

Molecular study of *Klebsiella* *Pneumoniae* antibiotic resistance genes isolates from wound infections

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genes
isolates from wound infections**

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ABSTRACT

K. pneumoniae, which produces Carbapenemases, is of public health importance because of its resistance to antimicrobials. *K. pneumoniae* is identified phenotypically by its large pink mucus appearance on MacConkey agar because of lactose fermentation and by bacteriological, microscopic, and biochemical tests using the VITK 2 device. Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion. *Klebsiella pneumoniae* represented 5.3% (n = 16). It is highly resistant to antibiotics used to treat wound infections and shows the highest rate of resistance with cefepime 11(68.8%), followed by Ertapenem 9(56.3%), Doripenem and Imipenem 7(43.8%), and meropenem 6(37.5%). Carbapenemases resistance was identified by phenotype through the Hodge test and also detection of Carbapenemases genes by polymerase chain reaction. The results showed the presence of the bla SHV 16 gene (100%) in *K. pneumoniae* isolates. The results showed there were 4 isolates (25%) positive for the bla AMP gene. The results also showed the presence of the bla KPC gene in two isolates (12.5%). The bla GES gene was not found in *K. pneumoniae* isolates. In general, the isolates were resistant to carbapenem and cefepime antibiotics. Five random samples were examined by publishing the results of the sequence analysis by BLAST in (NCBI) and the Mega 7 polymorphism detection program: it was found that there was no difference between the sequences, but rather the match was with the bacterium *Klebsiella pneumoniae*.

Keyword: *Klebsiella pneumoniae*, Modified Hodge Test, Carbapenemases class A., PCR, sequence

Introduction

K. pneumoniae is widely distributed in nature and poses a threat to human life in cases of infection [1]. Most bacteria belonging to the order *Enterobacteriaceae* have characteristics that can confer virulence, pathogenicity, and phenotypes. These characteristics can lead to serious health concerns, such as the development of multidrug resistance [2].

Antibiotic resistance has grown due to the expression of beta-lactamase enzymes, such as class A Carbapenemases and (AmpCs), in Gram negative bacteria. This has therapeutic implications. failure, extended hospital stays, rising medical expenses, and fatality [3]. Antimicrobial resistance (AMR) has been observed among Gram negative bacteria because beta-lactamases manifest themselves, and this is a global public health risk [4].

In Iraq Due to their low toxicity and high efficacy, beta-lactam antibiotics from the cephalosporin class are overused in this region and elsewhere in the world to treat infections, which has resulted to the phenomenon of resistance termed resistance The beta-lactam ring's active amide link is broken by beta-lactamases for them to function. Chromosome-encoded or acquired beta-lactams through mobile genetic elements (MGEs) like plasmids. MGEs function as vectors in the transfer of AMR β -lactamase (bla) and non- β -lactam genes between bacterial species, leading to limited therapeutic options for treating infections [5].

Plasmid-mediated *AmpC* β -lactamases are a group of clinically relevant cephalosporins. *AmpC* overproduction confers resistance to broad-spectrum cephalosporins including cefepime [6]. It has been widely documented that gut bacteria produce ampC. The overabundance of *AmpC* β -lactamases and the overexpression of efflux pumps are cause for concern as they could potentially give Gram negative bacteria more resistance against carbapenems. Crucially, bacteria that produce AmpC are a source of illnesses linked to healthcare that have difficult treatment choices [7].

Several carbapenems have been discovered, the most notable of which are the carbapenems *Klebsiella pneumoniae* Carbapenemases (KPC) (Class A Carbapenemases), Guiana-Extended Spectrum (*GES*), and *Sulphydryl variable (SHVs)* Carbapenemases and have been reported globally as a major contributor to healthcare-associated infections [8]. Class A enzymes are resistant to Carbapenems are effective in Iraq, although beta-lactamases containing Gram negative bacteria are more common. Studies have only investigated the occurrence of carbapenems in the intestines, concentrating on *Klebsiella pneumoniae* and *Escherichia coli*. [9].

Therefore, few investigations have documented the simultaneous existence of β -lactamases, such as *AmpC* and Carbapenemases, in the class of Gram-negative bacteria. Using medical sources, data on resistance of antimicrobial-producing organisms are crucial for directing the use of antibiotics, conducting efficient monitoring, and preventing and controlling infections. Thus, this study looked at the prevalence and antibacterial qualities of Resistance to Carbapenemases and ampicillins in a collection of recovered gram-negative bacteria from sources that are clinical A beta-lactam antibiotic called carbapenem inhibits peptidase, a protein that binds penicillin, and stops the synthesis of peptidoglycan, which results in lytic cell death [10].

Beta-lactam antibiotics are used to treat gram-negative infections. Carbapenems exhibit the widest range of inhibitory action against Gram negative bacteria among all beta-lactam antibiotics [11]. Carbapenems, such as Imipenem, meropenem, Ertapenem, and Doripenem, are currently used in clinical practice [12]. Carbapenem hydrolytic enzymes are carbapenems. Because genes for Carbapenemases class A (e.g., bla *KPC*, bla *GES*, bla *SHV*) are transported by highly disenable genetic elements, making this downstream method especially concerning. [13].

Methods

Collection of samples

During the period extending from September 2023 to December 2023, wound swabs were collected from Al-Hussein Teaching Hospital. Nasiriya General Hospital and Al-Haboubi Teaching Hospital in Dhi Qar, consisting of 300 samples from surgical patients. Samples were transferred using a sterile transport medium swab for preservation. Then, swabs were taken and transferred to the College of Science laboratory: to grow the bacteria on blood agar and MacConkey agar media for 24 hours at a temperature of 37°C for bacterial diagnosis.

Isolation and Diagnosis of *K. pneumoniae*

The bacterial isolate was diagnosed based on Morphology and microscopic and biochemical tests [14,15]. The VITEK 2 system has also been used to verify the diagnosis of *Klebsiella* at the species level and to prevent discrepancies in biochemical test results *K. pneumoniae* [16], as shown in Figure (1).

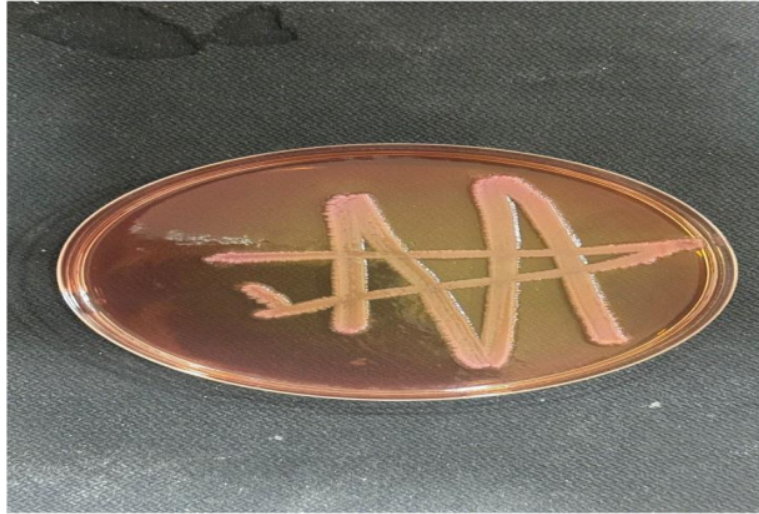


Figure 1: *Klebsiella pneumoniae* colonies on MacConkey agar

A-Phenotypic Method

Identification of Carbapenemases (Modified Hodge Test)

This test was used to detect *K. pneumoniae* Carbapenemases (*KPC*, *GES.SHV*, *AMPC*) according to the CLSI [17]. A diagnostic and sensitive *E. coli* isolate was taken to the carpenems and cefepime group and compared with the 0.5 McFarland standard. In terms of turbidity, then the Muller-Hinton agar plate was streaked with the *E. coli* sample, then 6 plates were left to dry for 3-10 minutes. Next, an antibiotic disc 6 carpenems or Cefepime (10 µg/ml) was placed in the center of the MHA plate. Then take a straight line from 17 the disc to the end of the dish from the *Klebsiella pneumoniae* colonies The Mueller-Hin 36 plates were then incubated at 37°C for 16-20 hours. A positive MHT 7 has a cloverleaf-like indentation of *E. coli* bacteria growing along the growth line of the test organism within the disc diffusion zone. A negative MHT test has no growth of *E. coli* along the growth line of the *K. pneumoniae* test within the disc diffusion. A positive MHT result indicates that this isolate produces Carbapenemases. However, a negative MHT indicates that this isolate is unable to produce Carbapenemases.

Genotypic Method (PCR technology)

Amplification of 16S r RNA gene

Using primers K 16S-F and K 16S-R, which stand for primers for PCR amplification of *K. pneumoniae* 16S r RNA, all 16 isolates were submitted to molecular testing. (16) isolates produced positive findings, all of which were identified as *K. pneumoniae*. The results of the PCR amplification confirmed this diagnosis as shown in Figure (2).

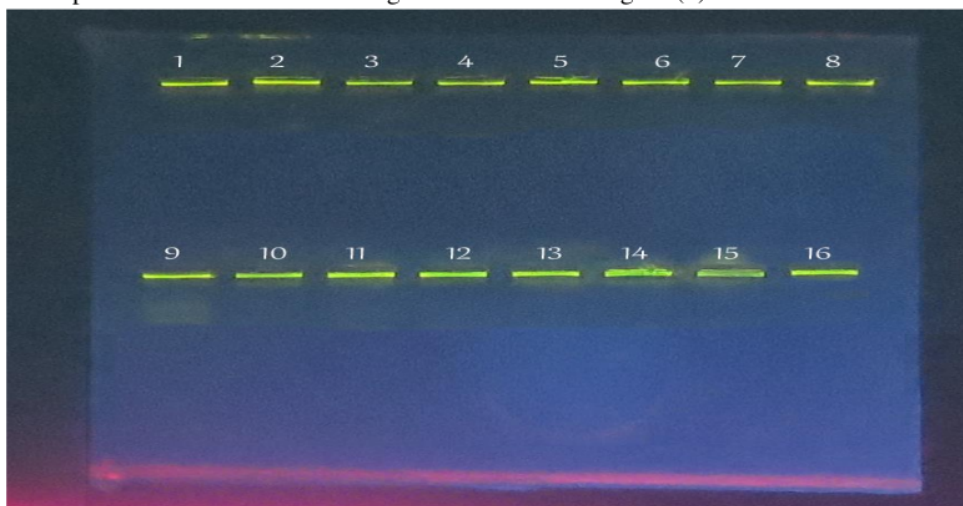


Figure 2: 16S r RNA gene of *K. pneumoniae*

Selection and preparation of primers

Forward and reverse primers (Macrogen Company/South Korea) were selected for detection (*bla KPC*, *GES*, *SHV*, *AMPC*). These primers were supplied in dried form and stored in the freezer until use. The sequences of the primers used in this study are listed in Table 1

Table (1) The primers used, annealing temperature and the products size for Genes of Macrogen Company/South Korea

No.	Gene	Primer Sequence ³ to ⁵	Product size. Annealing temp.	Reference
1	16 S r RNA	F- AGAGTTTGATCMTGGCTCAG	1500 bp, 56 C	[18]
		R- TACGGYTACCTTGTTACGACTT		
2	<i>KPC</i>	F- TGTCACGTGATCGCCGTCTAG	821 bp, 60 C	[19]
		R-TTACTGCCCGTTGACGCCCAATCC		
3	<i>SHV</i>	F- ATGCGTTATATTGCCTGTG	753 bp, 60 C	[20]
		R-TGCTTGTATTTCGGGCAA		
4	<i>GES</i>	F-GCGTTTTGCAATGTGCTCAAC	846 bp, 55 C	[21]
		R-CGCCGCCATAGAGGACTTTAG		
5	<i>AMPC</i>	F-ATCAAAACTGGCAGCCG	550 bp, 60 C	[22]
		R-GAGCCGTTTATGCACCCA		

Amplification reaction

Primers, PCR premix, and extracted DNA were vortexed and quickly centrifuged to force the material to the tubes' bottoms. Several attempts were conducted until the polymerase chain reaction was optimized. A 20 µl PCR mixture was created, including 2 µl of each primer and 6 µl of template DNA. Free water nuclease was added to finish the remaining volume. Everything was absent from the negative control except for the DNA, which had D.W. inserted in place of the template DNA. After being vortexed, PCR reaction tubes were eventually put inside the heat cycler PCR apparatus [23].

Agarose gel electrophoresis

Agarose gel electrophoresis (1.5% agarose, 70% V for 2–3 h) was used to separate DNA fragments and PCR products that were visualized with the help of ethidium bromide and a UV documentation system [24].

Results

Isolation and Diagnosis of *K. pneumoniae*

Based on the physical characteristics of the colonies on MacConkey agar, *K. pneumoniae* isolates were first identified. Because lactose ferments, *K. pneumoniae* isolates on MacConkey agar appeared mucous, big, and pink in color. and all isolates were identified by microscopic examination. Gram-negative bacteria are short or double-stranded bacilli and use chemical IMVC tests for Indole and methyl red. The results were negative, while the results with Voges-Proskauer and citrate were positive. The VITEK 2 system has also been used to verify *Klebsiella* diagnosis at the species level and to prevent discrepancies in biochemical test results.

Phenotypic Method

Identification of Carbapenemases (Modified Hodge Test)

Carbapenemases production by MHT is detected when the test isolate produces the enzyme that allows the growth of a carbapenem-sensitive *E. coli* strain. Three *K. pneumoniae* isolates were resistant to carbapenems (25.0%). Of the sixteen samples, the Hodge test result was positive in only three samples. The result is a distinct indentation that resembles a clover leaf. While Carbapenemases production by MHT is detected when the test isolate produces the enzyme that allows the growth of a cefepime sensitive *E. coli* strain. Five isolates were resistant to cefepime (31.25%). Of the 16 samples, only five samples tested positive for the Hodge test. The result is a distinct indentation that resembles a clover leaf. The result is shown in Figure (3).

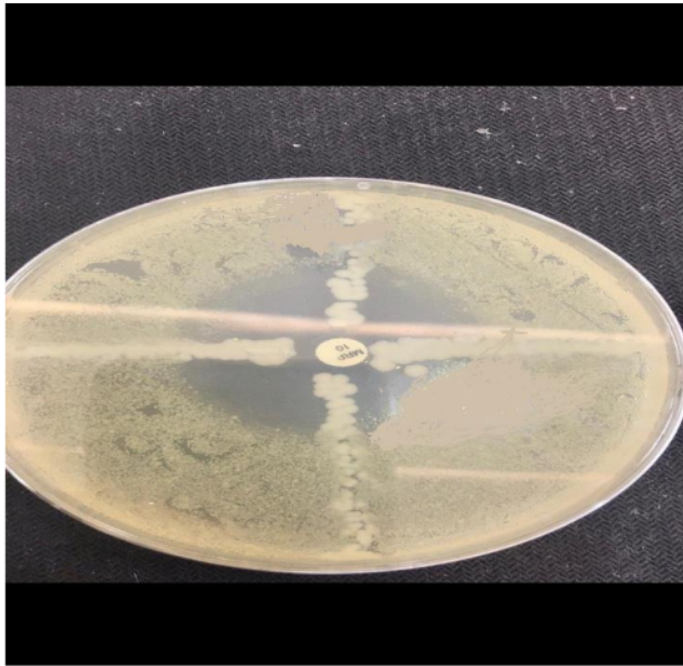


Figure 3: Hodge test of Carbapenemase resistant *K. pneumoniae*

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Molecular detection of Carbapenemase genes class A in clinical isolates of *Klebsiella pneumoniae*

1- Carbapenemase genes class A (*KPC*): It was detected by conventional PCR. Table 2 shows the distribution. Carbapenemase genes were identified by specific PCR primers. The results showed that some of the 16 isolates tested contained *Klebsiella pneumoniae* genes. However, two of the isolates tested were identified as possessing the *KPC* gene (12.5%), as shown in Figure (4)

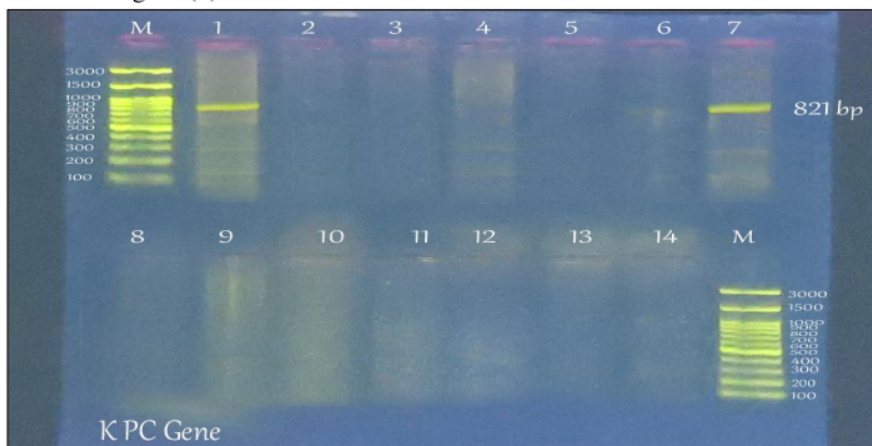


Figure 4: *KPC* gene of *K. pneumoniae*

Figure (4) Agarose gel electrophoresis of the bla *kpc* gene product (amplified size 821 bp) using the DNA template of carbapenem-resistant *K. pneumoniae* isolates extracted using the salting-out method. Lane (m). DNA molecule size marker (100 bp ladder). Lanes (K1 and K7) for *Klebsiella pneumoniae* isolates show positive results with the bla *kpc* gene, and lanes (k2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, and K16) show negative results. With bla *kpc* gene.

2- Carbapenemases genes class A (AMPC): It was detected by conventional PCR. Table 2 shows the distribution. Carbapenem genes were identified by specific PCR primers. The results showed that some of the 16 isolates tested contained *Klebsiella pneumoniae* genes. However, four of the isolates tested were identified as possessing the AMPC gene (25.0%). as shown in Figure (5).

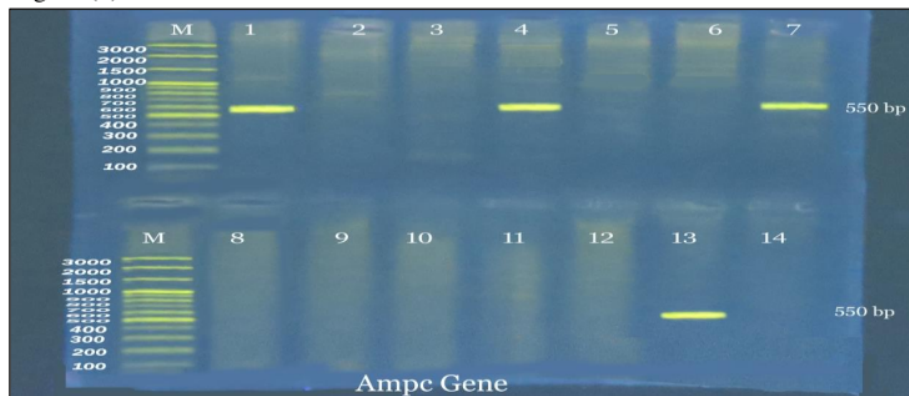


Figure 5: Ampc gene of *K. pneumoniae*

Figure (5) Agarose gel electrophoresis of the bla AMPC gene product (amplified size 550 bp) using the DNA template of cefepime-resistant *Klebsiella pneumoniae* isolates extracted using salting out. road. lane (3). DNA molecule size marker (100 bp ladder). Lanes (K1, K4, K7, and K13) of *Klebsiella pneumoniae* isolates show positive results with the bla AMPC gene, and lanes (k2, 3,5, 6,8, 9, 10, 11, 12, 14, 15, and K16) show negative results. With the bla AMPC gene.

3- Carbapenemases genes class A (SHV): It was detected by conventional PCR. Table 2 shows the distribution. Carbapenem genes were identified by specific PCR primers. The results showed that all 16 isolates tested contained the *Klebsiella pneumoniae*. SHV gene (100%). as shown in Figure (6).

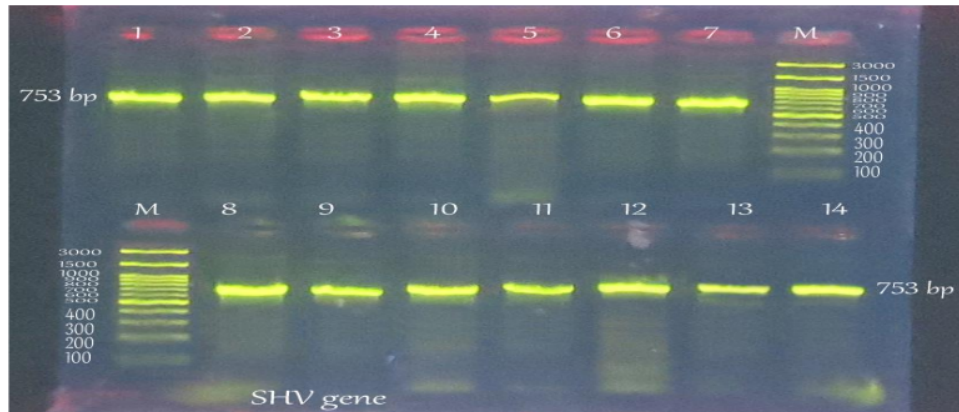


Figure 6: SHV gene of *K. pneumoniae*

Figure (6) Agarose gel electrophoresis of the bla SHV gene product (amplified size 753 bp) using the DNA template of carbapenem-resistant *Klebsiella pneumoniae* isolates extracted using the salting-out method. lane (m). DNA molecule size marker (100 bp ladder). Lanes (K1,2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and K16) of *Klebsiella pneumoniae* isolates show positive results with the bla SHV gene.

4- Carbapenemases genes class A (GES): It was detected by conventional PCR. Table 2 shows the distribution. Carbapenem genes were identified by specific PCR primers. The results showed that all 16 isolates tested did not contain *Klebsiella pneumoniae* genes. GES gene (0%). as shown in Figure (7).

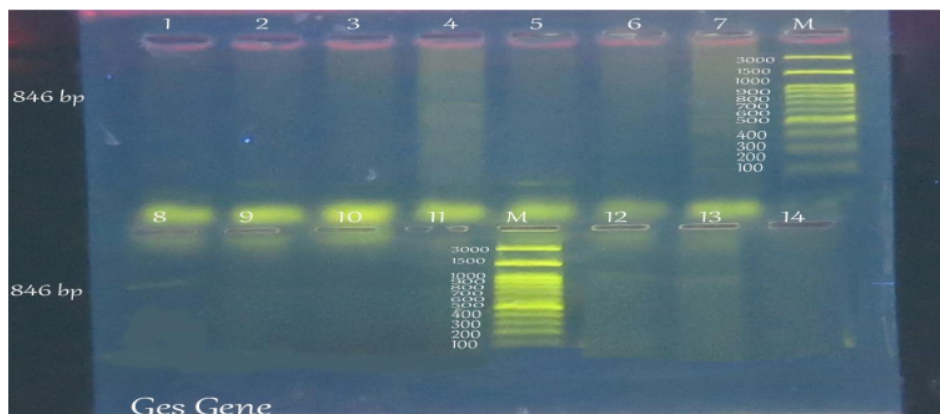


Figure 7: GES gene of *K. pneumoniae*

Figure (7) Agarose gel electrophoresis of the bla GES gene product (amplified size 846 bp) using the DNA template of carbapenem-resistant *Klebsiella pneumoniae* isolates extracted using salting out. road. lane (m). DNA molecule size marker (100 bp ladder). Lanes (K1,2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and K16) of *Klebsiella pneumoniae* isolates show negative results with the bla GES gene.

Comparison of the phenotype with the genotype of *Klebsiella pneumoniae*:

It was observed that isolates that produce Carbapenemases enzymes are resistant to the antibiotics of the carpenems group and cefepime, by comparing the gene type with the phenotype, where the production of Carbapenemases enzymes in the phenotype of the carpenems group was 25.0%, while it was 31.25% for cefepime. When comparing the results of the phenotype to produce Carbapenemases: With gene type, we find that the isolates that produce Carbapenemases are less resistant to the carpenems group compared to the isolates that produce Carbapenemases with the highest resistance to cefepime.

Table (2) Distribution of Carbapenemases phenotype isolated from carbapenem-resistant *Klebsiella pneumoniae*

No.	Meropenem	Imipenem	Ertapenem	Doripenem	Cefepime
K 1	+	-	-	-	-
K 2	-	-	-	-	-
K 3	-	-	-	-	+
K 4	-	-	-	-	-
K 5	+	-	+	-	+
K 6	-	-	-	-	-
K 7	-	-	-	-	+
K 8	-	-	-	-	+
K 9	-	-	-	-	+
K 10	+	-	-	-	-
K 11	-	-	-	-	-
K 12	-	-	-	-	-
K 13	-	-	-	-	-
K 14	-	-	-	-	-
K 15	-	-	-	-	-
K 16	-	-	-	-	-
Percentage	3 (18.75)	0 (0.0)	1 (6.25)	0 (0.0)	5 (31.25)
Total Positive	25.0				31.25

Table (3) Distribution Of Carbapenemases genes Carbapenem resistant *Klebsiella* isolate

No.	Types of Carbapenemases genes class A			
	KPC	SHV	GES	AMPC
K1	+	+	-	+
K2	-	+	-	-
K3	-	+	-	-
K4	-	+	-	+
K5	-	+	-	-
K6	-	+	-	-
K7	+	+	-	+
K8	-	+	-	-
K9	-	+	-	-
K10	-	+	-	-
K11	-	+	-	-
K12	-	+	-	-
K13	-	+	-	+
K14	-	+	-	-
K15	-	+	-	-
K16	-	+	-	-
Total Positive %	2 (12.5)	16 (100)	0 (0.0)	4 (25.0)

Discussion

Klebsiella pneumoniae is one of the most common bacteria causing multidrug-resistant nosocomial infections due to the presence of Carbapenemases and AMPC enzymes. Antimicrobial susceptibility assays, PCR and genetic Carbapenemases, and a high prevalence of highly virulent MDR *K. pneumoniae* isolates are present in clinical specimens. Due to multidrug resistance resulting from the emergence of drug-inactivating enzymes, especially beta-lactamases. Carbapenems are increasingly being identified in bacteria causing nosocomial infections [25].

Class A *Klebsiella pneumoniae* Carbapenemases include enzymes that inactivate beta-lactam antimicrobials in various ways. Beta-lactamases are produced in intestinal bacteria and have been detected in many parts of the world, these genes have been found in *K. pneumoniae*. In this study, the percentage of *SHV* genes in *Klebsiella pneumoniae* isolates was 100% in Iraq. The prevalence of *SHV* genes was higher than in clinical isolates of *Klebsiella*. bla *SHV* genes in Iran (87.5%). Likewise, *K. pneumoniae* isolates expressing *KPC* carbapenems are among the species of interest. Recently, several alarming cases of multi-antibiotic-resistant infections have emerged for years, due to their XDR phenotypes and their tendency to spread rapidly in nosocomial settings, with significant impact [26]. *KPCs* inactivate all beta-lactam antibiotics. The bla *KPC* gene has a higher prevalence, with the percentage of results of the current investigation in the city of Nasiriya being 2/16 (12.5%). The percentage of other studies is 1/40 (2.5%) [27].

The higher than the results of 1/22 (4.5%) isolated by [28]. The current study shows slightly lower results, 2/16 (12.5%), as the results also indicated that 12 (16.2%) of the *K. pneumoniae* isolates carried the bla*KPC* gene of those reported by global monitoring (Al-Sahlawi, 2011; Kiratsin et al., 2008; Lin et al., 2010; Shahid et al., 2011; Abraham et al., 2014). The PCR results revealed the *GES* gene, as there was a match between the current study in Iraq, where the *GES* genes did not appear, with another study in Egypt. The *GES* gene was not found in *Klebsiella pneumoniae* sites [29]. However, the current study in Iraq does not match other studies in which *GES* prevalence rates were determined. by conventional PCR [30]. In Iran, the *GES* gene was isolated (16%) [31]. The present study used PCR to identify the bla *AmpC* gene. In the current study conducted in Iraq, the results indicated the presence of the *AMPC* gene in a percentage of 4/16 (25.0%). This is lower than in previous studies of *AMPC* gene expression conducted by Al-Sahlawi in Najaf, where it was found that 27.4% of *K. pneumoniae* cases were amplified using bla *AmpC* primers. In (2011), compared with other research, the *AMPC* gene was lower than that conducted by Al-Hafnawi (2008) and Pham et al. (2013) in Egypt where *AmpC* occurs at 34% respectively [32].

The current study hypothesizes that identifying the *SHV*, *KPC*, *GES*, and *AmpC* genes may help combat infections in hospitals and the doctor's ability to prescribe the most appropriate antibiotic drug, thus reducing the selective pressure that generates antibiotic resistance. Through the current study, it was shown that by limiting entry into their external cell walls through porin channels, antibiotics may be unable to reach their areas of action, which could be linked to resistance to beta-lactam medications. Active efflux pumps may also play a role in this process. The act of removing an antibiotic from its site of action prior to its onset of action is considered resistance. Since the first-time antibiotics were used, one important

mechanism of antibiotic resistance has been the modification of antibiotics via hydrolysis. ¹⁹ In the current research, It was shown that bacteria exhibited a higher sensitivity of around 62.5% to meropenem, but a lower resistance of 37.5%. similar findings correspond with those of previous research conducted in Iraq, which identified similar microorganisms. Wound isolates showed a significant susceptibility to meropenem [33]. The increased prevalence of germs resistant to several drugs might be attributed to individuals who took antibiotics prior to surgical debridement [34].

Overuse of antibiotics, which is widespread among patients due to their ¹⁰ frequent hospitalizations, can also be attributed to antibiotic resistance [35], show that the microbial infection patterns in diabetic foot infections are variable; hence, it is necessary to repeatedly assess the microbiological properties and antibiotic sensitivity in order to choose the right antibiotics [36].

¹⁰ In the current investigation, the antibiotic meropenem induced the highest rate of sensitivity. Even though carbapenems are the most significant and effective antibiotics for treating MDR *K. pneumoniae* ²⁶ infections, carbapenem resistance has been more widely documented globally. Several mechanisms, including the production of Carbapenemases ³¹ deficit in outer membrane porin (OprD), contribute to carbapenem resistance. Moreover, the horizontal transmission of resistance to other species is a significant problem posed by Carbapenemases genes [37].

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