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The Prevalence of *Pks* Genotoxin Among *Klebsiella Pneumoniae* Isolated from Different Clinical Samples in Baghdad, Iraq

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Abstract

Klebsiella pneumoniae is a pathogenic bacterium that has been linked to several illnesses acquired in both the community and hospitals. This study aimed to determine the prevalence of the *clbA*, *K1*, and *K2* genes in *K. pneumoniae* and their relationship with antibiotic resistance. In this research, 130 clinical samples from patients in hospitals were collected from blood, urine, wounds, burns, and sputum, and only 61 of those samples were positively identified as *K. pneumoniae* using biochemical tests, selective media, and Vitek II. The method of disc diffusion was utilized to evaluate the 61 isolates' susceptibility to 10 antibiotics, including Imipenem, Meropenem, Cefixime, Ceftazidime, Amoxicillin-clavulanate, Gentamicin, Aztreonam, Azithromycin, Levofloxacin, and Trimethoprim-sulfamethoxazole. The results showed that the highest resistance was towards amoxicillin-clavulanate (98.36%) and the lowest towards meropenem (13.11%), and that 95.08% of the isolates were multidrug resistant (MDR). The *clbA*, *K1*, and *K2* genes were detected using conventional PCR; the results showed that out of 61 isolates, only 3 were positive for *K1*, 52 were positive for *K2*, and none of the isolates were *clbA*+. (2/3) of the *K1* and (51/52) of the *K2* isolates were MDR. The finding shows a substantial association between the *K1* and *K2* genes and resistance to antibiotics, as the capsule is the main virulence factor in this bacteria.

Keywords: pathogenic bacteria, colibactin, capsule, MDR, disc diffusion.

انتشار السم الجيني *Pks* بين *Klebsiella Pneumoniae* المعزولة من عينات سريرية مختلفة في بغداد، العراق

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الخلاصة

Klebsiella pneumoniae هي بكتيريا ممرضة تم ربطها بالعديد من الأمراض اكتسبت في كل من المجتمع والمستشفيات. تهدف هذا الدراسة إلى تحديد مدى انتشار جينات *clbA* و *K1* و *K2* في بكتيريا *K. pneumoniae* وعلاقتها بمقاومة المضادات الحيوية. في هذا البحث، تم جمع 130 عينة سريرية من المرضى في المستشفيات من الدم، الادرار، الجروح، الحروق واللعاب، وتم تشخيص 61 عينة فقط على أنها تعود لجنس *K. pneumoniae* باستعمال الاختبارات الكيميائية الحيوية، والوساط الانتقائية، و Vitek II. استعملت طريقة الانتشار القرصي لتقييم حساسية 61 عينة لـ 10 مضادات حيوية بما في ذلك Imipenem و

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و Gentamicin و Amoxicillin-clavulanate و Ceftazidime و Cefixime و Meropenem و Aztreonam و Levofloxacin و Azithromycin و Trimethoprim-sulfamethoxazole. أظهرت النتائج أن أعلى مقاومة كانت تجاه Amoxicillin-clavulanate 98.36% وأقل مقاومة تجاه Meropenem 13.11% وأن 95.08% من العزلات كانت مقاومة للأدوية المتعددة (MDR). تم الكشف عن جينات *clbA* و *K1* و *K2* باستعمال تفاعل البوليميراز المتسلسل التقليدي، وأظهرت النتائج أنه من أصل 61 عزلة فقط 3 عزلة كانت موجبة لـ *K1*، و 52 موجبة لـ *K2* ولم تكن أي من العزلات يحتوي على *clbA* +. (3/2) من عزلات *K1* و (52/51) من عزلات *K2* كانت MDR. تظهر النتائج ارتباطاً جوهرياً بين جينات *K1* و *K2* ومقاومة المضادات الحيوية لأن الكبسولة هي عامل الضراوة الرئيسي في هذه البكتيريا.

Introduction

One of the most antibiotic-resistant bacteria causing outbreaks in both the community and the hospital setting is *Klebsiella pneumoniae*, an *Enterobacteriaceae* member pathogen that causes health-associated illnesses [1]. *K. pneumoniae* is negative for gram stain, has a conspicuous polysaccharide capsule of substantial thickness, and appears shiny and mucoid on plates of agar [2]. Following *Escherichia coli*, this species is the second most frequent cause of Gram-negative bacteremia. Significant morbidity and mortality are brought on by *K. pneumoniae* bacteremia in the general population [3]. Nosocomial respiratory infections, urinary tract infections (UTIs), inflammation of wounds, and bloodstream infections are all caused by the common hospital-acquired bacterium *K. pneumoniae* [4]. Drug-resistant *K. pneumoniae* is regarded as a significant pathogen acquired in hospitals, particularly in intensive care units [5]. The most recent classification divides *K. pneumoniae* strains into two main pathotypes: classical *K. pneumoniae* and hypervirulent *K. pneumoniae* (HVKP).

The classic form has a limited capacity for virulence while being a prevalent infectious agent in relation to hospital-acquired pneumonia (HAP). In addition, the classical pathotype readily changes mobile genetic components like plasmids to produce MDR strains, whereas HVKP is known to be the primary cause of fulminant and invasive illnesses and infections in populations. The HVKP pathotype can also have plasmids that are resistant to carbapenem or are hypervirulent [6]. The pathogenesis of *K. pneumoniae* depends heavily on the expression of a number of virulence factors, such as the capsule, endotoxin, siderophores, iron scavenging system, and fimbriae [7]. Just a few of the more than 80 capsular serotypes that have been recognized in *K. pneumoniae* are usually found in serious infections. The polysaccharide capsule is the primary pathogenic component of this bacteria. Under laboratory circumstances, it has been shown that the capsule increases the bacterial ability to resist phagocytosis, antibacterial peptides, and complement deposition [8]. Serotypes K1 and K2 of these are related to bacteremia and have been linked to high rates of mortality in Taiwan, Europe, and North America [9]. Hypermucoviscosity appears to be linked to capsular serotype K1 and, to a lesser extent, serotype K2 [10]. Toxins secreted by bacteria that are pathogenic may have cytotoxic or cytostatic impacts on the cells they invade. *Enterobacteriaceae* with polyketide synthase (PKS) genes are known to produce the toxin colibactin. The 54 kb-long PKS island was discovered in multiple *Enterobacteriaceae* family members [11]. Colibactin is another virulence factor, a secondary metabolite that interferes with eukaryotic cells' cycles, is synthesized in the genetic island PKS, and has been associated with human colon cancer [12]. This study aimed to determine the prevalence of the *clbA*, *K1*, and *K2* genes in *K. pneumoniae* and their relationship with antibiotic resistance.

Materials and methods

Bacterial isolation and identification

A total of 130 samples, including sputum, blood, burns, urine, and wounds, were collected by culturing them in Brain Heart Infusion Broth (BHI Broth) as shown in Figure (1) from the educational laboratory/medical city, Baghdad teaching hospital, Burns specialist hospital, and Ghazi Al-Hariri Hospital in Baghdad, Iraq, from September 2022 to December 2022. Utilizing selective media (MacConkey agar, Eosin methylene blue agar (EMB), and CHROMagar) and biochemical tests (urease, simmon citrate, oxidase, catalase, and indole), all isolates were identified and also confirmed by Vitek II.

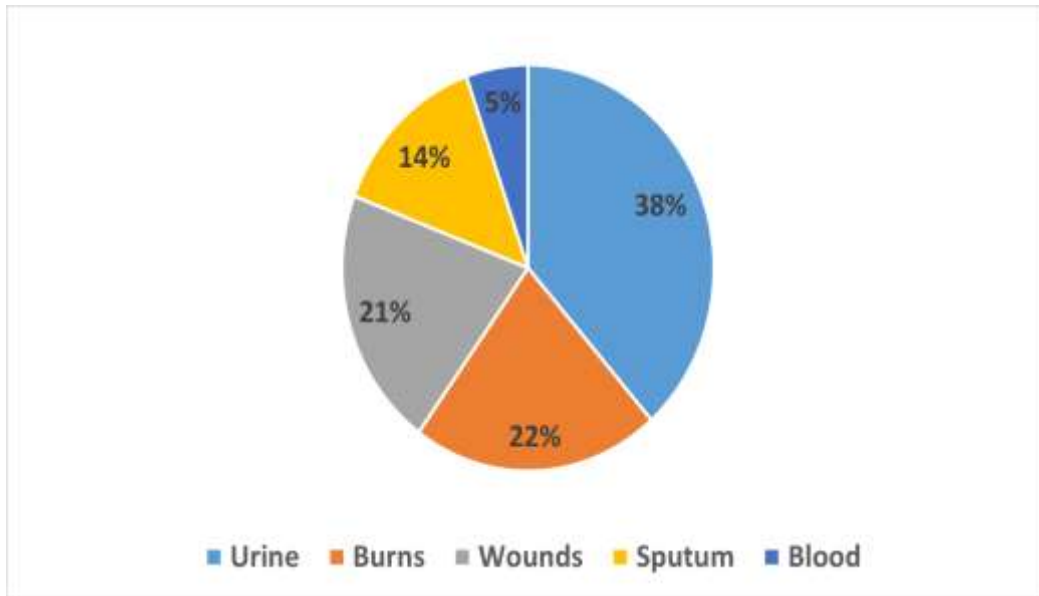


Figure 1: The percentage of clinical samples that were examined

Antibiotics susceptibility test

According to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), samples were evaluated for resistance to antibiotics. Ten antimicrobial agents were used to examine each sample, and the antibiotics used are presented in Table 1. The results were evaluated in accordance with CLSI (2022) guidelines [13].

Table 1: Antibiotics used in this study

Antibiotic	Symbol	concentration (µg/disc)
Imipenem	IPM	10
Meropenem	MEM	10
Amoxicillin-clavulanate	AMC	20/10
Ceftazidime	CAZ	30
Cefixime	CFM	5
Aztreonam	ATM	30
Gentamicin	GEN	10
Azithromycin	AZM	15
Levofloxacin	LVX	5
Trimethoprim-sulfamethoxazole	SXT	1.25/23.75

DNA extraction

Genomic DNA was extracted from bacterial growth in accordance with the directions on the ABIopure Extraction Genomic DNA Mini Kit (ABIopure, USA). Before use, the isolated DNA was stored at -20°C.

Oligonucleotides and PCR amplification

Conventional PCR was used to detect whether the genes were present. The sequence of primers used in this research is presented in Table 2.

Table 2: Primers sequences used in this research for the detection of genes

Gene	Primer sequence 5'→3'		Product size (base pair)	Reference
<i>K1</i>	F	CCTTTCTTAAATCAGCATCG	329 bp	This study
	R	CTGGAGGAGTTGGGAATAAAT		
<i>K2</i>	F	AAAGGCAATTCCAAAGGAGA	466 bp	This study
	R	GACGGAGTATTCGGAGAGAT		
<i>ClbA</i>	F	CTAGATTATCCGTGGCGATTC	1002 bp	[14]
	R	CAGATACACAGATACCATTCA		

Specific primers were used for the detection of the *ClbA*, *K1*, and *K2* genes by using the extracted DNA from *K. pneumoniae*. The volume of the reaction mixture used was 20 µl: 10 µl Master Mix, 1 µl forward primer, 1 µl reverse primer, 2 µl DNA, and then 6 µl of nuclease-free water were added to reach the 20 µl volume. In the thermal block, the PCR reaction was conducted.

The PCR program for detecting the genes started with initial denaturation at 95 °C for one cycle for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing for 30 seconds at 54 °C, 48 °C, and 49 °C for the genes (*ClbA*, *K1*, *K2*), extension for 30 seconds at 72 °C, and finally, final extension for 7 minutes at 72 °C for one cycle. On a 1.5% agarose gel that includes Red Safe Dye, the PCR products were observed. The gel was then inspected under ultraviolet (UV) light after that.

Statistical analysis

The Statistical Analysis System [15] program was used to detect the effect of different factors on study parameters. The chi-square test was used to compare the significance between percentages (0.05 and 0.01 probability) in this study. A P-value ≤ 0.05 was considered statistically significant, and a P-value ≤ 0.01 was considered highly significant.

Results and discussion

Klebsiella pneumoniae isolation and identification

The bacterial isolates were collected from hospitals by culturing them in BHI Broth, and then they were cultured and identified using different selective media (MacConkey agar, Eosin methylene blue agar (EMB), and CHROMagar). *K. pneumoniae* isolates showed up as big, mucoid, pink colonies on MacConkey agar due to lactose fermentation. While on Eosin methylene blue agar, they appeared as a dark pink, mucoid, smooth, and large colony, and metallic blue colonies on CHROM agar. After that, the isolates were tested by different biochemical tests (urease, indole, oxidase, simmon citrate, and catalase). The bacteria showed negative results for indole and oxidase, while they were positive for urease, simmon citrate, and catalase.

The results showed that only 61 of the 130 bacterial specimens were recognized to be *Klebsiella pneumoniae*; the results were also confirmed by Vitek II. Figure 2 shows *K. pneumoniae* growth on different selective media. Figure 3 shows the Vitek II results.

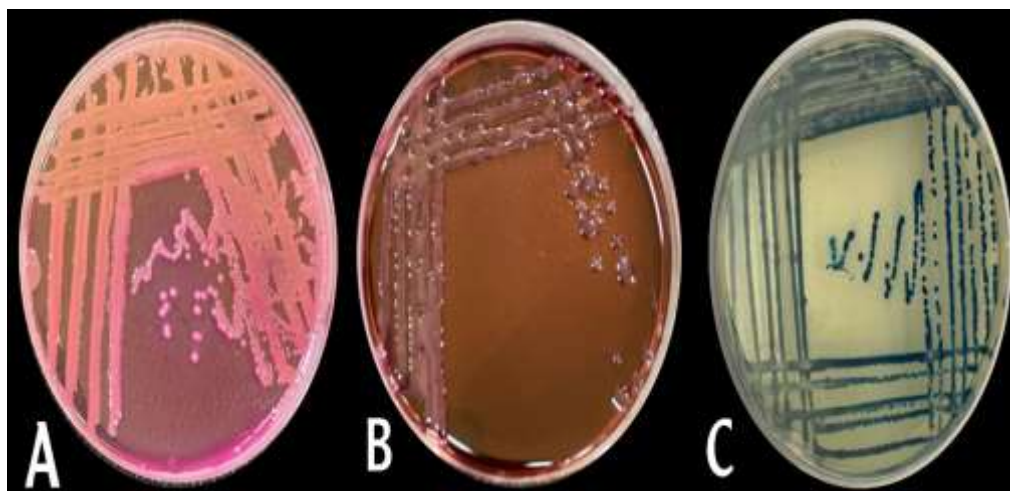


Figure 2: *K. pneumoniae* appearance on A) MacConkey agar, B) EMB agar, and C) CHROMagar.

bioMérieux Customer:		Microbiology Chart Report		Printed March 11, 2023 1:27:44 PM CST													
Patient Name: mustafa jassim, 2			Patient ID: 47														
Location:			Physician:														
Lab ID: 47			Isolate Number: 1														
Organism Quantity:																	
Selected Organism : <i>Klebsiella pneumoniae</i> ssp <i>pneumoniae</i>																	
Source:			Collected:														
Comments:																	
Identification Information		Analysis Time: 3.87 hours		Status: Final													
Selected Organism		99% Probability <i>Klebsiella pneumoniae</i> ssp <i>pneumoniae</i>															
ID Analysis Messages		Bionumber: 6607735773564010															
Biochemical Details																	
2	APPA	-	3	ADO	+	4	PyrA	+	5	lARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	SKG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	+	53	lHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLT#	-	62	ELLM	-	64	ILATa	-			

Figure 3: Vitek II results

Antibiotic susceptibility of *K. pneumoniae*

The percentages of *K. pneumoniae* resistance were arranged as follows: Amoxicillin-clavulanate’s percentage is 98.36%, the resistance is slightly lower for ceftazidime (96.72%), and the level of resistance to other antibiotics also kept on decreasing as the susceptibility of bacteria lowered to 81.96% for cefixime, 72.13% for both azithromycin and trimethoprim-sulfamethoxazole, while for gentamicin it was 47.54%, aztreonam 40.98%, levofloxacin 32.78%, and imipenem 19.67%. Lastly, it was 13.11% for meropenem. The two antibiotics that were shown to be the most effective against *K. pneumoniae* were meropenem and

imipenem, while amoxicillin-clavulanate and ceftazidime were the least efficient. The results revealed that 95.08% of the isolates were MDR. MDR is characterized as having developed resistance to a minimum of one agent in three or more antibiotic groups [16]. This resistance may be due to a variety of reasons, but the major cause is that it contains many virulence factors such as capsules, lipopolysaccharides (LPSs), adhesins, exopolysaccharides linked to mucoviscosity, and iron uptake mechanisms that cause high antibiotic resistance [7]. This study is consistent with previous research by [17], who reported that *K. pneumoniae* is resistant to amoxicillin-clavulanate (98.57%), and meropenem (22.85%), and according to the study, our result was inconsistent with the other tested antibiotics.

Another report by [18] recorded that the resistance to imipenem was 21.5%, trimethoprim-sulfamethoxazole was 70%, which is consistent with our study, and gentamicin was 26.5%, which disagrees with our study. In the research of [19], which agreed with our study, the resistance to gentamicin was 45% and levofloxacin was 29%, but was inconsistent at the same time with the results of meropenem (33%). The resistance to ceftazidime in our study was 96.72%, which agreed with [20], as they recorded that 100% of the isolates are resistant, but was inconsistent in that the resistance to aztreonam was 95.1%, while the study of [1] reported that it was 46.77%, which is convergent with our study. In addition, the study [20] agreed with our results, and they reported that the resistance to cefixime was 82.2% and azithromycin was 81.6%. Also, the study [21] was consistent with our azithromycin results, as they reported 92.5% resistance. Through the statistical analysis of this result, we found the P-values for all the antibiotics were highly significant except for levofloxacin, which was non-significant (0.341). Figure 4 shows the *K. pneumoniae* antibiotic resistance pattern.

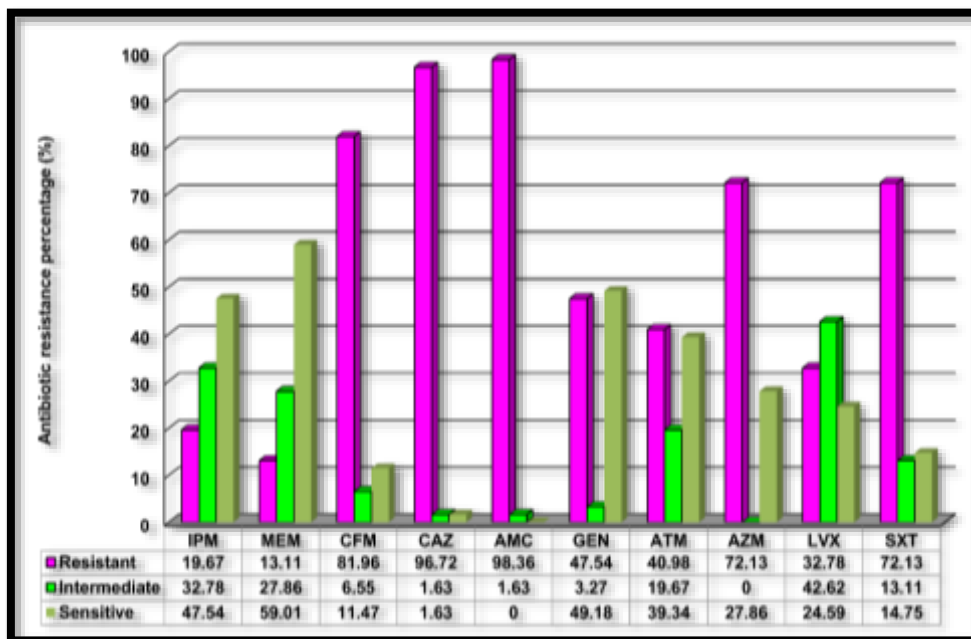


Figure 4: *K. pneumoniae* antibiotic resistance pattern

Imipenem (IPM), Meropenem (MEM), Cefixime (CFM), Ceftazidime (CAZ), Amoxicillin-clavulanate (AMC), Gentamicin (GEN), Aztreonam (ATM), Azithromycin (AZM), Levofloxacin (LVX), and Trimethoprim-sulfamethoxazole (SXT)

Molecular detection of *ClbA*, *K1*, *K2* genes

Conventional PCR was used for the amplification of the genes, and it was conducted on 61 isolates using the specific primers listed in Table 1. The results showed that the *K1* gene was present in only 3 isolates (4.91%), while the *K2* gene was present in 52 isolates (85.24%). The other 6 isolates are non-*K1/K2* (9.83%). None of the isolates were positive for the *ClbA* gene. In the study of [22], 17.39% of the isolates were positive for *K1* and 30.43% were positive for *K2*, which agrees with our study that *K2* is the predominant serotype, while in the research of [23], the prevalence of *K1* was 21.1% and the *K2* serotype was 11.7%. Another study in Iraq revealed that the prevalence of the *K1* gene was 12% and the *K2* gene was 8% [24]. In the research [25], only 7.14% of *K. pneumoniae* isolates were pks+ (*ClbA*+). The primary virulence components of *K. pneumoniae* are thought to be the *K1* and *K2* capsular antigens and the mucoid capsular phenotype [26]. Figures (5) and (6) show the results of the PCR.



Figure 5: Agarose gel electrophoresis of PCR amplification products of the (329 bp) *K1* gene (1.5% agarose gel electrophoresis stained with Red Safe Dye, 70 volts for 60 min, L: 100 bp ladder marker)

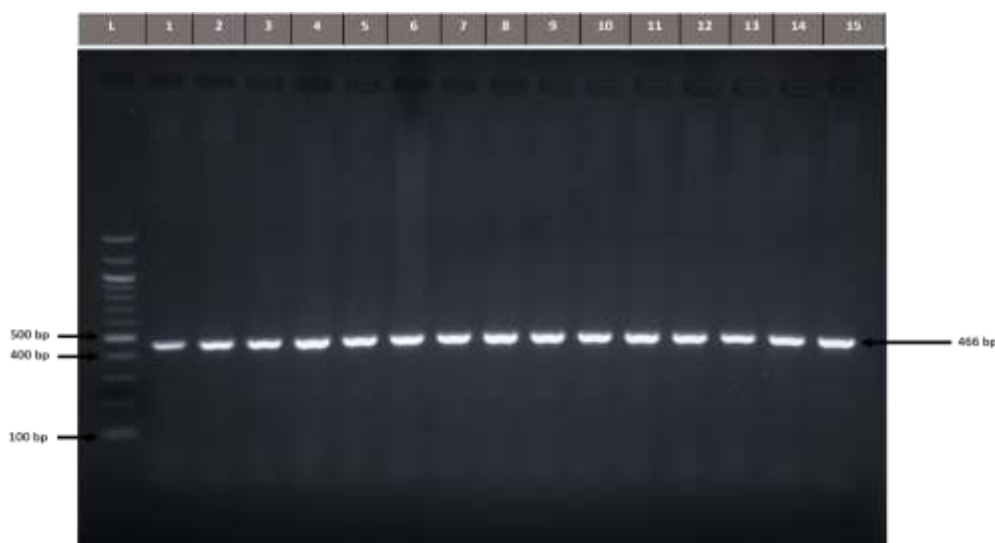


Figure 6: Agarose gel electrophoresis of PCR amplification products of the (466 bp) *K2* gene (1.5% agarose gel electrophoresis stained with Red Safe Dye, 70 volts for 60 min, L: 100 bp ladder marker)

Conclusion

This study concludes that *K. pneumoniae* has strong antibiotic resistance, that the *K1* and *K2* genes are widely distributed, especially the *K2* gene, and that 2/3 of the *K1* and 51/52 of the *K2* isolates were MDR. In addition, none of the isolates were *ClbA+*.

Ethical clearance

This study was approved by the ethical committee of the College of Science, University of Baghdad, according to the reference number (CSEC/0123/0001).

Conflict of interest:

The authors declare that they have no conflict of interest.

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