profile. Another study by Abdallah et al. (2023) reported increased resistance in *K. pneumoniae* to cefotaxime and ceftazidime associated with the presence of phenotypic and genotypic resistance profiles of ESBLs such as *bla*$_{SHV}$ [24]. The visualization of the *bla*$_{SHV}$ DNA band is presented in Figure 5.

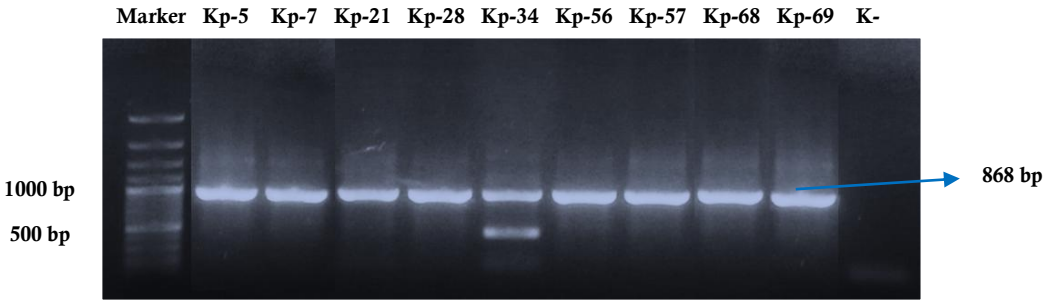
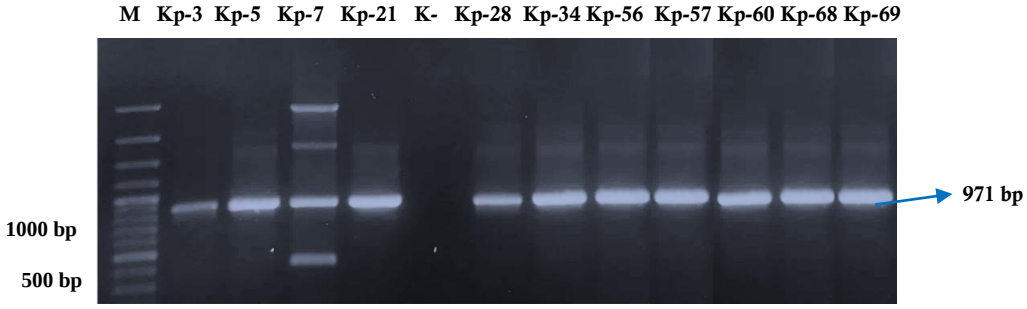


Figure 5. Detection of the *bla*$_{SHV}$ gene in *Klebsiella pneumoniae* isolates with a size of 868 bp

The DNA band corresponding to the *bla*$_{TEM}$ gene was detected in 11 out of 12 *Klebsiella pneumoniae* isolates (91.67%), specifically in isolates Kp-3, Kp-5, Kp-7, Kp-21, Kp-28, Kp-34, Kp-56, Kp-57, Kp-60, Kp-68, and Kp-69, with the size of the amplified DNA band measuring approximately 971 bp. The *bla*$_{TEM}$ gene, as reported in GenBank has a length of 861 bp, indicating that the PCR product was larger than the target gene. The presence of *bla*$_{TEM}$ in this research will show the match between the resistance to fenotif ceftriaxone and the genotype profile. The study conducted by Nargesian et al. (2023) also reported a high prevalence of extended-spectrum beta-lactamase (ESBL) encoding genes in urinary tract infection (UTI) patients in the city of Qom, including *bla*$_{SHV}$ and *bla*$_{TEM}$, which was found at a rate of 74.54% [25]. The visualization of the *bla*$_{TEM}$ DNA band is presented in Figure 6.



Note: M = mark K- = negative control

Figure 6. Detection of the *bla*$_{TEM}$ gene in *Klebsiella pneumoniae* isolates with a size of 971 bp

Distribution patterns of resistance genes in *Klebsiella pneumoniae* isolates with respect to ceftriaxone MIC values and inhibition zone diameters showed that two isolates were detected to possess the *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes, specifically Kp-3 and Kp-60. One isolate, Kp-34, was found to harbor both the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes. The genes *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ were simultaneously detected in eight isolates: Kp-5, Kp-7, Kp-21, Kp-28, Kp-56, Kp-57, Kp-68, and Kp-69, all presenting an MIC value of ≥ 64 µg/mL. In these cases, no inhibition zone was observed for seven isolates. The distribution patterns of the *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes are presented in Table 3.

Table 3. Distribution patterns of *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes in relation to ceftriaxone MIC values and inhibition zones

Gene	Isolate Code	Sampel Type	MIC Ceftriaxone (µg/mL)	Inhibition Zone (mm)
bla$_{CTX-M}$ + *bla*$_{TEM}$	Kp-3	Tissue	32	8
	Kp-60	Sputum	≥ 64	0
bla$_{SHV}$ + *bla*$_{TEM}$	Kp-34	Pus	16	13
bla$_{CTX-M}$ + *bla*$_{SHV}$ + *bla*$_{TEM}$	Kp-5	Tissue	≥ 64	0
	Kp-7	Tissue	≥ 64	9
	Kp-21	BAL	≥ 64	0
	Kp-28	Urine	≥ 64	0
	Kp-56	Sputum	≥ 64	0
	Kp-57	Urine	≥ 64	0
	Kp-68	Feses	≥ 64	0
	Kp-69	Feses	≥ 64	0

Note : BAL (Bronchoalveolar Lavage)

The co-detection of these genes in the *Klebsiella pneumoniae* isolates corresponds to the MIC values and the inhibition zones formed, indicating that isolates with MIC values ranging from 16 to ≥ 64 µg/mL and inhibition zones of ≤ 13 mm are likely to harbor multiple resistance genes. The presence of *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes together in *K. pneumoniae* isolates represents a potential risk for resistance to other β-lactam antibiotics [26]. Consistent with the study conducted by Dirar et al. (2020), *E. coli* and *K. pneumoniae* isolates obtained from clinical patients were detected to harbor the *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes, which may contribute to resistance within the β-lactam antibiotic group including ceftriaxone [27]. Isolates that detected only one or two ESBL genes in this study may be due to the fact that not all *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes are expressed simultaneously or to the presence of repressor genes that may result in different levels of enzyme expression [28].

K. pneumoniae is a member of the Enterobacteriaceae family that is well adapted for horizontal gene transfer. Resistance can occur due to the presence of mobile resistance genes mediated by plasmids. Various genetic elements play a role in transferring resistance genes from different bacterial species, facilitating horizontal gene transfer and thereby posing a potential threat for resistance development [29-30].

4. Conclusion

The *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes were detected in *Klebsiella pneumoniae* isolates, with *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ identified in 11 isolates (91.67%) and *bla*$_{\text{SHV}}$ in 9 isolates (75%). There was a conformity between the phenotypic test results and the genotypic findings of the *K. pneumoniae* isolates, indicating that higher minimum inhibitory concentration (MIC) values were associated with the simultaneous detection of the *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ genes.

5. Acknowledgements

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