

# Resistance Profiles, Virulence Factors, Genotypes and Clinical Outcomes of Klebsiella Pneumonia Isolated from Patients with Community-Acquired Pneumonia

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**Background and study aim:** Klebsiella pneumonia, particularly the hypervirulent (hvKP) strains, are resistant to antibiotics and cause infections difficult to treat. This study aimed to compare clinical presentation, severity of infection, resistance profiles, virulence factors, genotypes and clinical outcomes of the classical (CKP) and the (HvKP) isolates in patients with Community acquired pneumonia (CAP).

**Patients and Methods:** Ninety nine patients with CAP were enrolled in this study, sputum samples were taken, processed and cultured for isolation of *K. pneumoniae*. Isolated *K. pneumoniae* were identified by conventional methods and PCR. Hypervirulence was detected by string test and PCR. Virulence genes were detected using multiplex PCR and finally Genotyping was done using ERIC PCR.

**Results:** A total of 99 *K. pneumoniae* isolates were recovered, 29 (29.3%) were identified as hvKP while 70 (70.7%) were cKP. Pneumonia caused by Hvkp isolates showed more intensive care unit

admission, vasopressor use, and need for mechanical ventilation while mortality doesn't differ between Ckp and Hvkp isolates. Capsular serotypes K1, K2 and K5 were more prevalent in hvKP. However, capsular serotype K20 was more prevalent in cKP. Among all isolates, entB and ycfm (89.8% and 94.9%, respectively) were the most common virulence genes. rmpA, wcaG, uge and kfu were more predominant in hvKP isolates. Isolates were categorized into 12 ERIC types. **Conclusion:** Our findings support the effectiveness and applicability of the ERIC-PCR technique for molecular typing, epidemiological investigation of nosocomial infections, and analysis of genetic diversity among hospital pathogens, including *K. pneumoniae* strains. *K. pneumoniae* strains obtained from Egyptian hospitals displayed a considerable amount of variability in their antibiotic resistance and ERIC profiles.

## INTRODUCTION

In Enterobacteriaceae family, *Klebsiella pneumoniae* is a significant opportunistic Gram-negative infection that primarily causes pneumonia [1]. Moreover, it plays a significant role in global nosocomial infections [2, 3]. A combination of phenotypic and genotypic characteristics distinguishes hypervirulent *Klebsiella pneumoniae* (hvKP) from classic *Klebsiella pneumoniae* (cKP). A string test on agar plates shows that hvKP bacteria

overproduce a polysaccharide capsule, which causes their distinctive hypermucoviscosity. HvKP strains have this quality, which has previously been used to characterize the disease [4, 5]. Aerobactin, a siderophore that aids the bacterium in its battle with the host for iron and was used to discover hvKP [6], is one of the primary virulence factors in *K. pneumoniae*. PCR and the string test were used to determine whether strains of hvKP were aerobactin-positive and hypermucoviscous [7].

HvKP has caused serious invasive infections over the last two decades. As a clinically relevant pathogen, it differs from the cKP seen in both healthy and immunocompromised individuals [8]. In addition, unlike hvKP strains, the majority of cKP strains seldom exhibit common antimicrobial agent resistance, with the exception of ampicillin [9].

Variable virulence factor genes in *K. pneumoniae* play important roles in disease progression. One of the typical virulence factors is the capsule polysaccharide (K antigen) on its surface. There are at least 77 identified capsular serotypes, and each has a different range of sickness severity. The most virulent strains of the 77 capsular serotypes (K) have been identified, and mouse models have demonstrated this virulence in strains having capsular serotypes K1 and K2 [10]. According to several researches, K1 and K2 capsular serotypes have a significant incidence of hvKP isolates [11].

A number of virulence genes, such as regulator of mucoid phenotype A (rmpA) and wcaG (encoding GDP-fucose synthetase), have been identified as contributing to the hypervirulent phenotype [12-14]. RmpA is a transcriptional activator of Capsular polysaccharide synthesis (CPS) gene transcription, CPS synthesis, and hypervirulence in *K. pneumoniae* K1/K2 [15].

Among the important virulence factors in *K. pneumoniae* is the kfu gene, which encodes for an iron uptake system and is strongly linked to purulent tissue infections and hypervirulence [10]. Furthermore, iron acquisition systems are encoded by the iutA, iroN, and entB genes, whereas lipopolysaccharides are encoded by the uge, wabG, ycfM, and allS genes [16]. These elements all have a role in virulence and are crucial for invasion, colonisation, and pathogenicity. *Klebsiella* spp. showed one of the most dramatic recent increases in multidrug-resistant (MDR) from 2001 to 2011 [17]. It is worth noting that patients with multidrug-resistant infections, such as *K. pneumoniae*, have a high mortality rate [18, 19]. Typically, these MDR infections are challenging to treat since there are so few antibiotics like tigecycline, colistin, Fosfomycin, and aminoglycosides available [20].

There is little information known about the clinical and microbiological aspects of community-acquired pneumonia (CAP) brought on by hvKP. This study compared the clinical

presentation, resistance profiles, virulence factors, genotypes, and outcomes of the conventional and hypervirulent *K. pneumoniae* isolates in patients with community-acquired pneumonia (CAP) admitted to Assiut University hospitals due to the growing significance of hvKP and their impact on patients' morbidity and mortality.

## PATIENTS/MATERIALS AND METHODS

For this prospective cohort research, 99 adult patients with CAP were hospitalized to the Chest Department at Assiut University Hospital in Egypt. CAP was described as the appearance of a new pulmonary infiltrate on chest radiography together with pneumonia-like symptoms such coughing, shortness of breath, a rise in body temperature, and/or chest discomfort that were not picked up in a hospital [21]. Patients who had non *K. pneumoniae* or mixed positive sputum cultures were excluded from the study. The study was carried out in compliance with the Declaration of Helsinki.

Acute Physiology and Chronic Health Evaluation score (APACHE II), Sequential Organ Failure Assessment score (SOFA), complete blood count, liver and kidney function tests, history, clinical examination, chest radiography, arterial blood gases (ABG) analysis, and bacteriologic examination of sputum samples were all obtained from each patient. For each enrolled patient a high-quality sputum (i.e. thick, purulent, at least 1ml volume, and showing < 10 squamous epithelial cells and > 25 leukocytes per optical microscopy field) were collected and processed for bacterial cultivation on culture media [22].

*K. pneumoniae* was identified by Gram-staining, mucoid pink colonies on MacConkey, lactose fermenting. The identification of *Klebsiella pneumoniae* was further carried out using Vitek 2 system and confirmed using Polymerase Chain Reaction (PCR) [23].

### 2. Testing for hypervirulence

Aerobactin-positive and hypermucoviscous isolates were recognized as hvKp by PCR and a string test [24]. CKP and hvKP strains were distinguished using the string test. A viscous string longer than 5 mm is considered a positive string test when it is applied to a bacterial colony that has been stretched after being grown overnight on an agar plate at 37°C [25].

### 3. Antimicrobial susceptibility testing

The Kirby-Bauer technique was employed for assessing antibiotic susceptibility, in accordance with the Clinical and Laboratory Standards Institute (CLSI) on Antimicrobial Susceptibility Testing. Each *K. pneumoniae* isolate was grown in Mueller Hinton broth from Oxoid in the UK for a whole night at 37°C. Bacterial cultures were adjusted to 0.5 on the MacFarland nephelometer scale (1.5 10<sup>8</sup> CFU/ml), then streaked onto Mueller Hinton agar (Oxoid, UK). Antimicrobial susceptibility and resistance were determined using the Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Tests. Antibiotic discs were provided by Bioanalysis in Turkey [26].

Multidrug-resistant (MDR) isolates were those with resistance to three or more antimicrobial classes, whereas extremely drug-resistant (XDR) isolates were alarmingly likely to be resistant to all, or nearly all, approved antimicrobials, making them epidemiologically significant in addition to their multiple antimicrobial resistances. The term "pan drug-resistant" (PDR) refers to bacteria that are resistant to all classes of antibiotics that can be used as empirical treatments as well as all commercially accessible antimicrobial groups. These organizations were created in accordance with a suggestion made by an international expert for temporary uniform definitions of acquired resistance [27].

### 4. Analysis of the virulence genes by Multiplex PCR

The existence of several types of virulence-associated genes was investigated in *K. pneumoniae* strains using three distinct sets of multiplex PCR techniques. For group 1, the PCR conditions were as follows: a 15-minute initial activation at 95°C, followed by 35 cycles of 94°C for 30 seconds, 58°C for 90 seconds, and 72°C for 90 seconds, and then an extension at 72°C for 10 minutes [28]. The amplification of groups 2 and 3 was carried out under the following thermal cycling conditions: after five minutes of 95°C pre-denaturation, 30 cycles (Applied Bio systems, USA) [29]. 10 minutes of final elongation at 72°C following 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C. A 1.8% agarose gel was used for the electrophoresis analysis of PCR results. Table 1 includes a summary of the oligonucleotide primers utilized.

### 5. Genotyping by Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR)

To ascertain the genotypes of our isolated isolates, virulence gene profiles and ERIC-PCR were coupled. The primers ERIC1 (5'-ATGTAAGCTCCTGGGATTAC-3') and ERIC2(5'-AAGTAAGTGACTGGGGTGAGCG-3') were used for ERIC-PCR [30]. In a 25 L solution, 40ng of the template genomic DNA, 1 U of Taq DNA polymerase, 2.5 mM MgCl<sub>2</sub>, 1.0 mM of each primer, and 0.2 mM of each dNTP were added. Using an Applied Bio-systems Bio-Rad PTC-200 Thermal Cycler, the following amplification methods were carried out: a 5-minute initial denaturation at 94°C, 35 cycles of 1 minute at 94°C, 1 minute at 49°C, and 3 minutes at 72°C, and a 10-minute final extension at 72°C. 2.0% agarose gel electrophoresis was used to separate the products of the ERIC-PCR.

#### Data analysis:

The obtained PCR products were gel electrophoresed (described before), the agarose gel photos were scanned by the Gene Profiler 4.03 computer software program, that uses automatic lane and peak finding for detecting the presence of banding patterns, to calculate the bands molecular weight, and a binary data matrix recording the presence (1) or the absence (0) of bands was made.

Then, the software package MVSP (Multi-Variate Statistical Package) was used to draw the different dendrograms and to calculate the genetic similarities using the Dice coefficient of similarity of **Nei and Li [31]**.

$$2 * n11$$

$$\text{Similarity} = \frac{\quad}{\quad}$$

$$(2 * n11) + n01 + n10$$

Where:

n11 - designates the number of common bands for the two compared samples,

n10 - cases where the bands were visible only in the first sample,

n01 - when bands were visible in the other sample only [32].

#### Statistical analysis

All statistical calculations were completed using MedCalc statistical software. The descriptive data were presented using the mean, standard

division (SD), median, and percentage. Chi-square tests (and, when applicable, Fisher's exact tests) were used to compare categorical measurements between groups. Further statistical data were rapidly examined using independent sample testing. Probability (P) values < 0.05 were considered significant in statistics.

## RESULTS

### 1. Clinical characteristics of the cohort

This study included 99 CAP patients with microbiologically confirmed *Klebsiella pneumoniae*, cKP and hvKP were detected in 70.7% and 29.3% of cases, respectively. SOFA score, APACHE II score and shock at diagnosis of CAP were significantly higher in hvKP pneumonia cases ( $p=0.001$ ,  $0.002$ , and  $0.001$ , respectively). Derangement in pH and  $SPO_2$ , intensive care unit (ICU) admission, vasopressor use, and need for mechanical ventilation were significantly predominant in hvKP pneumonia cases ( $p=0.001$ ,  $0.042$ ,  $0.002$ ,  $0.001$  and  $0.009$ , respectively), as shown in Tables 2 and 3.

Non-survivor's group had significantly higher mean age  $72.07 \pm 8.8$  versus  $53.9 \pm 12.3$ , SOFA score  $11.1 \pm 1.9$  versus  $8.7 \pm 1.8$  and APACHE II score  $19.3 \pm 3.4$  versus  $14.8 \pm 2.3$  ( $p=0.001$  for all). Moreover, shock at diagnosis, altered mental status, tachypnea, and elevated serum urea were also higher in the non-survivor groups ( $p=0.001$ ,  $0.007$ ,  $0.001$  and  $0.033$ , respectively). Distribution of the virulence associated genes among survivors and non survivors did not show significant differences except for the capsular gene (K20) which was significantly ( $p=0.041$ ) higher in the survivor's group (Table 4). Table 4 lists the characteristics of patients with hvKP and cKP infections.

### 2. Antimicrobial resistance

There was no discernible difference between the cKP strains and the hvKP strains in terms of their resistance to the tested antimicrobials. Amoxicillin, Amoxicillin-clavulanic acid, and piperacillin were all ineffective against all hvKP and cKP isolates (fig.1). Similarly, 20% (14/70) of the classical *K. pneumoniae* isolates were MDR. 55.7% (39/70) were XDR and 24.2% (17/70) were PDR. In addition, 17.2% (5/29) of the hvKP isolates were MDR, 55.1% (16/29) were XDR and 27.5% (8/29) were PDR.

### 3. HvKP and CKP isolates' molecular features and genes related to pathogenicity

#### 3.1. Genetic comparison between hvKP and cKP isolates

Each *K. pneumoniae* isolate was examined for the presence of genes encoding the capsule K antigen. 40/70, or 56.1%, of the cKP isolates lacked the *K1*, *K2*, *K5*, *K20*, *K54*, or *K57* capsular types. Ten of the isolates were all *K1* capsular types and were all hvKP. *K2* and hvKP also had a good connection (6 out of the 7 isolates that belonged to capsular serotype *K2* were hvKP). Similarly, 5 isolates were *K5* type and belonged to the cKP group. In the cKP group, the *K20* serotype accounted for 32.3% (32/99) of isolates, making it the most common capsular type. Table 5 compares the prevalence of genes and capsular serotypes associated with virulence in hvKP and cKP isolates.

Generally, *ycfm* 94/99 (94.5%) and *entB* 89/99 (89.8%) were the most common detected virulence genes among isolated *K. pneumoniae* isolates. Interestingly, the virulence genes *rmpA*, *wcaG*, *uge* and *kfu* were detected at a higher frequency in hvKP group compared to the cKP isolates ( $p$  values= less than 0.001 in all cases) (Table 5).

#### 3.2. Genotyping of *Klebsiella pneumoniae* isolates by virulence markers.

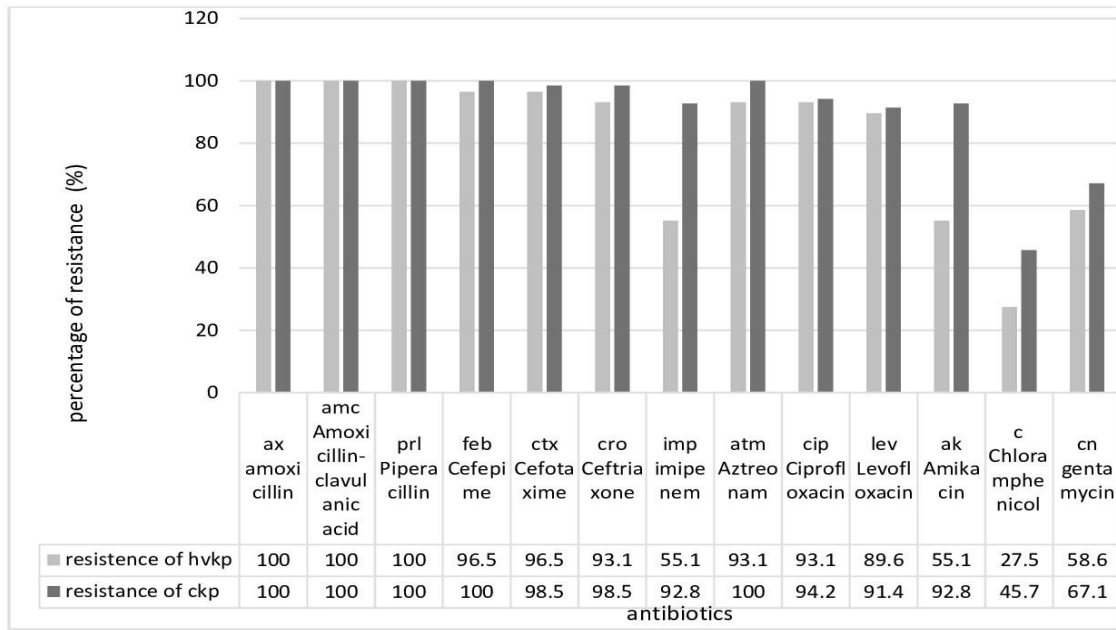
Dendrograms generated based on the presence of the isolates with 21 additional virulence markers revealed that the isolates were divided into five different clusters, with clusters A and B having three identical strains based on virulence genes included (79,17), (34,19), (62,40), (42,37), (53,51), and (54,52) respectively. The cluster C contains two stains that have the same (39, 12), (Figure 2). The remaining 12 isolates all produced singletons.

#### 3.3. Genotyping of *Klebsiella pneumoniae* isolates by ERIC-PCR

Sizes of the bands, which ranged from 200 bp to more than 1 kb, ranged from 3 to 13. The results of the software analysis and electrophoresis reveal that *K. pneumoniae* isolates exhibit a high degree of genetic diversity. Based on their ERIC-PCR patterns, the isolates were categorized into 12 ERIC-types. Isolates allocated to the same clusters in the dendrogram shared more than 95% of the bands with other isolates (Figure 3). A (n=10), B (n=6), five isolates in each type (C/D), four isolates in each type (E/F), three

isolates in each type (G/H/J), and two isolates in each type (K/L) were all identified by ERIC. The remaining 52 isolates (n=52) were unrelated, had a 95% similarity, and were of the categories M1–M52. Three clusters (A–D) were identified by dendrogram analysis of the ERIC genotyping:

cluster B comprised seven different ERIC-types (A, B, C, D, E, G, and H), while cluster C contained four different ERIC-types (F, J, K, and L). These isolates were quite similar to one another, indicating that they are part of a clonal lineage.



Amoxicillin (AX) (25 µg), Amoxicillin + Clavulanic Acid (AMC) (30 µg), Piperacillin( PRL) (30 µg), Cefepime (FEB) (30 µg), Cefotaxime (CTX) (30 µg), Ceftriaxone (CRO) (30 µg), Imipenem (IPM) (10 µg), Aztreonam(ATM) (30 µg), Ciprofloxacin (CIP) (5 µg), Levofloxacin (LEV) (5 µg), Amikacin (AK) (30 µg), Chloramphenicol (C) (30 µg), Gentamycin (CN) (30 µg), Multi Drug Resistant (MDR), Extensive Drug Resistant (XDR), and Pan Drug Resistant (PDR).

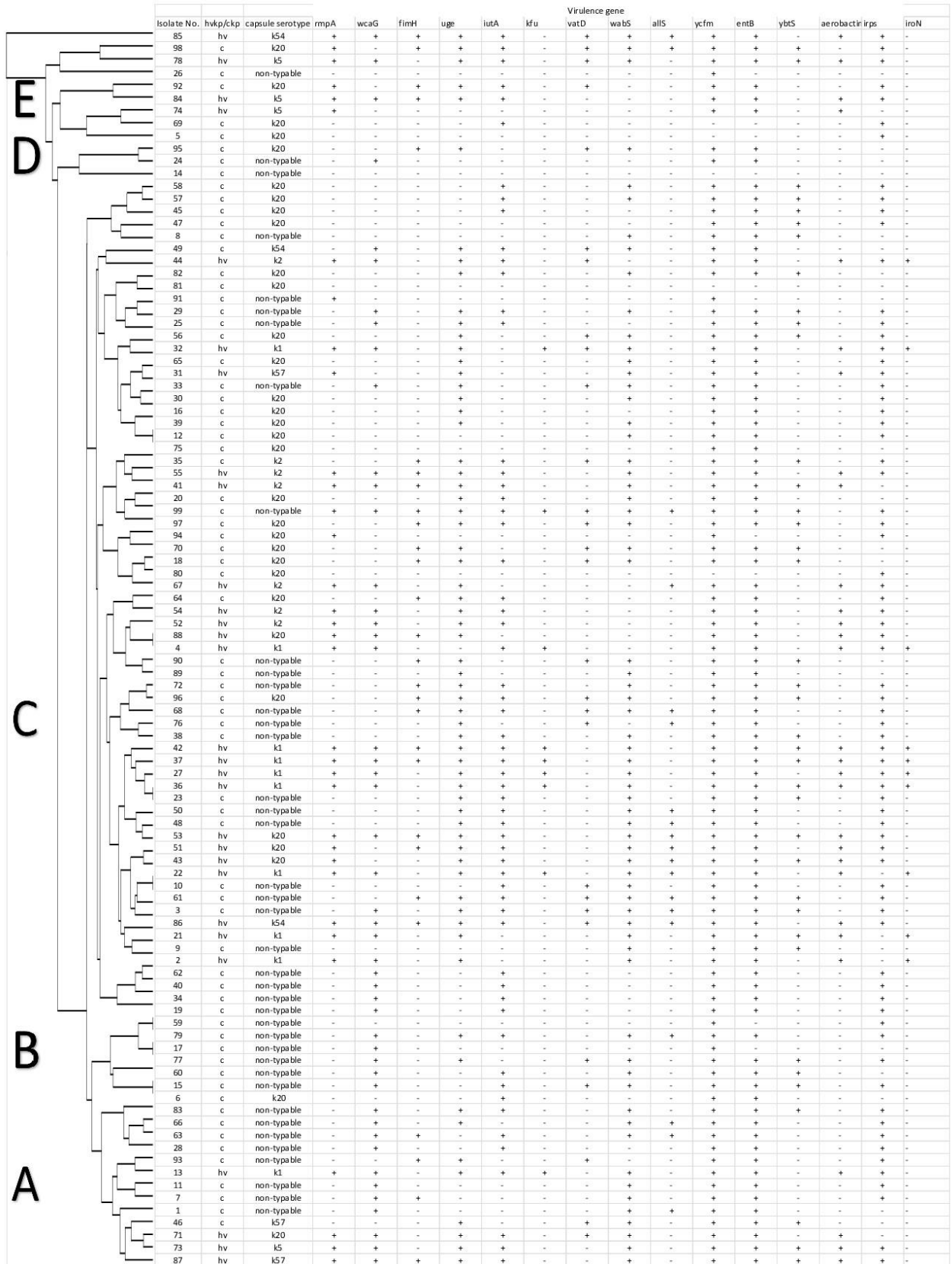
**Figure 1.** Antibiotic resistance of *K. pneumoniae* isolates.

**Table 1.** Multiplex PCR primers

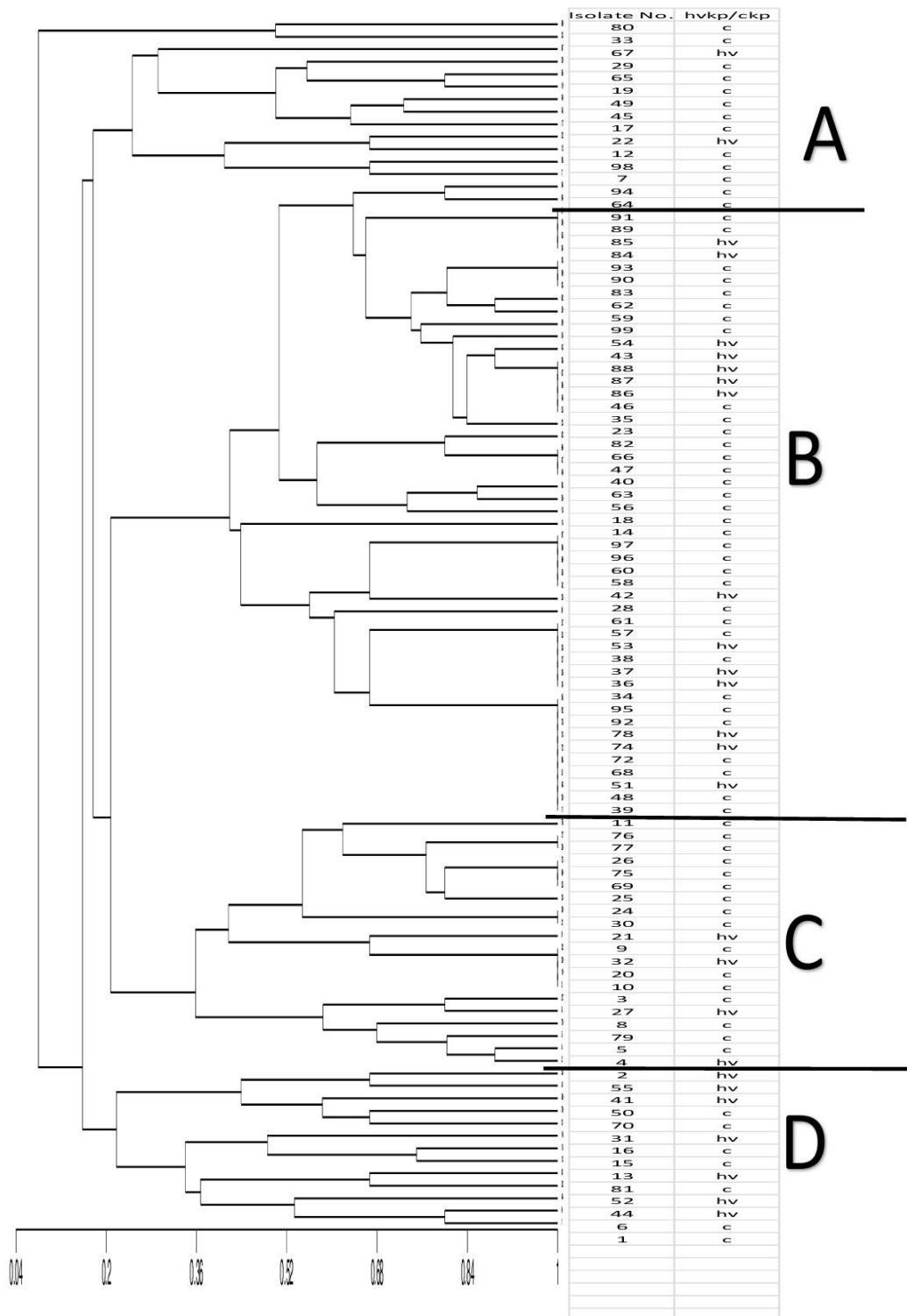
	Target	Primer	Sequence (5' to 3')	Size (bp)	Ref.
Group 1	Capsular type K1	MagAF1	GGTGCTCTTTACATCATTCG	1283	[85]
		MagAR1	GCAATGGCCATTTGCGTTAG		
	Capsular type K2	K2wzy-F1	GACCCGATATTCATACTTGACAGAG	641	[86]
		K2wzy-R1	CCTGAAGTAAAATCGTAAATAGATGGC		
	Capsular type K5	K5wzxR360	TGGTAGTGATGCTCGCGA	280	[86]
		K5wzxR639	CCTGAACCCACCCCAATC		
	Capsular type K54	wzxK54F	CATTAGCTCAGTGGTTGGCT	881	[11]
		wzxK54R	GCTTGACAAACACCATAGCAG		
	Capsular type K57	wzyK57F	CTCAGGGCTAGAAGTGTCAT	1037	[11]
		wzyK57R	CACTAACCCAGAAAGTCGAG		
Capsular type K20	wzyK20F	CGGTGCTACAGTGCATCATT	741	[11]	

		<i>wzyK20R</i>	GTTATACGATGCTCAGTCGC		
	<i>RmpA</i>	<i>RmpAF</i>	ACGACTTTCAAGAGAAATGA	434	[87]
		<i>RmpAR</i>	CATAGATGTCATAATCACAC		
	<i>WcaG</i>	<i>WcaGF</i>	GGTTGGKTCAGCAATCGTA	169	[88]
		<i>WcaGR</i>	ACTATCCGCCAACTTTGC		
<b>Group 2</b>	<i>iutA</i>	<i>iutA-F</i>	GGCTGGACATCATGGGAAGTGG	300	[89]
		<i>iutA-R</i>	CGTCGGGAACGGGTAGAATCG		
	<i>uge</i>	<i>uge-F</i>	TCTCACGCCTTCCTTCACT	534	[24]
		<i>uge-R</i>	GATCATCCGGTCTCCCTGTA		
	<i>allS</i>	<i>allS-F</i>	CCGAAACATTACGCACCTTT	508	[24]
		<i>allS-R</i>	ATCACGAAGAGCCAGGTCAC		
	<i>fimH</i>	<i>fimH-F</i>	TGCTGCTGGGCTGGTCGATG	688	[24]
		<i>fimH-R</i>	GGGAGGGTGACGGTGACATC		
	<i>VatD</i>	<i>VatD-F</i>	GAAGGAAACAAATCAGTA	463	[29]
	<i>VatD-R</i>	GTTTTATTTCGTTAGCAG			
<b>Group 3</b>	<i>wabG</i>	<i>wabG-F</i>	ACCATCGGCCATTTGATAGA	683	[90]
		<i>wabG-R</i>	CGGACTGGCAGATCCATATC		
	<i>ycfM</i>	<i>ycf-F</i>	ATCAGCAGTCGGGTCAGC	160	[91]
		<i>ycf-R</i>	CTTCTCCAGCATTACAGCG		
	<i>entB</i>	<i>entB-F</i>	ATTTCTCAACTTCTGGGGC	371	[91]
		<i>entB-R</i>	AGCATCGGTGGCGGTGGTCA		
	<i>Aerobactin</i>	<i>AerobactinF</i>	GCATAGGCGGATACGAACAT	556	[92]
		<i>AerobactinR</i>	CACAGGGCAATTGCTTACCT		
	<i>IroNB</i>	<i>iroNB-F</i>	GGCTACTGATACTTGACTATTC	992	[93]
		<i>iroNB-R</i>	CAGGATAACAATAGCCCATAG		
	<i>YbtS</i>	<i>YbtS-F</i>	CACCGCAAACGCAATCTG	782	[29]
		<i>YbtS-R</i>	GCCATAGACGCTGTTGTGA		
<i>irp-2</i>	<i>irp-2-F</i>	TCCCTCAATAAAGCCCACGCT	287	[94]	
	<i>irp-2-R</i>	TCGTCGGCAGCGTTTCTTCT			

**Figure 2.** Using the Nei, Li's coefficient and the Unweighted Pair Group Method with Arithmetic (UPGMA) clustering method, a dendrogram was created to demonstrate the genetic similarities of *K. pneumoniae* isolates based on markers for their virulence:



**Figure 3.** Enterobacterial Repetitive Intergenic Consensus (ERIC) genotyping-generated dendrogram demonstrating the genetic similarity among *K. pneumoniae* isolates using Nei, Li's coefficient, and the UPGMA clustering approach:





**Table 2.** Characteristics of CAP patients in relation to classical and hypervirulent K. pneumonia.

Variable		Demographic data and health status			
		Ckp (n=70)	Hvcp (n=29)	DF	P value
Age		58.9±15.03	58.1±12.04	97	0.799
Gender	Male	35(50%)	16(55%)	1	0.804
	Female	35 (50%)	13(44%)		
DM		19(27%)	11(37%)	1	0.455
HTN		26(37%)	19(65%)	1	<b>0.020*</b>
IHD		1(1.4%)	2(6%)	1	0.549
Hepatic disesses		1(1.4%)LC/1(1.4%)HPS	0(0%)		-
SOFA score		8.9± 2.1	10.6±1.7	97	<b>0.001*</b>
APACHE II score		15.4±3.14	17.7±3.7	97	<b>0.002*</b>
Initial appropriate antibiotics	R	54 (70%)	23 (79%)	1	0.505
	S	5 (7%)	2 (6%)	1	0.795
Shock at diagnosis		18(25%)	23(79%)	1	<b>0.001*</b>
Laboratory investigations					
WBCs (10 <sup>3</sup> /ul)		9.1 ±3.1	10.6 ± 5.2	1	0.079
Hb (gm/dl)		12.7± 2.4	11.5 ± 2.4	97	<b>0.026*</b>
Creatinine (umol/l)		72.5 ± 32.4	70.2 ± 36.1	97	0.757
Urea (mmol/l)		6.3± 5.7	7.7 ± 5.1	97	0.255
AST (U/L)		51.9± 33.6	51.5± 17.3	97	0.952
ALT (U/L)		44.8± 20.4	45.8± 18.8	97	0.821
Albumin (g/dl)		3.2± 3.7	2.8± 0.9	97	0.568
Na (mmol/l)		136± 7.5	139.2 ± 9	97	0.072
K (mmol/l)		3.9± 0.8	4.1 ± 0.6	97	0.229
PH		7.40± 0.04	7.30 ± 0.04	97	<b>0.001*</b>
PaO2 (mmHg)		56.7± 7.3	54.7 ± 7.7	97	0.225
PaCO2 (mmHg)		49.3± 12.9	44.6 ± 8.1	97	0.073
SPO2 (%)		90±2.05	83±3.47	97	<b>0.042*</b>

**Table 3.** Clinical outcomes of patients with pneumonia caused by K. pneumonia.

Variable	Ckp (n=70)	Hvcp (n=29)	DF	P value
Length of hospital stay (mean±SD)	9.3 ± 4.1	9.5 ± 4.02	97	0.825
ICU Admission (No,%)	35(50%)	25(86%)	1	<b>0.002*</b>
Vasopressor use (No,%)	17(24%)	23(79%)	1	<b>&lt;0.001*</b>
Mechanical ventilation (No, %)	34(48%)	22(79%)	1	<b>0.009*</b>
Early mortality (≤48 h) (No, %)	6(8%)	3(10%)	1	0.944
In-hospital mortality (No, %)	12(17%)	6(20%)	1	0.947

\* Statistically significant difference (p<0.05), DF (is the degree of freedom).

**Table 4.** Variables associated with in hospital mortality in CAP patients

Variable	Demographic data and health status (No, %)			
	Survivors (n=72)	Non survivors (n=27)	DF	P value
Age (mean±SD)	53.9±12.3	72.07 ±8.8	97	<b>0.001*</b>
Male (No,%)	38(52.7%)	13(48%)	1	0.849
Female (No,%)	34(47.2%)	14(51%)	1	0.912
SOFA score (mean±SD)	8.7±1.8	11.1±1.9	97	<b>0.001*</b>
APACHE II score (mean±SD)	14.8±2.3	19.3±3.4	97	<b>0.001*</b>
Shock at diagnosis	7 (9.7%)	11 (40.7%)	1	<b>0.001*</b>

Altered mental status	14 (19.4%)	21 (77.7%)	1	0.007*
Respiratory rate $\geq$ 30 breath/min	15 (20.8%)	19 (70.3%)	1	0.001*
Arterial PH < 7.35	12 (16%)	17 (62.3%)	1	0.010*
Serum urea > 10 mmol/L	20 (27.7%)	18 (66.6%)	1	0.033*
<b>Virulence genes (No, %)</b>				
<i>k54</i>	2(2%)	1(3%)	1	0.646
<i>k57</i>	2(2%)	0(0%)	1	-
<i>RmpA</i>	23(31%)	10(37%)	1	0.744
<i>WcaG</i>	34(47%)	17(62%)	1	0.270
<i>FimH</i>	19(26%)	6(22%)	1	0.882
<i>Uge</i>	48(66%)	15(55%)	1	0.438
<i>IutA</i>	44(61%)	15(55%)	1	0.755
<i>Kfu</i>	6(8%)	4(14%)	1	0.604
<i>VatD</i>	21(29%)	5(18%)	1	0.393
<i>WabG</i>	46(63%)	17(62%)	1	0.887
<i>AllS</i>	13(18%)	7(25%)	1	0.621
<i>Ycfm</i>	67(93%)	25(92%)	1	0.792
<i>IroN</i>	6(8%)	5(18%)	1	0.286
<i>YbtS</i>	25(34%)	9(33%)	1	0.885
<i>Aerobactin</i>	20(27%)	9(33%)	1	0.735
<i>IrpS</i>	54(75%)	17(62%)	1	0.305
<i>EntB</i>	65(90%)	25(92%)	1	0.934
Multidrug resistance strain (No,%)	7 ( 5 – 10 )	7 ( 5 – 11 )	97	0.975
Extensive drug resistance strain (No,%)	16(22%)	3(11%)	1	0.340
Pan drug resistance strain (No,%)	38(52%)	17(62%)	1	0.507
Multidrug resistance strain (No,%)	18(25%)	7(25.9%)	1	0.794

\* Statistically significant difference ( $p < 0.05$ ), APACHE, Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Failure Assessment, DF (is the degree of freedom)

**Table 5.** Virulence genes in relation to hypervirulent and classical *Klebsiella pneumoniae* isolates.

Virulence related genes	hvKP (N= 29)	cKP (N= 70)	DF	P value
<i>K1</i>	10(34.4%)	0(0%)	1	-
<i>K2</i>	6(20.6%)	1(1.4%)	1	0.003*
<i>K5</i>	4(17.2%)	0(0%)	1	-
<i>K20</i>	5(13.7%)	27(38.5%)	1	0.029*
<i>K54</i>	2(6.8%)	1(1.4%)	1	0.430
<i>K57</i>	2(6.8%)	1(1.4%)	1	0.430
<i>rmpA</i>	29(100%)	5(7.1%)	1	<0.001*
<i>wcaG</i>	25(86.2%)	32(45.7%)	1	<0.001*
<i>fimH</i>	11(37.9%)	16(22.8%)	1	0.198
<i>Uge</i>	27(93.1%)	38(54.2%)	1	<0.001*

<i>iutA</i>	22(75.8%)	37(52.8%)	1	0.058
<i>Kfu</i>	8(27.5%)	1(1.4%)	1	<0.001*
<i>vatD</i>	6(20.6%)	21(30%)	1	0.479
<i>WabG</i>	21(72.4%)	43(61.4%)	1	0.418
<i>AllS</i>	7(24.1%)	12(17.1%)	1	0.599
<i>Ycfm</i>	29(100%)	65(92.8%)	1	0.327
<i>IroN</i>	10(34.4%)	0(0%)	1	-
<i>YbtS</i>	10(34.4%)	27(38.5%)	1	0.876
<i>Aerobactin</i>	29(100%)	0(0%)	1	-
<i>IrpS</i>	23(79.3%)	48(68.5%)	1	0.400
<i>EntB</i>	29(100%)	60(85.7%)	1	0.075

Chi-square test, \* Statistically significant difference ( $p < 0.05$ ), DF (is the degree of freedom).

## DISCUSSION

### Bacterial strains and HV strains of *K. pneumoniae*.

A significant pathogen in both hospitals and the general population, *K. pneumoniae* is a Gram-negative, conditionally pathogenic bacillus. The lungs are the most frequently affected area of the human body, but it can affect numerous other areas as well.

Community-acquired pneumonia (CAP) caused by hvKP is uncommon, and there is little information available about its clinical and microbiological characteristics. The purpose of this study was to evaluate and compare the resistance profiles, virulence factors, and genotypes of classic and hypervirulent *Klebsiella pneumoniae* strains isolated from community acquired pneumonia (CAP) patients admitted to Assiut University Hospital's Chest Department. The clinical presentation and outcome of the patients who were enrolled were also evaluated. In Okinawa, Japan, Jun Hirai et al 2020 reported the first case of CAP caused by the capsular genotype K2-ST86 HV-KP [33].

According to the current findings, 70.7% of the isolates were cKP and 29.3% were hvKP. According to Jeong-Hwan Hwang et al., 2020, the majority of his isolates were Ckp (54% for Ckp versus 45.9% for hvkp) [34]. Yamamoto Hiroyuki, 2020 discovered an APACHE II score of 20 and septic shock at hvkp pneumonia diagnosis in his study [35], which was consistent with the current results, Chih-Han Juan et al 2020 reported a median APACHE II score of 23 and 29.4% of cases had septic shock [36]. Jeong-

Hwan Hwang et al, 2020 found no difference in Shock at Diagnosis between Ckp and Hvkp pneumonia ( $p=0.474$ ) [34]. Pneumonia caused by hypervirulent strains resulted in an increased need for mechanical ventilation (75.8%) and vasopressor use (79.3%), which was consistent with previous findings. According to [34, 36], hypervirulence determinants do not have a significant impact on mortality, but shock at diagnosis, altered mental status, respiratory rate 30 breath/min, arterial PH 7.35, serum urea 10 mmol/L were all significantly higher in hvkp pneumonia.

There is no denying that the internet has changed the way people communicate. In the past, a positive string test has been used to define hypermucoviscosity, but more recent studies have shown that the two phenotypes are separate from one another [37]. Studies are finding more and more clinical isolates of *K. pneumoniae* that cause pyogenic liver abscesses without the typical hypermucoviscous appearance [38-40]. These findings suggested that hypermucoviscosity is not necessary for the hypervirulent phenotype of *K. pneumoniae*. Thus, employing hypermucoviscosity to define hvKP might not be accurate. A major virulence factor in human hvKP infection and survival is the siderophore aerobactin [6]. It is located on the large virulence phenotype of *K. pneumoniae* plasmid (pLVPK), which is present in the great majority of hvKP isolates but absent in cKP strains, and is assumed to represent a genetic factor for both hypermucoviscous and hypervirulent phenotypes [37, 41]. Because of its importance in the disease, *aerobactin* positivity

was thought to be a defining genetic trait for hvKP [13]. *Aerobactin* was more sensitive but less specific than traditional hypermucoviscosity designation criteria for defining hvKP, which was consistent with previous research findings [13, 14].

The *rmpA* gene, which is found on the large virulence plasmid pLVPK and is known to enhance capsular formation [42], has previously been shown to be a significant virulence-associated component in hvKP isolates [13, 14, 43]. A high correlation between *rmpA* and hypervirulence was identified in our investigation, where the *rmpA* gene was found in 100% (29/29) of hvKP isolates but only in 5 of 70 cKP isolates. Also, it was looked at how *rmpA* affected the pathogenicity of cKP isolates that were *rmpA* positive.

The majority of hvKP bloodstream infections are acquired in the community, in contrast to traditional *K. pneumoniae* bloodstream infections, suggesting that hvKP isolates are crucial in the development of community-acquired [44]. Despite being a well-known common pathogen, *K. pneumoniae* has been associated with blood and urinary tract infections, community and hospital-acquired pneumonia, and all of these other illnesses [45]. Our results suggested a connection between hvKP and respiratory illnesses. This shows that respiratory infections are the primary cause of cKP strains. These numbers are consistent with earlier findings [4, 46]. The study is compatible with [47], in that hvKP is not correlated with either gender or age. Yet, other research revealed that hvKP patients were younger and more likely to be infected than female patients [44].

### Antimicrobial resistance

The excessive use of antimicrobials get to high incidence of resistance in *K. pneumoniae* [48]. All of the samples included in our investigation were made up of *K. pneumoniae* isolates that were multidrug resistant. Other studies [30, 49] discovered rates of more than 66.7% of MDR *K. pneumoniae* isolates. High rates of antimicrobial resistance were discovered in our study, and this can be related to Egypt's lax antibiotic use regulations [3]. MDR was found in 20% (14/70) of classical *K. pneumoniae* isolates, XDR in 55.7% (39/70), and PDR in 24.2% (17/70). In contrast, 17.2% (5/29) of hypervirulent *K. pneumoniae* isolates tested positive for MDR, 55.1% (16/70) tested positive for XDR, and

27.5% (8/29) tested positive for PDR. In [26], 84.37% (27/32) of *K. pneumoniae* isolates were MDR, 12.5% (4/32) were XDR, and 12% (1/32) were PDR. In contrast, 84.37% (27/32) of *K. pneumoniae* isolates were MDR.

The rate of *K. pneumoniae* resistance to fluoroquinolones varies according to the geographical distribution, e.g., resistance rates are 71.4% in Iran, 93.1% in Egypt, and 89% in India [50].

In this work, we compared the features of cKP and hvKP isolates' antibiotic resistance. Recent research has discovered that these strains are not only less related with antibiotic resistance but also less resistant to antibiotics than hvKP strains, which have previously been proven to be more resistant to antibiotics than cKP strains [4]. As a result, these data are inconclusive. In the current study, antibiotic resistance between hvKP strains and cKP strains was not significantly different.

### Molecular characteristics and virulence-associated genes of hvKP and cKP isolates

In a model of rat subcutaneous abscess, the genetic features of virulence for hvKP and cKP showed that hvKP was more virulent than cKP [51]. Six serotypes (*K1*, *K2*, *K5*, *K20*, *K54*, and *K57*) known to be extremely virulent and linked to serious infections in humans were examined in this work [52]. *K. pneumoniae* serotypes *K1* and *K2* cause pyogenic liver abscess and have been linked to community-acquired pneumonia [53]. *K. pneumoniae* has been classified into more than 70 capsular serotypes [54]. Hypermucoviscous, or HV, strains are those with the *K1* and *K2* capsular serotypes that predominantly cause liver abscess and correspond to certain clones [55,56,57, 58]. Mucoviscosity-associated gene, or *MagA* The genes *k1A* (unique to *K1* capsule serotype) and *k2A* play significant roles in the pathogenesis of liver abscess (specific to *K2* capsule serotype) [59]. Several HV serotypes, including as *K5*, *K20*, *K54*, and *K57*, have been discovered in addition to *K1* and *K2* [25]. Our findings suggest that additional serotypes besides *K1/K2/K5/K20/K54/K57* may play a significant role in hvKP infections. *K20* was the most frequent capsular type in our study, however it was almost exclusively recovered from cKP isolates. This outcome is consistent with past studies where the *K20* was more commonly found in strains other than hvKP [4, 60, 61]. As a

consequence, it seems that the *K20* capsule plays a less significant role in the pathogenesis of hypervirulent *K. pneumoniae* than was previously thought [11]. *K1*, *K2* capsular genes are typically identified in hvKP strains and may also be present in cKP strains [10]; hvKP strains may also contain non-*K1/K2* genes [57].

According to the findings, *K1* and *K2* were the most common capsule serotypes among hvKP isolates (34./\*4%, 10/29), (20.6%, 6/29), which is consistent with previous research [44, 62].

In virulent strains of *K. pneumoniae*, the capsular serotypes *K1* and *K2* predominate [30]. According to Feizabadi et al., 11.2% of all *K. pneumoniae* isolates were *K2* serotypes [63].

A high viscosity phenotype was connected to the *rmpA* gene, a plasmid gene that controlled extracellular polysaccharide production [64-66]. In this investigation, the proportion of hypervirulent isolates containing the *rmpA* gene was 100%, which is much higher than in other findings from Beijing, China [13,44]. The findings support the hypothesis that *rmpA*-positive HV phenotypes are more resistant to phagocytosis by neutrophils and macrophages (65). It was determined that the capsular antigens (*K1* and *K2*) and *rmpA* had a significant relationship to hvKP strains ( $P = 0.001$  and  $P = 0.003$ , respectively). These findings support previous research that identified *rmpA* ( $P = .025$ ) and the capsular antigens (*K1* and *K2*) ( $P = .024$  and  $.039$ , respectively) [4].

*WabG*, *uge*, and *ycfM*, genes related with capsules, enhance resistance to phagocytosis [67]. *K. pneumoniae* isolates frequently included these genes, which seem to be the cause of the pathogenicity of *K. pneumoniae*. The virulence gene areas discovered in this research were *uge* 65.6% (65/99), *wabG* 64.6% (64/99), and *ycfm* 94.8% (94/99), each of which encodes a component of the capsule, a lipoprotein component of the capsule, and an exterior membrane protein. These rates are in line with earlier studies showing that clinical strains of *K. pneumoniae* develop virulence factors [68].

Camarota et al. (2003) [26,69] demonstrated that *K. pneumoniae* strains with a mutant *wab G* gene are non-capsulated and less virulent. This demonstrates how crucial the *wab G* gene is for the pathogenicity of *K. pneumoniae*. By demonstrating that mutant strains of *K. pneumoniae* (without the *uge* gene) were not

virulent in laboratory animals, Regué et al. (2004) demonstrated the importance of the *uge* gene in *K. pneumoniae* pathogenicity [70,71].

Type 1 fimbriae and fimbrial adhesins are expressed in clinical strains of *K. pneumoniae*. Whereas type 1 fimbriae, which encode *fimH*, are crucial for the urinary tract infections (UTIs) caused by these strains [59]. Moreover, the siderophores encoding the iron-binding proteins *entB*, *iutA*, and *iroN* promote the development of biofilms [68, 72]. Fimbrial adhesins H (*fimH*) were found in 27% (27/99) of the samples, while siderophores (*iutA*, *entB*) were found in 59.5% (59/99) and 89.8% (89/99), respectively. Several microorganisms, including fungi and bacteria, create and release siderophores, which are bio-synthetic molecules that are selective chelators and synthesized for the uptake of iron (Fe+3). These ions are only slightly soluble and are present in practically all oxygenated situations. In this study, the *irp-2* gene was found in 71.7% (71/99) of our isolates. These findings were consistent with previous research that found these chelators in almost all *K. pneumoniae* clinical isolates [68,73].

Bacteria create the siderophore substances enterobactin (*entB*) and yersiniabactin (*ybtS*) to absorb iron (Fe+3) from host iron-binding proteins. They contribute significantly to bacterial pathogenesis and virulence as high-affinity extracellular ferric chelators. Furthermore, enterobactin expression promoted biofilm formation [72]. During bacterial infection, the expression of iron-enterobactin appears to be activated [68].

It has been shown in mice that the *kfu*, which facilitates ferric iron absorption, is a virulence factor (10), and that hvKP strains tend to have more of it than cKP strains. The *kfu* gene, which codes for an iron absorption pathway, is thought to be pathogenic. The *kfu* gene is linked to purulent tissue infections, capsule formation, and virulent hypermucoviscosity. Thus, it is believed that this gene is crucial for the host cell's absorption of iron [74].

In comparison to cKP strains, hvKP strains produced more *aerobactin* [75,6]. Kim et al. determined that *kfu* and *aerobactin* are necessary for bacterial pathogenicity. The intake of iron is related to their protein products [76]. It is consistent with earlier studies [24,44] that *Kfu* and *aerobactin* were present in 100% (29/29) and 27.5% (8/29) of the hvKP isolates in this study,

respectively. The discovery of *wcaG*-positive strains in patients with severe and invasive illnesses raises the possibility that *wcaG* may be involved in the emergence of these strains. Fucose, a component of the polysaccharide capsule of *K. pneumoniae*, has been linked to bacterial pathogenicity in mice, and it is produced in part by the *wcaG* protein [77]. This study's conclusion that the *wcaG* gene is extremely relevant for hvKP is in line with earlier studies [44].

Furthermore, a study found a strong correlation between the *kfuB*, *allS* gene, K1 serotype, and hvKP isolates [78]. There was no clear link between hypervirulence and the *allS* gene in this study. In comparison to cKP strains, hvKP strains have a higher prevalence of virulence-related genes, including K1, K2, *rmpA*, aerobactin, *iutA*, *wcaG*, *iorN*, *irpS*, *uge*, and *kfu*. The genes for FIM-1, *ycfM*, *ybtS*, *wabS*, and *vatD* did not significantly change between the two.

#### Genotyping of *Klebsiella pneumoniae* isolates by ERIC analyses

*E. coli* and, more recently, *K. pneumoniae*, members of the Enterobacteriaceae family, have both been detected using ERIC-PCR [79, 80]. The current investigation discovered a significant degree of genetic variability among various *K. pneumoniae* strains. In Ramazanzadeh et al (2013)'s investigation on the genetic diversity in clinical isolates of *E. coli* taken from hospitals in significant cities in western Iran, ERIC-PCR permitted typing of the 230 isolates into 205 ERIC types, which were further clustered into twenty (C01-C20) as principal clusters. Seifi et al. discovered that *K. pneumoniae* strains in a Tehran hospital had a high level of genetic diversity [79, 80]. In a hospital in Borujerd, Mehr et al. (2017) found that *K. pneumoniae* strains had a significant degree of genetic diversity [81-83]. In a hospital in Borujerd, Mehr et al. (2017) found that *K. pneumoniae* strains had a significant degree of genetic diversity [84]. 99 isolates from this investigation were discovered to exhibit 63 distinct ERIC profiles. As a result, 52 *K. pneumoniae* isolates generated separate profiles and were not grouped. Indeed, the non-clonal distribution of the bacterium in the hospitals under inquiry is reflected in the genetic diversity of *K. pneumoniae* strains.

## CONCLUSION

Our research revealed no clinically meaningful differences in mortality amongst hvKP strains. Further studies are necessary to clarify the relationship between each distinct virulence factor and antibiotic resistance, as well as their influence on the clinical consequences of CAP caused by *K. pneumoniae*. Strict infection control measures must be put in place, together with good monitoring, to stop the spread of multidrug resistant hvKP. Our findings support the effectiveness and applicability of the ERIC-PCR technique for molecular typing, epidemiological investigation of nosocomial infections, and analysis of genetic diversity among hospital pathogens, including *K. pneumoniae* strains. *K. pneumoniae* strains obtained from Egyptian hospitals displayed a considerable amount of variability in their antibiotic resistance and ERIC profiles. Hospital infections caused by *K. pneumoniae* strains are difficult to cure when this variation is present.

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**Conflict of Interest:** The authors declare no conflict of interest.

**Ethical approval** The study was carried out in compliance with the Declaration of Helsinki and authorised by the Faculty of Medicine Ethics Committee, Assiut University's Institutional Review Board" (ID: 17101223).

**Author contributions:** Each author made a contribution to the project's planning, patient research, sample collection, data processing, or data interpretation. Each contributor contributed to the drafting and editing of the manuscript.

**Abbreviations:** HvKP: hypervirulent *Klebsiella pneumoniae*, CKP: classic *Klebsiella pneumoniae*, CPS: Capsular polysaccharide synthesis, MDR: multidrug-resistant, CAP: community-acquired, APACHE II: pneumonia Acute Physiology and Chronic Health Evaluation score, SOFA: Sequential Organ Failure Assessment score, ABG: arterial blood gases, PCR: Polymerase Chain Reaction, CLSI: Clinical and Laboratory Standards Institute, MDR: Multidrug-resistant, XDR: extremely drug-resistant, PDR: pan drug-resistant, ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction, MVSP: Multi-Variate Statistical Package, SD: standard deviation, P: Probability, ICU: intensive care unit,

pLVPK: phenotype of *K. pneumoniae* plasmid, UTIs: urinary tract infections.

## HIGHLIGHTS

- *Klebsiella pneumoniae*, particularly the hypervirulent (hvKP) strains, are resistant to antibiotics and cause infections difficult to treat.
- This study showed that pneumonia caused by Hvkp isolates showed more intensive care unit admission, vasopressor use, and need for mechanical ventilation while mortality doesn't differ between Ckp and Hvkp isolates.
- Capsular serotypes K1, K2 and K5 were more prevalent in hvKP. However, capsular serotype K20 was more prevalent in cKP.
- *rmpA*, *wcaG*, *uge* and *kfu* were the predominant virulence genes in hvKP

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