زانکۆی سەلاحەدین - هەولێر
Salahaddin University-Erbil

Antibiotic Resistance Pattern and Extended Spectrum Beta Lactamase Producing *Klebsiella pneumoniae* Isolated From clinical samples

Research Project

Submitted to the Department of (Biology) in partial fulfillment of the requirements for the degree of **BSc.** in **Biology**

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April – 2023

CERTIFICATE

This research project has been written under my supervision and has been submitted for the award of the **BSc.** degree in **Biology** with my approval as a supervisor.

Signature
Name:

Date: **April , 2023**

DEDICATION

This effort I dedicate to **Allah** Almighty, my lord, my powerful foundation, my source of inspiration, wisdom, knowledge, and understanding. Throughout this project, he was the source of my energy.

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ACKNOWLEDGMENTS

To begin with, I thank (**Allah**) for His blessing, which made me able to complete and perform this study with success, the lord of the universe, blessing, and peace be on **Muhammad** (Allah's peace and prayers be upon him).

Finally, I want to say thanks to all those I forgot them here to mention his/her name, who assisted me even by one useful scientific word directly or indirectly.

SUMMARY

Background: Due to the recent appearance of organisms that are resistant to several drugs (multidrug-resistant) like Enterobacteriaceae that produce extended-spectrum β-lactamase (ESBL, concerns have remarkably increased regarding the suitable treatment of infections. The present study was an investigation into ESBL molecular characteristics among clinical isolates of *Klebsiella pneumoniae* and their pattern of antimicrobial resistance in order to come up with helpful information on the epidemiology of these infections and risk factors accompanied with them.

Methods: In order to conduct the study, 10 *K. pneumoniae* were isolated and retrieved from Central and Internal Lab. of Teaching Hospital hospitals in Erbil, Iraq during October, 2022 and February, 2023. The collected strains were analyzed, and the profile of their antimicrobial susceptibility was specified. In order to spot β-lactamase genes (i.e., *bla*TEM, *bla*SHV, and *bla*CTX-M), a polymerase chain reaction was conducted.

Results: The findings obtained from multiplex PCR assay showed that out of the collected strains of ESBL-producing *K. pneumoniae* had 100% *bla*TEM, 80% *bla*CTX-M, and 70% *bla*SHV genes. Antibiotic resistance pattern of *K. pneumoniae* isolates to 10 antibiotics varied widely. However, the majority of the *K. pneumoniae* isolates were multi-drug resistant (MDR).

Conclusion: TEM prevalence was high among other types of ESBLs. Overall, the most active antimicrobial agents *in vitro* remained to be the Amikacin.

Keywords: *Klebsiella pneumoniae*, ESBL, bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$

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1. INTRODUCTION

The genus *Klebsiella* was named by Edwin Klebs. The bacillus which known as *Klebsiella* was described by Carl Friedlander and for many years the "Friedlander bacillus" was well known as a cause of severe, often fatal pneumonia (Luce, 2010). They are widely distributed in nature, occurring both as commensals in human and animal intestines as well as saprophytes in soil, water and vegetation. *Klebsiella* are non-motile, Gram-negative, straight rods and possess a polysaccharides capsule. The capsule is responsible for the mucoid appearance of the bacterial colonies and enhanced virulence of the organism *in vivo* (Chakraborty, 2005).

The genus *Klebsiella* is an important human pathogen, which has been associated in recent decades with nosocomial outbreaks. As opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus, chronic pulmonary obstruction, urinary tract infections and pneumonia. Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, the medically most important species of the genus (Podschun and Ullmann, 1998).

Bacterial resistance to antimicrobial agents is a threat to public health throughout the world. The presence of antibiotic resistance genes on bacterial plasmids and transposons have further helped in the transmission and spread of drug resistance among pathogenic bacteria. During the last years, outbreaks of diseases due to multi-resistant strains of bacterial pathogens have occurred (Reichling et al., 2009). The discovery of antimicrobial agents had a major impact on the rate of survival from infections. However, the changing patterns of antimicrobial resistance caused a demand for new antibacterial agents. Routine monitoring of antibiotic resistance provides data for antibiotic therapy and resistance control, prescription programs, making policy decisions and assessing the effectiveness of both (Olowe et al., 2008).

K. pneumonia is resistant to a number of antibiotics mainly extended-spectrum cephalosporin's and penicillin's due to acquisition of plasmid that encode for the

production extended spectrum β-lactamase (ESβL) enzyme (Samaha-Kfoury and Araj, 2003). ESBLs are a group of enzymes that lead to resistance increase in Aztreonam, Ceftazidime, Cefotaxime, related Oxyimino-β-lactams, cephalosporins, and penicillins, but Clavulanic acid inhibits them. TEM, SHV, and CTX-M are the 3 main types of ESBLs. CTX-M, which has become more prevalent than SHV and TEM, includes a rapidly expanding family which has spread among a wide range of clinically important bacteria and over wide geographic areas (Paterson and Bonomo, 2005). Furthermore, strains that produce ESBL often demonstrate resistance to antibiotics belonging to other classes (i.e. aminoglycosides, quinolones, and sulfonamides), which makes strategies of treatment more complex (Liao et al., 2017).

In addition, Enterobacteriaceae family members such as *Klebsiella pneumoniae* and *Escherichia coli* often produce ESBLs; however, other genera of the *Enterobacteriaceae* family have recently been reported to contain some other enzymes. A higher level of resistance in such organisms was first observed in patients with prolonged hospital stays in intensive care units in Europe. However, isolates were identified in Africa, Asia, the Middle East, and South and North Americas, and ESBL GNB soon became a global problem and concern (Malloy and Campos, 2011).

Common ESBL genes coding for isolates of *K. pneumoniae* and *E. coli* were determined as CTX-M (cefotaximase that preferentially hydrolyzes cefotaxime), TEM (found and isolated in the early 80s from Teminora who was a Greek patient), and SHV (for variable of sulphydryl which was first observed in a single *Klebsiella ozaenae* strain retrieved in Germany). These genes which are mediated by transposons, plasmids, or chromosomes are all sporadically described all over the world (Akpaka et al., 2010).

The aims of this study are to isolate and identify *K. pneumonia* from different clinical samples as well as determining the susceptibility pattern and Extended Spectrum Beta Lactamase of the isolates.

2. MATERIALS AND METHODS

Specimen's collection

A total of (10) isolates of K. pneumoniae were collected from urine and wound, within the period between October, 2022 and February, 2023 in Central and Internal Lab. of Teaching Hospital in Erbil city.

Culture media

Different culture media such as Nutrient agar, MacConkey agar and Blood agar were used to isolate and identification of this above pathogens, these media were prepared as described by (Atlas, 2010).

Isolation and identification of bacteria

All isolates which collected were cultured on different media (Nutrient agar, MacConkey agar and Blood agar) by streaking method and incubated at 37 ^{0}C for 18-24hours. Discrete colonies were sub-culture into nutrient agar aseptically to obtain pure culture of the isolates, then transfer single colonies to MacConkey agar directly and incubated for 24 hours for screening of E. coli. Size, shape and fermenting of lactose were studied, and then the colonies that fermenting lactose sugar and carried K. pneumoniae features were purified more than one time to obtain purified culture. Then all isolates were identified according to morphology, cultural and biochemical tests. Besides, 16S rRNA-based molecular identification of clinical isolates was utilized to re-identify them.

Susceptibility studies

The isolates were subjected to antibiotic sensitivity test to 10 antibiotics which include Amoxiclave (AMC 20 µg), Amikacin (AK 30 µg), Cefoxitin (FOX 30 µg), Gentamycin (G 10 µg), Nitrofurantoin (F 300 µg), Pipercillin (PIP 100 µg), Tetracycline (TE 30 µg), Lincomycin (MY 10 µg), Trimethoprim-Sulfamethoxazole (SXT 1.25+23.75 µg) and Rifampin (RA 5 µg) as clarified in Table (1) was determined according to NCCLS (National Committee for Clinical Laboratory Standards) standards. Twenty ml of Muller Hinton agar melted and cooled at 45 °C was poured into sterile petri plates and allowed to solidify completely. A lawn of test

pathogen was prepared by evenly spreading 100µl inoculums (1.5 x 108 CFU/ml) (according to 0.5 McFarland standard solutions) with the help of a sterilized swab onto the entire surface of agar plate. The plates were allowed to dry before applying antibiotic disc. The discs were firmly applied to the surface of agar plates within 15 minutes of inoculation (Wayne, 2012).

Table (1): Antibiotics name, abbreviations, and standard diameter of inhibition zone

No.	Antibiotics*	Symbol	Concentration (µg)	Zone diameter, Nearest Whole (mm)		
				R **	I or M	S
1	Amoxiclave	AMC	20	≤ 19	…..	≥ 20
2	Amikacin	AK	30	≤ 14	15-16	≥ 17
3	Cefoxitin	FOX	30	≤ 14	15-17	≥ 18
4	Gentamycin	G	10	≤ 12	13-14	≥ 15
5	Nitrofurantoin	F	300	≤ 14	15-16	≥ 17
6	Pipercillin	PIP	100	≤ 17	…..	≥ 18
7	Tetracycline	TE	30	≤ 11	12-14	≥ 15
8	Lincomycin	MY	10	≤ 12	13-16	≥ 17
9	Trimethoprim-Sulfamethoxazole	SXT	1.25+23.75	≤ 10	11-15	≥ 16
10	Oxacillin	CO	1	≤ 19	20	≥ 21

Amplification of 16S gene

Universal primers 1492R (1) (5′- GGTTACCTTGTTACGACTT -3′) as reverse primer and 8F (5′- AGAGTTTGATCCTGGCTCAG -3′) as forward were used to carry out PCR for the amplification of the 16S rRNA gene (Turner et al., 1999). All reactions of PCR were conducted by utilizing 2 µl DNA template (density of 10 ng/µl), the Master Mix consisting of 3 mM MgCl2, 0.2% Tween® 20, 20 mM Tris-HCl pH 8.5, (NH4)2S04, 0.2 units/µl Ampliqon Taq DNA polymerase, 0.4 µM of each primer, and 0.4 mM of each dNTP. The conditions of PCR included primary denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 45 s, at 55 °C for 45 s, and at 72 °C for 90 s, and a last extension at 72 °C for 6 min. After safe staining, the product of the reaction was visualized under UV light on a 1% agarose gel (Russell and Sambrook, 2001) (Fig. 1). The PCR products were used for sequencing the 16S rRNA of all 78 bacterial isolates.

Detection of ESBL genotypes by multiplex PCR amplification

Using the method utilized by (Monstein et al., 2007) with slight modifications, multiplex PCR was employed to examine the positive isolates in the initial screening test for ESBL production for the existence of blaSHV, blaCTX-M, and blaTEM genes. Freshly cultured isolates bacteria were used to prepare template deoxyribonucleic acid (DNA) was prepared using PrestoTM Mini gDNA bacterial kit. All reactions of PCR were conducted by utilizing 2 µl DNA template (density of 10 ng/µl), the Master Mix consisting of 3 mM MgCl2, 0.2% Tween® 20, 20 mM Tris-HCl pH 8.5, (NH4)2S04, 0.4 mM of each dNTP, 0.4 µM of each primer, and 0.2 units/µl Ampliqon Taq DNA polymerase. The conditions of polymerase chain reaction amplification were set up as follow: primary denaturation step for 10 minutes at 95° C; 30 denaturation cycles for 30 seconds at 94° C, annealing 30 seconds at 60° C for, extension for 2 minutes at 72° C, and a final extension step for 10 minutes at 72° C. Using agarose gel electrophoresis, size separation PCR amplicons were utilized to detect respective genes **(Table2).**

Table 2. List of primers used for Multiplex PCR amplification

Target gene	Primer	Sequence (5'-3')	Amplicon size	References
*bla*TEM	Forward	TCG CCG CAT ACA CTA TTC TCA GAA TGA	445-bp	(Al-Ouqaili, 2018)
	Reverse	ACG CTC ACC GGC TCC AGA TTT AT		
*bla*SHV	Forward	ATG CGT TATATT CGC CTG TG	747-bp	(Paterson et al., 2003)
	Reverse	TGC TTT GTT ATT CGG GCC AA		
*bla*CTX-M	Forward	ATG TGC AGY ACC AGT AAR GTK ATG GC	593-bp	(Al-Ouqaili, 2018)
	Reverse	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

3. RESULTS AND DISCUSSION

Ten isolates of *K. pneumoniae* were collected from urine and wound of human infections, from Central and Internal Lab. of Teaching Hospital. The isolates were initially been identified using conventional (which is depend on cultural, morphological, and biochemical tests). The characteristics of isolates were studied, through culturing it on MacConkey agar the results showed that the colonies of *K. pneumoniae* were circular, lactose fermenter, mucoid and rose-pink in color, as shown by (Brooks et al., 2007). Also, it is cleared from the Gram stain procedure that the bacterial cell of *K. pneumoniae* from smear preparation are gram negative rods, arranged singly in pairs or short chain and presumptively as described in (Brooks et al., 2007).

After isolates had initially been identified using conventional, to emphasize the results of isolates under study the molecular method was used for isolate identification based on the sequence of 16S ribosomal RNA. The 16S rRNA gene was amplified for each strain to confidently identify bacteria. And the results were recorded in Table (3).

Table3: Morphological and biochemical characteristics of *E. coli* isolates.

Parameters	**Results**
Gram stain	Negative
Cellular morphology	Rods
Growth on Blood agar	Large grayish-white
Growth on MacConky agar	Rose-pink mucoid
Indole test	Negative
Citrate test	Positive
Urease test	Positive
Catalase test	Positive

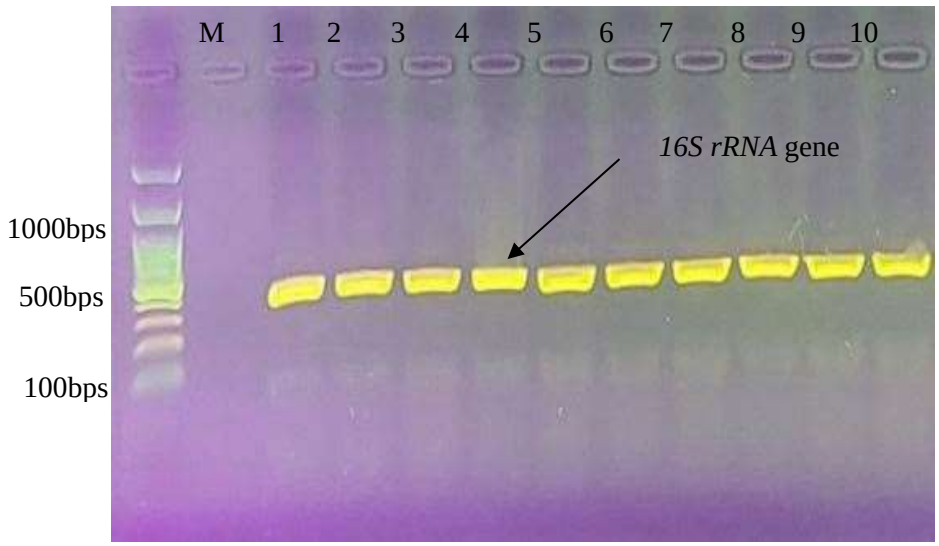

Figure 1 Agarose gel electrophoresis of PCR amplification products for the 16S ribosomal RNA of *E. coli*. **M**: The DNA marker (100 bp ladder), lanes **1, 2, 3, 4, 5, 6, 7, 8, 9,** and **10** positive amplification of **16S ribosomal RNA**.

Antibiotic susceptibility assay

In the present study table (4) shows the susceptibilities of 10 isolates to different antibiotics and the result showed that all *K. pneumoniae* isolates were sensitive to Amikacin and 90% of isolates were sensitive to Gentamicin, Cefoxitin, and Oxacillin, followed by Trimethoprim-Sulfamethoxazole and Tetracycline (60% and 50%) respectively. On the other hand, this study revealed that all the isolates (100%) were resistant to Pipercillin and Lincomycin, and 60% of isolates were resistant to Amoxiclave and Nitrofurantoin. The resistance of *K. pneumoniae* to different antibiotics in this study confirms similar findings reported by several workers. A study done by (Khadri and Alzohairy, 2009) reported that 75%, 69%, 50% and 48% of their *K. pneumoniae* isolates were sensitive to AK, G, SXT and TE respectively. (Kaftandzhieva et al., 2009) also found in their study that 100% and 60% of the isolates were resistance to PIP and AMC respectively. Also, a study by Feizabadi *et al*., [(26)] found that 42% of the isolates were resistance to F. And finally, (Khadri et al., 2007) reported that 28% of the isolates were resistance to FOX. The high resistance of the bacteria to different antibiotics may be related to the presence and dissemination of plasmids within heterogeneous population of these bacteria. Our findings agree

with other observations done by (Sharma et al., 2007) who demonstrated that using inaccurate concentration of antibiotics or drug or unnecessary of medicine appointment leads to the resistance of sensitive bacteria, in addition to weakling immunity system in some human due to poor nutrition or heredity factors make bacteria to be more resist. On the other hand the results may regard to the increase resistance of *K. pneumoniae* to antibiotics due to the evolution and spread of plasmid encoded ESβL enzyme and other genes conferring cross-resistance to other antibiotics. This is of concern due to the increasing cost of antibiotic treatment and the spread of multi-drug resistance to more pathogenic microorganisms (Kamatchi et al., 2009). ESβL are enzymes which can hydrolyze Penicillins as well as Cephalosporins and Monobactam. The majority of ESβL isolates are also resistant to other antibiotics such as Fluroquinolones and Aminoglycosides. This finding was supported with those previously recorded by (Chaudhary and Aggarwal, 2004).

Table (4): Percentage Resistance of *K. pneumoniae* to antibiotics

No.	Antibiotics*	No. of resistant isolates	% of resistant
1	AMC	6	60
2	AK	0	0
3	FOX	1	10
4	G	1	10
5	F	6	60
6	PIP	10	100
7	TE	5	50
8	MY	10	100
9	SXT	4	40
10	CO	1	10

Also, the table (5) shows a detailed resistance pattern to antimicrobial agents. All isolates of *K. pneumoniae* show difference in susceptibility against these antibiotics which used in this study started from 30% of resistant as low percentage and high percentage of resistance was recorded by isolate no. 8 which represent 70% to all antibiotics.

Table (5): Antibiotic Susceptibility Results of *K. pneumoniae*

Bacterial isolate no.	Antibiotics										% resistance
	AMC	AK	FOX	G	F	PIP	TE	MY	SXT	CO	
ISO 1	R	S	S	S	R	R	S	R	S	S	40
ISO 2	S	S	S	S	S	R	R	R	S	S	30
ISO 3	S	S	S	S	R	R	R	R	S	S	40
ISO 4	S	S	S	S	R	R	S	R	S	S	30
ISO 5	R	S	R	S	R	R	S	R	R	S	60
ISO 6	R	S	S	S	R	R	R	R	R	S	60
ISO 7	R	S	S	S	S	R	S	R	R	S	40
ISO 8	R	S	S	R	S	R	R	R	R	R	70
ISO 9	S	S	S	S	R	R	R	R	S	S	40
ISO 10	R	S	S	S	S	R	S	R	S	S	30

In PCR detection of ESBL genotypes, it was found that all of the ESBL screening positive K. pneumoniae isolates had one or more ESBL genes that were tested in the present study. Overall, 85% (17/20) of K. pneumoniae isolates were positive for one or more ESBL genes. The multiplex PCR assay results indicated that 100% blaTEM genes, 80% blaCTX-M, and 70% blaSHV genes were detected in the *E. coli* isolates genes were detected in the K. pneumoniae. The overall incidence of ESBL genotypes in *K. pneumoniae* isolates is illustrated in fig. 2.

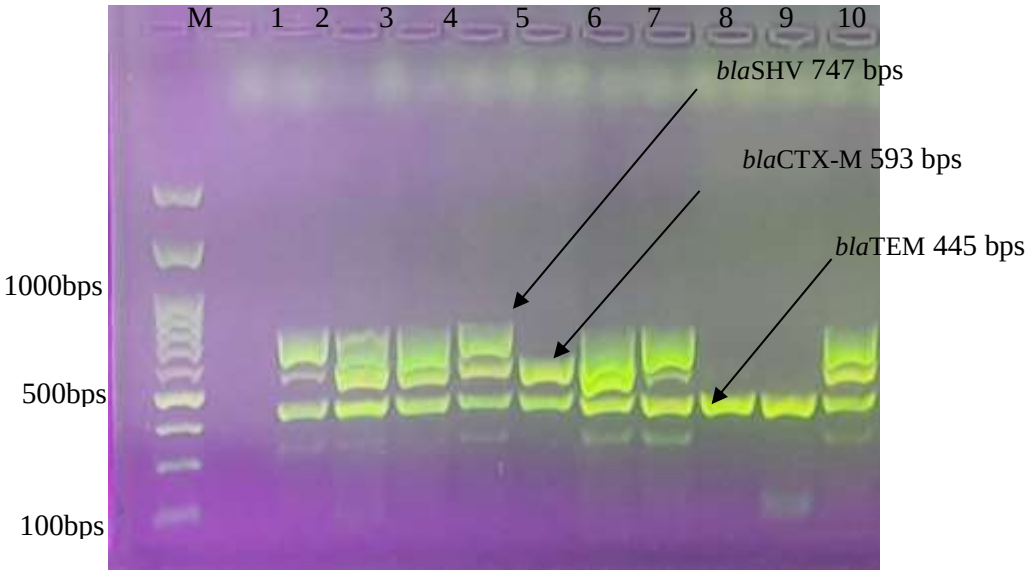

Figure 2. PCR products of SHV, CTX-M and TEM genes with expected sizes of 747 bp, 593 bp and 445 bp respectively against 100 bp DNA Ladder on a 1% agarose gel.

Phenotypic tests for detection of ESBL can only confirm ESBL production but fail to recognize the subtypes of ESBL. As reported by (Nüesch-Inderbinen et al., 1996), molecular methods have been proved to be sensitive, but they costly and conducting them requires a long time, expertise, and specialized equipment. Ultimate identification is only probable through methods of molecular detection. The results of a study conducted by (Navon-Venezia et al., 2003) revealed that it is necessary to periodically evaluate these phenotypic tests because introduction of new enzyme can change their performance. In their study of phenotypic and genotypic methods of ESBL detection, (Grover et al., 2006) stated that PCR is a reliable method for detecting ESBL. In the present study, multiplex PCR amplification assay was utilized to detect blaCTX-M, blaSHV, and blaTEM genes in the retrieved clinical isolates of K. pneumoniae because one of the advantages of this assay rapid screening of large numbers of clinical isolates, moreover, if it is required, further molecular epidemiological studies can take advantage of the DNA that is isolated via this assay (Monstein et al., 2007).

Furthermore, it is essential to identify beta lactamase in order to conduct a reliable epidemiological investigation into antimicrobial resistance. The current study was conducted to survey antimicrobial drug resistance, ESBL phenotypes, and blaSHV, blaTEM and blaCTX-M genes detection in K. pneumoniae isolates retrieved from urinary tract infections in Erbil.

The most globally common type of ESBL appeared to be CTX-M-type ESBLs with their higher incidence in most locations compared to SHV and TEM ESBLs (Jorgensen et al., 2010). Among the three ESBL genotypes included in this study, the most prevalent one was found to be blaTEM (81%) in ESBL-producing isolates of K. pneumoniae. The less prevalent ESBL genotype was blaSHV, and the prevalence rate of blaSHV in ESBL-producing K. pneumoniae isolates (16.2%). A study conducted by (Manoharan et al., 2011) reported similar findings. Furthermore, in another study, (Moghnieh et al., 2018) have reported that E. coli and Klebsiella spp resistance to

third-generation cephalosporins is usual in whole countries, with outbreak reaching over 50% in Egypt and Syria.

4. CONCLUSIONS

Knowledge of the antimicrobial resistance patterns and resistance genes of bacterial pathogens in a geographical area is important for control and surveillance of antibiotic resistance. The results of the present study revealed that MDR was highly prevalent. In addition, the Carbapenems, Amikacin, and Ciprofloxacin were found to be the most to the least active antimicrobial agents in vitro. Based on the results obtained in the present study, TEM was highly prevalent among other types of ESBLs.

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