

# Genetic fingerprinting and profile analysis of virulence genes in XDR clinical isolates of *Klebsiella Pneumoniae*

L.I. BADGER-EMEKA<sup>1</sup>, P.M. EMEKA<sup>2</sup>

<sup>1</sup>College of Medicine, <sup>2</sup>College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia

**Abstract. – OBJECTIVE:** *Klebsiella pneumoniae*, a leading cause of hospital- and community-acquired infections, requires the acquisition of drug resistance and virulence for survival in evading the host's immune responses. The investigation looks at the molecular diversity of 30 clinical isolates using rep-PCR DNA fingerprinting and the association between drug resistance and virulence genes.

**MATERIALS AND METHODS:** Isolates were cultured using basic bacteriological techniques, while antimicrobial susceptibility test and ID confirmation were made by Vitek 2 Compact Automated System (BioMerieux, Marcy L'Etoile, France). DNA used for PCR reactions was extracted with Qiagen DNA extraction kit according to the manufacturer's guidelines, while molecular genotyping by REP-PCR was carried out according to previously described methods. Some virulence genes and capsular serotypes, *ybtS*, *mrkD*, *entB*, *rmpA*, *K2*, *Kfu*, *alls*, *iutA*, and *magA*, were amplified by multiplex PCR.

**RESULTS:** The majority (60%) of the isolates were MDR, others were XDR (37%) and susceptible strains (3%). Additionally, resistance was high (73%) for meropenem and lower (40%) for imipenem, while 97% were susceptible to ertapenem and azithromycin. DNA fingerprinting by rep-PCR showed polymorphic loci sizes that ranged from 100-2000 bp-, and phylogenetic analysis placed the isolates in seven clades with 40-96%. There was a 75% phylogenetic relatedness to antimicrobial resistance, but no specific pattern of relatedness was seen between virulence genes for the *K. pneumoniae* strain. Regulator mucoid phenotype A (*rmpA*) gene was not amplified in any of the isolates.

**CONCLUSIONS:** This study further confirms the presence of both MDR and XDR *K. pneumoniae* strains. Isolates exhibited genetic diversity in phylogenetic patterns and carriage of virulence with relatedness by susceptibility to antimicrobials. Therefore, circulation could pose a risk to public health.

*Key Words:*

*Klebsiella pneumoniae*, Virulence gene, Antimicrobial resistance, PCR, XDR.

## Introduction

*Klebsiella pneumoniae* (*K. pneumoniae*) is universally considered as a nosocomial pathogen, a leading cause of pneumonia, bacteremia, and urinary tract infections. Additionally, it has been shown that a third of infections resulting from Gram negative bacteria, such as cystitis, UTIs, septicaemia, and others, are those caused by *K. pneumoniae*<sup>1</sup>. Resultant infections are known to contribute to long hospital stays and high mortality rates<sup>1-3</sup>. This *Enterobacteriaceae*, which is generally considered as an opportunistic pathogen, has become a bacterial superbug of great clinical importance<sup>4,5</sup>. Hence, it emerges as a pathogen of concern in this era of high antimicrobial resistance and is thus classified as an ESKAPE bacteria, an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species<sup>6,7</sup>. Generally, the threat to antimicrobial resistance is a global problem, especially with the dramatic rise in infections of multi and extreme drug resistant (MDR, XDR) bacterial strains<sup>1</sup>. It is thought that to be successfully resistant to antibiotics, bacteria strains must also be a highly effective channel for disseminating antibiotic resistance. This usually occurs through drug resistance and virulence traits transferred either vertically to daughter cells or horizontally to other species and strains<sup>8</sup>.

Therefore, the pathogenesis of *K. pneumoniae* in the acquisition of both virulence and hence

drug resistance is necessary for survival. It is worth noting that the pathogenicity of this opportunistic pathogen is mediated by a wide range of virulence factors used by the bacterium to evade the innate immune responses of the host. These virulence factors are diverse, some of which include the capsule, lipopolysaccharides, iron acquisition systems, and adhesins<sup>9</sup>. It is postulated that knowledge of the associated *K. pneumoniae* virulence factors could assist in characterizing strains involved in outbreaks of infection in hospital settings<sup>10</sup>. Furthermore, detecting virulence genes in clinical *K. pneumoniae* isolates, as well as ascertaining their molecular diversity, could provide profound insights into strains of the bacterium that are multi-drug resistant (MDR)<sup>2</sup>. However, there is a paucity of information on *K. pneumoniae*-related virulence genes and their link to antimicrobial resistance, as expressed earlier<sup>2</sup>. Basically, two subtypes of *Klebsiella pneumoniae* are categorized as either classical or non-classical (cKP, ncKp). They differ in terms of antimicrobial resistance and virulence profiles with ncKp associated with severe and difficult-to-treat infections. Epidemiologically, *K. pneumoniae* infections could be specific to localities, varying between countries while also exhibiting similarities intercontinentally. This study uses repetitive extragenic palindromic PCR (rep-PCR) DNA fingerprinting to characterize MDR/XDR *K. pneumoniae* clinical isolates, as well as analyze the virulence genes profile associated with them by multiplex PCR. This method was needed to establish any correlation between phylogenetic profiles, antimicrobial resistance, and virulence determinants in *K. pneumoniae* isolates in this region of study. Additionally, the investigation looks at the molecular diversity of thirty clinical *K. pneumoniae* isolates, the resistance to antimicrobials, and the associated virulence genes.

## Materials and Methods

### **Ethical Consideration**

*Klebsiella pneumoniae* clinical bacterial isolates were stored in the microbank of microbiology laboratory at the College of Medicine, at King Faisal University. No patients were engaged in the study.

### **Bacteria Isolates, Antimicrobial Resistance Assay, and Extraction of Genomic DNA**

Thirty randomly selected *K. pneumoniae* isolates were retrieved from the -80°C microbank

freezer in the microbiology laboratory at the College of medicine, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia. They were plated out on MacConkey agar, and incubated overnight at 37°C. The resultant overnight bacterial growth was re-cultured on MacConkey agar for 24 hours at 37°C, and pure colonies were used for the confirmation of bacterial ID. Antimicrobial assay was performed using ID and AST cards of the Vitek 2 Compact Automated System (BioMerieux, Marcy L'Etoile, France) according to the guidelines of the manufacturers. The following antibiotics were used for the antibiogram assay: amoxicillin (Am), ampicillin/sulbactam (Ams), amoxicillin/clavulanic acid (Aug), piperacillin/tazobactam (Ptz), ceftazidime (Caz), cefepime (Pime), cephalotin (Kf), cefoxitin (Ctt), ceftriaxone (Cro), cefuroxime (Cxm), aztreonam (Azt), ertapenem (Etp), imipenem (Imp), meropenem (Mer), amikacin (Amk), gentamicin (Gn), tobramycin (To), ciprofloxacin (Cp), minocycline (Min), norfloxacin (Nor), levofloxacin (Levo), nitrofurantoin (Fd), tigecycline (Tig), and trimethoprim/sulfamethoxazole (Ts). Minimum inhibitory concentrations were also determined using the Vitek 2 Compact Automated System (BioMerieux, Marcy L'Etoile, France). Additionally, fresh overnight cultures of the 30 *K. pneumoniae* strains were used for molecular DNA extraction using a Qiagen DNA extraction kit (QIAGEN Inc., Valencia, CA, USA), according to the recommended guidelines of the manufacturers. The extracted DNA from each isolate was used as a template for performing PCR reactions.

### **Genotyping of *K. Pneumoniae* by Repetitive Extragenic Palindromic (REP)-PCR**

Isolates were genotyped by REP-PCR with the primer pair REP 1 (5'-IIIGCGCCGICAT-CAGGC-3') and REP 2 (5'-ACGTCTTATCAG-GCCTAC-3')<sup>11</sup>, according to previously described methods for amplification by PCR<sup>12</sup>. Briefly, 25 µL final reaction volume of amplification reaction made up of 45 pmol (1.8 µmol. L-1) and 36 pmol (1.44 µmol. L-1) of each of the REP primers was added to 100 ng of template DNA and 1X PCR master mix (Taq DNA Polymerase Master Mix RED, Ampliqon, Denmark). The PCR thermal cycler amplification process was used as previously described<sup>13</sup>. The resultant amplified DNA products were separated by gel electrophoresis on 2% agarose gel with a 1kb DNA ladder as marker and stained with ethidium bromide. Images

were captured with a Uvitec firereader Max gel documentation unit (UViTEC Cambridge, UK). REP-PCR fingerprinting of the strains was photographed and compared. Band-matching was performed with an error rate of 0.02. Code 0 (no band) and 1 (band) were transferred from the program to PAST software and dendrogram of Dice similarity coefficient created with the Complete Linkage algorithm.

### Detection of Associated Virulence Genes by Multiplex PCR

Qiagen Multiplex PCR Kit for 100 × 50 µL multiplex reactions was used for the detection of virulence genes, according to the manufacturer's guidelines on standard multiplex PCR. The primers used for the investigation are shown in Table I. The reaction mix, which consisted of 2 × Qiagen Multiplex PCR Master Mix [providing a final concentration of 3 mM MgCl<sub>2</sub> (3 × 0.85 mL), 5 × Q-Solution (1 × 2.0 mL), and RNase-Free Water (2 × 1.7 mL)], was added to 1 µL of DNA template of each bacterial isolate to obtain a final reaction volume of 50 µL as earlier described<sup>14</sup>. Cycling conditions were based on the recommendations of the manufacturers: initial 15 mins of HotStar Tag DNA polymerase heat activation at 95°C, followed by 3 cycling steps involving 30 s of denaturation at 94°C and annealing for 90 s at 63°C, followed by extension at 72°C for 90 s. With a total of 45 cycles, a 10 min final extension was

achieved at a temperature of 72°C; details are as described in the manufacturer's guide ([www.qiagen.com/HB-0453](http://www.qiagen.com/HB-0453), accessed on 19 June 2021). A 2% agarose gel (sigma) stained with ethidium bromide (0.5 µg/ml) in 1 × TAE buffer was used for gel electrophoresis analysis and visualized with a UV transilluminator.

### Statistical Analysis

GraphPad prism version 9.2.0 (283) was used for data analysis. Antimicrobial results are presented as percentages, while two sample *t*-test of statistics calculator (StatPac version 4.0) was used for comparing significant differences between percentages. Significance was taken at *p* < 0.05. REP-PCR, gel images were transferred to Uvitec firereader Max gel documentation unit (UViTEC Cambridge, UK) for imaging, bands were done to match with error rate of 0.02. With codes of 0 and 1, representing no band and band respectively, the program was transferred to the PAST software, and a dendrogram of the Dice similarity coefficient was drawn with the Complete Linkage algorithm.

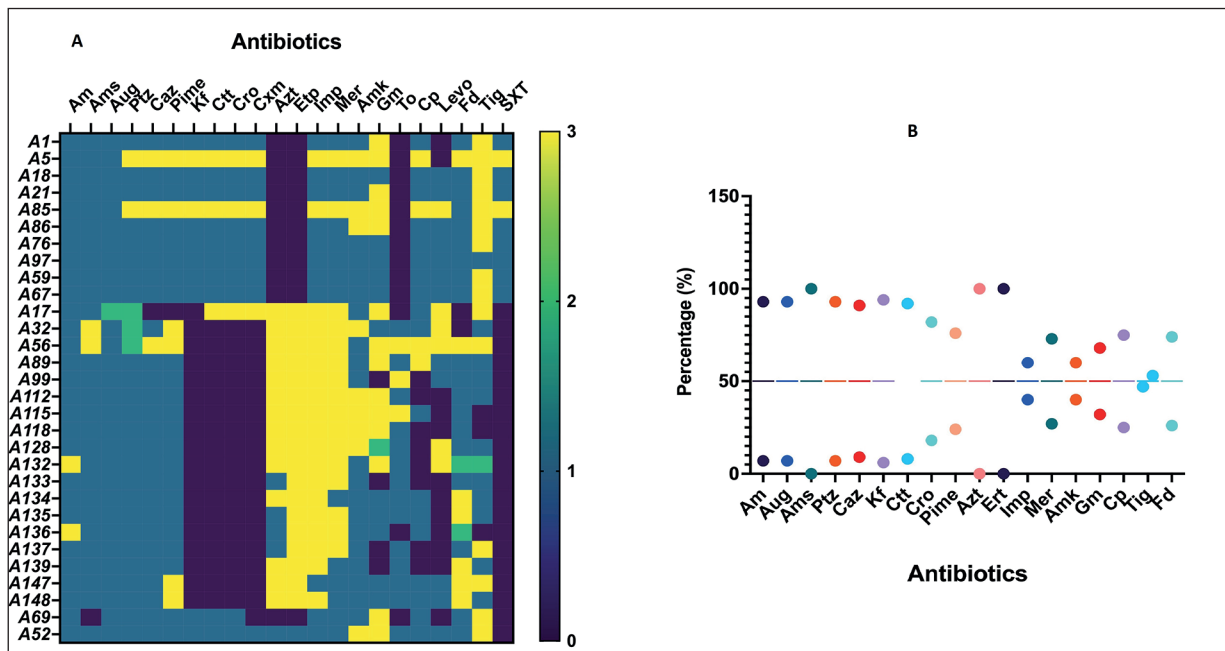
## Results

### Antimicrobial Susceptibility

The percentage frequency distribution of antimicrobial predisposition against twenty-two antibiotics and the susceptibility or of the thirty

**Table I.** Primers, oligonucleotide sequences and amplicon sizes (bp) used for the study.

Primer	DNA sequence (5' to 3')	Target gene product/function	Amplicon size (bp)	Reference
ybtS_FP	GACGGAAACAGCACGGTAAA	Siderophore	242	
ybtS_RP	GAGCATAATAAGGCGAAAGA			
mrkD_FP	AAGCTATCGCTGTACTTCCGGCA	Adhesin type 3 fimbriae	340	
mrkD_RP	GGCGTTGGCGCTCAGATAGG			
entB_FP	GTCAACTGGGCCTTTGAGCCGTC	Siderophore	400	Compain et al <sup>43</sup>
entB_RP	TATGGGCGTAAACGCCGGTGAT			
rmpA_FP	CATAAGAGTATTGGTTGACAG	Regulator of mucoid phenotype A	461	
rmpA_RP	CTTGCATGAGCCATCTTTCA			
K2_FP	CAACCATGGTGGTCGATTAG	Capsular serotype K2 and hypermucoviscosity phenotype	531	
K2_RP	TGGTAGCCATATCCCTTTGG			
Kfu_FP	GGCCTTTGTCCAGAGCTACG	Iron transport and phosphotransferase function	638	
Kfu_RP	GGGTCTGGCGCAGAGTATGC			
alls_FP	CATTACGCACCTTTGTCAGC	Allantoin metabolism	764	
alls_RP	GAATGTGTCCGGCAGTCACTT			
iutA_FP	GGGAAAGGCTTCTCTGCCAT	Siderophore	920	
iutA_RP	TTATTCGCCACCACGCTCTT			
magA_FP	GGTGCTCTTTACATCATTGC	Capsular serotype K1 and hypermucoviscosity phenotype	1283	



**Figure 1.** Antibiotic susceptibility of the isolates. **A**, shows the distribution of the isolate susceptibility to general antimicrobial agents (1 = Resistant, 2 = Intermediate, 3 = Sensitive). **B**, shows the percentage resistance and sensitivity of the specific antibiotics used for treatment of the *K. pneumoniae* infection. Amoxicillin (Am), Amoxicillin/clavulanic acid (Aug), Ampicillin/sulbactam (AMS), piperacillin/tazobactam (Ptz), ceftazidime (Caz), cefepime (Pime), cephalotin (Kf), ceftaxime (Ctt), ceftriaxone (Cro), cefuroxime (Cxm), aztreonam (Azt), ertapenem (Etp), imipenem (Imp), meropenem (Mer), amikacin (Amk), gentamicin (Gm), tobramycin (To), ciprofloxacin (Cp), levofloxacin (Levo), nitrofurantoin (Fd), and trimethoprim/sulfamethoxazole (Ts).

strains of *Klebsiella pneumoniae* are shown in Figure 1A-B. Figure 1A displays the heatmap distribution showing the isolate ID and the percentage of antimicrobial susceptibility of isolates against the tested drugs. An overall resistance to antibiotics, such as amoxicillin (Am), augmentin (Aug), piperacillin/tazobactam (Ptz), ampicillin/sulbactam (Ams), ceftazidime (Caz), and ceftaxime (Ctt), was high, ranging between 91-100% (Figure 1B). Results showed isolates to be significantly more MDR (60%), as they were resistant to one of the three antibiotics categories, while one isolate (3.3%) was a susceptible strain and the remaining (36.67%) were XDR, being susceptible to two or fewer antimicrobial categories (Table II). Antimicrobial susceptibility varied among the isolates. 97% of the isolates examined in this study were sensitive to ertapenem and azithromycin; however, for the rest of the carbapenems, there was high resistance to meropenem (73%), with imipenem being less resistant (40%). Additionally, variations were noticed in the susceptibility to the antibiotics for individual isolates, as displayed in Table II, which shows that the *K. pneumoniae* isolate (A97) was not sensitive to any of the tested drugs, while other isolates (A59, A67, A18, A76)

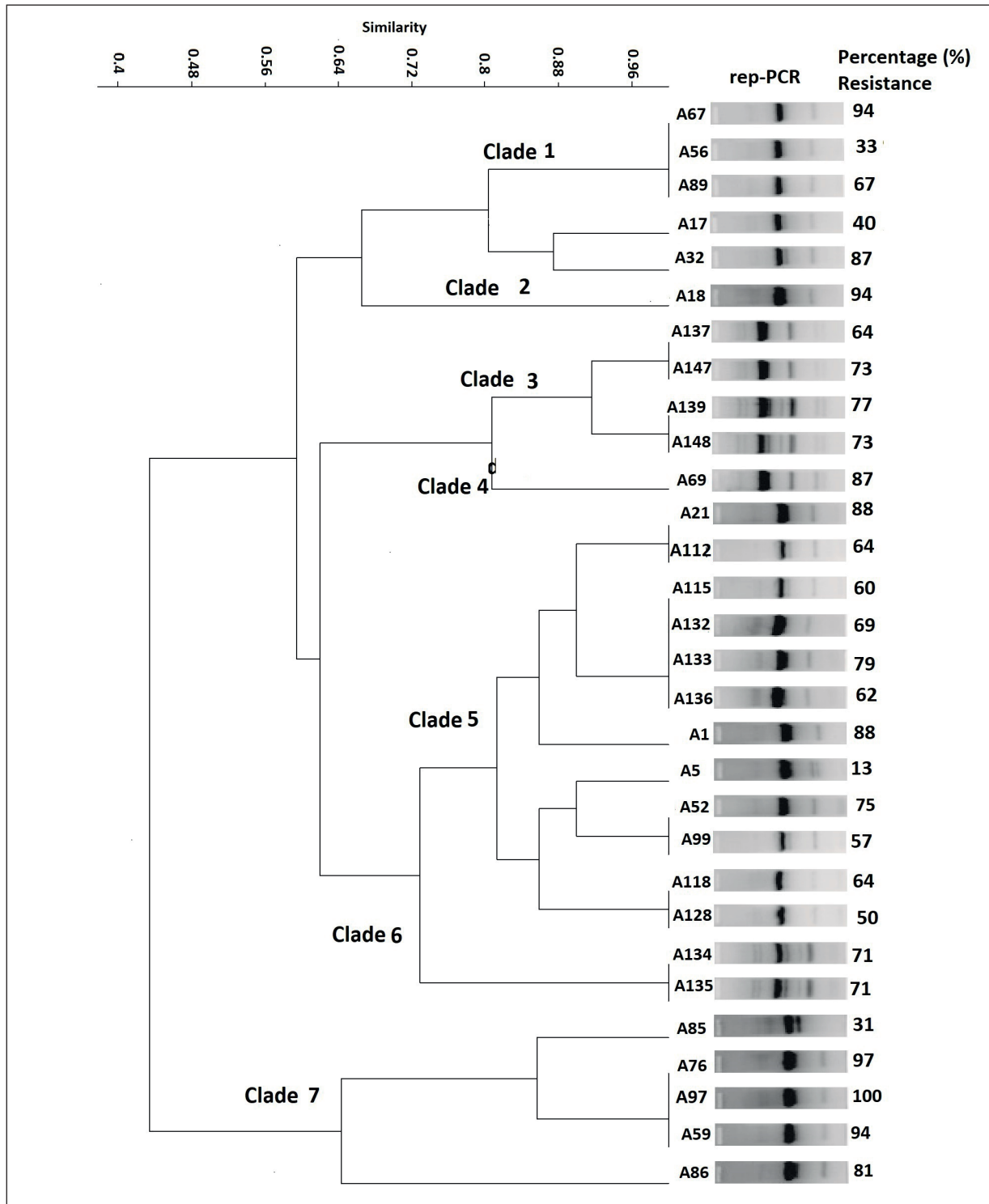
were sensitive either to one antibiotic or (A21, A5, A1, A69) to two of the tested drugs (tigecycline and gentamicin).

#### DNA Fingerprinting by Rep-PCR of *Klebsiella pneumoniae* Isolates and Antimicrobial Resistance

Rep-PCR DNA fingerprinting results of the amplified product showed polymorphic loci for the thirty clinical isolates (Supplementary Figure 1) with loci sizes that ranged from 100- 2000 bp p. A dendrogram comparing the phylogenetic relatedness of the isolates and the percentage resistance is shown in Figure 2. The phylogenetic tree places the thirty isolates into seven (7) clades with 40-96 % similarity. Clade 1 has two clusters with five isolates (A67, A56, A89, A17, and A32). While Clade 2 had one strain of *K. pneumoniae* (A18), there were only clusters with four isolates in Clade 3. The next lowest number of isolates was seen in Clade 4, which consisted of one strain of *K. pneumoniae* (A69). The highest number isolates were seen in Clade 5, with 12 isolates, distributed into 6 clusters (Figure 3). Clade 6 displays only two isolates (A34 and A35), while Clade 7 showed five *K. pneumoniae* isolates grouped into

three clusters. Figure 2 also displays the percentage resistance for each of the isolates in the different clades, and it seems to vary between phylogenetic clades. Resistance to antimicrobial drugs by

*K. pneumoniae* isolates in the two clusters within Clade 1 showed diversity and no relatedness with resistance patterns (Figure 2). However, there is a phylogenetic relatedness to drug resistance in



**Figure 2.** Phylogenetic tree analysis of rep-PCR DNA fingerprinting of thirty *Klebsiella pneumoniae* isolates. Showing isolates grouped into seven clades and relatedness to antimicrobial resistance.

**Table II.** The antibiotics that each of the *K. pneumoniae* isolates were sensitive to, the antimicrobial resistance phenotypes, and the amplified virulence genes for each isolate (+ = present, - = absent). XDR = Extensively drug-resistant, CRE = Carbapenem-resistant Enterobacteriaceae, MDR = multi-drug-resistant, SS = susceptible strain.

Isolate ID	Susceptible antibiotics	Resistant Characteristics	ybtS	mrkD	entB	rmpA	K2	Kfu	alls	iutA	magA
A99	Azt, Ert, Imp, Mer, To.	XDR/CRE	-	+	+	-	-	-	+	+	+
A112	Azt, Ert, Imp, Mer, Amk, Gm.	MDR	-	+	+	-	-	-	+	+	+
A115	Azt, Ert, Imp, Mer, Amk, Gm.	MDR	-	+	+	-	-	-	+	+	+
A118	Azt, Ert, Imp, Mer, Amk.	MDR	-	+	+	-	-	-	+	+	+
A128	Azt, Ert, Imp, Mer, Amk, Gm, Fd.	MDR	-	+	+	-	-	-	+	+	+
A132	Am, Azt, Ert, Imp, Gm, Levo.	MDR	-	+	+	-	-	-	+	+	+
A133	Azt, Ert, Imp	MDR	-	+	+	-	-	-	+	+	+
A56	Aug, Caz, Pime Azt, Ert, Imp, To, Gm, To, Cp, Levo, Tig, Fd.	MDR	+	-	+	-	-	+	+	+	+
A21	Gm, Tig.	XDR/CRE	+	+	+	-	+	+	-	+	+
A59	Tig.	XDR/CRE	+	-	+	-	-	+	-	+	+
A67	Tig.	XDR/CRE	+	-	+	-	-	+	-	+	+
A5	Gm, Tig	SS	+	+	+	-	-	+	-	+	+
A18	Tig.	XDR/CRE	+	+	+	-	-	+	-	+	+
A76	Tig.	XDR/CRE	+	+	+	-	-	+	-	+	+
A17	Pime, Azt, Ert, Imp, Mer, Amk, Gm, To, Levo, Tig.	MDR	+	+	+	-	-	+	-	+	+
A97	None	XDR/CRE	+	+	+	-	-	+	-	+	+
A52	Amk, Gm, Tig, Ts	XDR/CRE	+	+	+	-	+	+	-	+	+
A89	Azt, Ert, Imp, Mer, Amk, Gm.	MDR	+	+	+	-	-	+	+	-	+
A135	Azt, Ert, Imp, Fd.	MDR	+	+	+	-	-	+	+	-	+
A139	Azt, Ert, Imp, Fd.	MDR	+	+	+	-	+	+	+	-	+
A147	Pime, Azt, Ert, Tig, Fd.	MDR	+	+	+	-	+	+	+	-	+
A148	Pime, Azt, Ert, Fd.	MDR	+	+	+	-	+	+	+	+	+
A1	Gm, Tig.	XDR/CRE	+	+	+	-	+	+	-	+	+
A85	Ptz, Caz, Cro, Pime, Imp, Mer, Amk, Gm, Cp, Tig.	MDR	+	+	-	-	+	+	-	+	+
A136	Am, Azt, Ert, Imp.	MDR	+	+	+	-	+	+	+	+	+
A86	Amk, Gm, Tig.	XDR/CRE	+	+	+	-	+	+	+	+	+
A137	Azt, Ert, Imp, Fd.	MDR	+	+	+	-	+	+	+	+	+
A69	Gm, Tig.	XDR/CRE	+	+	+	-	+	+	+	+	+
A134	Azt, Ert, Imp, Fd.	MDR	+	+	+	-	-	+	+	+	+
A32	Aug, Pime, Azt, Ert, Imp, Mer, Amk. Cp, Levo.	MDR	+	+	+	-	+	-	-	+	+
MDR 18 [60 %] XDR/CRE 11 [36.67 %] SS 1[3.33 %].			<i>p</i> -value 0.0182								

Clade 3 with 75%, while eight clusters in Clade 5 had 67% similarity. Isolates in Clade 6 had 100% relatedness, whereas three clusters of Clade 7 showed 60% similarity. Thus, the results for phylogeny relatedness with antimicrobial resistance showed 75.5% overall similarity.

### Amplified Virulence Genes

Virulence genes were characterized by their molecular weights with amplicon sizes that ranged from 242- 1283 bp and gel electrophoresis image documentation (**Supplementary Figure 2**). Nine virulence genes with primers listed in Table I were investigated. A maximum of eight virulence genes were seen in some isolates, and

the distribution of amplified genes is shown in Table I. A minimum of five combinations of virulence genes were amplified in ten (33%) of the isolates (A99, A112, A115, A118, A128, A132, A133, A67, A59, and A5). Additionally, in other ten (33%) isolates, six combinations of virulence genes were amplified (A76, A17, A18, A97, A32, A56, A89, A135, A139, and A147), while seven combinations of genes were amplified in six of the clinical *K. pneumoniae* isolates (A21, A52, A85, A1, A134, and A148). A maximum of eight genes were amplified in five (17%) of the isolates (A148, A136 A86, A137, and A69), as shown in Table II.

Absent in all the amplified virulence genes is the Regulator of mucoid phenotype A (*rmpA*)

**Table III.** The antibiotics that each of the *K. pneumoniae* isolates were sensitive to, the antimicrobial resistance phenotypes, and the amplified virulence genes for each isolate (+ = present, - = absent). XDR = Extensively drug-resistant, CRE = Carbapenem-resistant Enterobacteriaceae, MDR = multi-drug-resistant, SS = susceptible strain.

S/N	Gene product/function	Primer	Number of isolates tested	Number positive (%)	Number negative (%)	p-value
1	Siderophore.	ybtS	30	23 (77)	7 (23)	0.0001
2	Adhesin type 3 fimbriae	mrkD	30	26 (87)	4 (13)	0.0001
3	Siderophore	entB	30	28 (93)	2 (7)	0.0001
4	Regulator of mucoid phenotype A	rmpA	30	0 (0)	30 (100)	0.0001
5	Capsular serotype K2 and hypermucoviscosity phenotype	K2	30	12 (40)	18 (60)	0.0439
6	Iron transport and phosphotransferase Function	Kfu	30	22 (73)	8 (27)	0.0001
7	Allantoin metabolism	alls	30	11 (37)	19 (63)	0.0083
8	Siderophore	iutA	30	25 (83)	5 (17)	0.0001
9	Capsular serotype K1 and hypermucoviscosity phenotype	magA	30	29 (97)	1 (3)	0.0001
10	Presence Both K1/K2			12 (40%)		
11	Absence of Both K1 and K2			1 (3%)		

gene. The capsular serotype and hypermucoviscosity phenotype gene (MagA) and siderophore (entB) were the most amplified at percentage frequencies of 97% and 93%, respectively (Table III). The least number of amplified virulence genes occurred in capsular serotype K2/hypermucoviscosity phenotype (K2) and allantoin metabolism (alls), which were amplified in 40% and 37% of the clinical *K. pneumoniae* isolates, respectively. The Z Score calculation was used to compare the presence and absence of amplified virulence in two population portions showed significant differences (Table III).

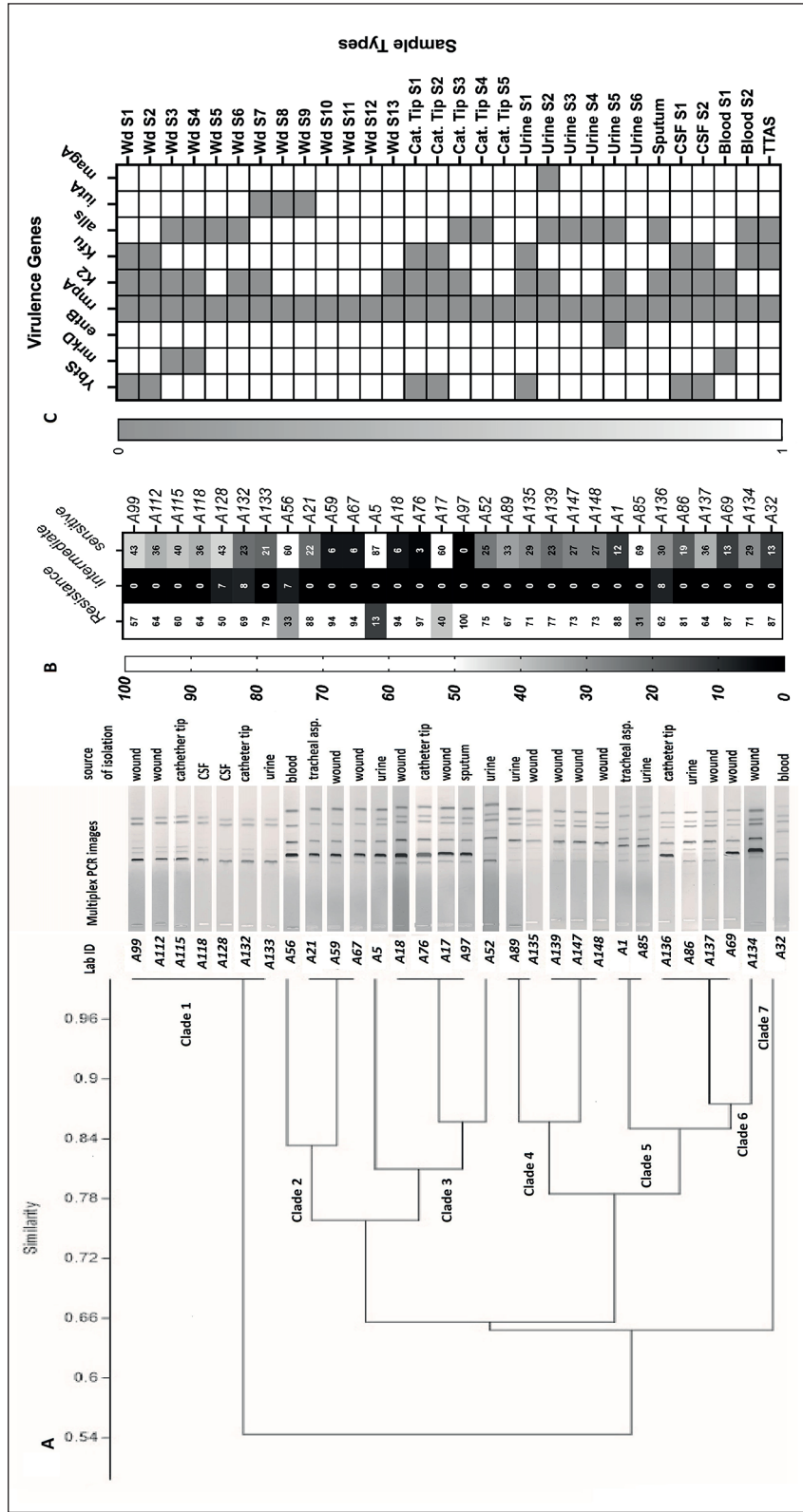
#### Comparison of Phylogenetic Relatedness of Virulence Genes

The phylogenetic tree from the amplified virulence genes analysis grouped the isolates into seven clades (Figure 3A). The first clade had one cluster of seven isolates, and sources of samples were wound swab, CSF, urine, and catheter tip. In Clade 2, there were four *K. pneumoniae* strains from wound, blood, and transtracheal aspirates samples. Clade 3 had three clusters of six *K. pneumoniae* isolates from urine, wound (two), sputum (one), and catheter tip (one) samples. Additionally, Clade 4 consisted of five isolated grouped into two clusters obtained from wound (four) and urine samples (one). The two isolates in Clade 5 were from transtracheal aspirates and urine samples. In addition, isolates in Clade 6 were grouped into two clusters, including three samples from wound swabs and the remaining from catheter tips (one) and a urine sample (one). The minimum

was found in Clade 7, with one *K. pneumoniae* strain from the blood specimen, as shown in Figure 3A. The figure also compares the virulence phylogeny and the antimicrobial resistance (Figure 3B). No specific pattern of relatedness was shown between the virulence genes and the percentage resistance to antibiotics for the *K. pneumoniae* strain. Isolates in Clade 1 with similar amplified virulence gene (mrkD, entB, alls, iutA and MagA) and percentage antimicrobial resistance ranged from 50% to 79%. Findings for other clades were similar. However, in most clades where most of the isolates were highly resistant (80-100%) to antimicrobials, there were some outliers with susceptible or less resistant strains among them, such as in Clade 2, A56 (33%) and A5 (15%); in Clade 3, A17 (40%); and in Clade 5, A85 (31%). The figure also shows that identical virulence gene were amplified in CSF specimens: in wounds in Specimens 1 and 2, in wounds in Specimens 3 and 4, and in urine in Specimens 3 and 4 (Figure 3C).

#### Discussion

The present study examined isolates of *K. pneumoniae* strains associated with infections in immunocompromised patients. Sources of samples in this investigation were from urine, CSF, catheter tips, sputum, tracheal, wounds, and blood specimens, which were like the samples used in an earlier report<sup>15</sup>. Our observation in this study indicates that resistance against antimicro-



**Figure 3.** A, Phylogenetic relatedness of *K. pneumoniae* isolates by amplified virulence genes. B, source of samples heatmaps of antimicrobial susceptibility. C, virulence genes distribution by specimens' type.



bials was high revealing MDR and XDR isolates in the majority, but with a high sensitivity to erapenem and azithromycin. However, the high percentage resistance to ampicillin here agrees with earlier reports<sup>16-18</sup>. Additionally, our findings are consistent with the high imipenem resistance documented in other studies<sup>19</sup>. However, carbapenem-resistant *K. pneumoniae* (CRE-KP) phenotypes differ geographically<sup>20</sup>. The percentage of CRE-KP seen in this study is higher, compared to that of others<sup>21,22</sup>; this could be due to the global rise in carbapenem-resistant *K. pneumoniae*, which has led to high global mortality rates<sup>23</sup>.

Rep-PCR has been widely used for genotyping bacterial strains in general and was also used in this investigation. Our results, which placed the thirty *K. pneumoniae* isolates into seven phylogenetic clades with varying clusters, showed a diversity of strains. Generally, the heterogenous characteristics of strains of pathogenic *K. pneumoniae* are documented<sup>24,25</sup> with differences in patterns of rep-PCR results, attributed to the massive clonal diversity geographically<sup>25,26</sup>. Additionally, variations as seen in this research between rep-PCR pattern phylogeny and any correlation with antimicrobial resistance further highlight clonal diversity. However, some reports point to a correlation between antimicrobial resistance profile patterns and rep-PCR. A non-significant correlation between rep-PCR pattern and multi-drug resistance was recently described<sup>25</sup>.

Most (60%) of the *K. pneumoniae* isolates in our results were MDR, while 37% were XDR; these findings corroborate other reports<sup>27</sup> for MDR and XDR<sup>28</sup> in hvKP strains. These results all point to a possible global rise in MDR and XDR hvKP strains, which has been credited to an increase in the use of antimicrobials in hospitalized patients, consequently promoting selection pressure for resistance to antimicrobials<sup>29,30</sup>. However, this report showed that the arrangement of *K. pneumoniae* isolates' phylogenetic analysis by rep-PCR was different for the distribution of isolates according to amplified virulence genes. Additionally, our findings show the non-relatedness of the rep-PCR pattern with the associated virulence factors, thus agreeing with a recent report findings<sup>25</sup>.

The classical *Klebsiella pneumoniae* (cKP) is considered to be linked to infections in the immunocompromised<sup>31</sup>. The majority (97%) of the *K. pneumoniae* strains in the current study were confirmed by PCR amplification to be K1 and hyperviscosity phenotypes, while 40% of them were capsular serotype K2 and hypermucoviscosity

phenotypes. Therefore, isolates examined in the present study here can thus be considered as both cKP and hyperviscosity KP (hvKP). Previous reports associated both MDR and XDR with cKP isolates, rather than with hvKP strains<sup>32,33</sup>.

Furthermore, in defining hvKP phenotypes, our report shows that it has no single façade<sup>20</sup>. However, numerous efforts have been proposed to establish a unanimous hvKP definition based on the suggestions of other researchers<sup>28,34,35</sup>. Additionally, reports on the prevalence of hvKP strains vary geographically and range between 37.8% in infections acquired in hospitals in Mainland China<sup>36</sup> and 2.8% in Spain<sup>30</sup>. The percentages of 97 (K1), 40% each for K2 and K1/K2 as found in the present investigation, can be considered high. There could be a number of factors responsible for this finding, one of which is the geographical position, as suggested by Rastegar et al<sup>32</sup>, as well as differences in molecular analysis methodologies. K1 and K2 are the two serotypes investigated in this study, and only in one *K. pneumoniae* isolate (A5) both serotypes were not amplified. The lower percentage of the K2 serotype among the *K. pneumoniae* strains in this study could point to the presence of other types of serotypes. However, both K1 and K2 serotypes are associated with invasive infections and meningitis<sup>37</sup>. They are also considered to be more virulent<sup>38</sup>, making them a major cause of hypervirulent infection<sup>39,40</sup>. In addition, according to some reports, there are other virulence factors that promote the pathogenicity of hvKP strains, including mucoviscosity-associated gene A (*magA*) and regulator of mucoid phenotype A (*rmpA*), both of which were examined in the present study. Regulator of mucoid phenotype A (*rmpA*) is, however, described as the main virulence *K. pneumoniae* factor<sup>41</sup>. In this study, it was not amplified in any of the isolates, showing the same results as other reports of sparse numbers in terms of the prevalence of this virulence factor<sup>32</sup> in hvKP isolates.

The absence of *rmpA* genes in this investigation is consistent with the study by Remya et al<sup>42</sup>. With *rmpA*-associated invasiveness and poor patient outcomes<sup>20</sup>, their absence in this study could mean that samples were from non-invasive syndromes or simply the presence of other types of mucoid factors besides *magA* and *rmpA*. This could be due to variations in the constitution of lipopolysaccharide might exist<sup>30</sup>. Fimbriae-virulence-related genes are also involved in the virulence of *K. pneumoniae*, including adhesin type 3 fimbriae (*mrkD*), which was amplified in a high percentage of the isolates (87%) in our study. These findings are similar to those of others<sup>16,34</sup>. In addition to

this, high percentages of amplified iron acquisition factors enterobactin (entB 98%), aerobactin receptor (iutA 83%), yersiniabactin, and kfu (ybtS 77%) have been detected, all of which are also in line with previous reports<sup>16,18,43</sup>. Although hypermucoviscous phenotype *K. pneumoniae* indicates virulence, discrepancies between the hvKP genotype and the phenotypic markers of virulence factors have been highlighted<sup>44</sup>.

The reason for the higher susceptibility of hvKP to antimicrobials is postulated to be the loss of resistance genes, which causes them to become hypervirulent<sup>45</sup>. The present findings suggest acquired resistance over time, as has been recently advocated<sup>20</sup>.

## Conclusions

The present study further highlights the global challenges that *K. pneumoniae* bacterial pathogens pose to public health. The presence of MDR and XDR strains is once again shown among the examined isolates. Genetic diversity is seen in this study within the rep-PCR phylogenetic pattern, which showed some relatedness to drug resistance, rather than an association with virulence genes. The genetic diversity observed from these clinical *K. pneumoniae* isolates, in terms of resistance to antimicrobials and virulence genes, could be detrimental to the management of infections caused by this bacterial pathogen.

---

### Institutional Review Board Statement

Not applicable.

---

### Informed Consent Statement

Not applicable. No Patients participated in the investigation.

---

### Data Availability Statement

Data are available in the research. Should there be a need, the corresponding author can be contacted.

---

### Conflicts of Interest

The authors declare no conflicts of interest.

---

### Acknowledgments

This work was supported through the Annual Funding track by the Deanship of Scientific Research, Vice

Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia [Project No. AN000689]. We thank Krishnaraj Thirugnanasambantham for his help in analyzing the data.

---

### Funding

This research was funded by Deanship for Scientific Research, King Faisal University, grant number AN000689.

---

### Authors' Contributions

Conceptualization, L.I.B-E and P.M.E; methodology, L.I.B-E.; software, L.I.B-E and P.M.E; validation, L.I.B-E and P.M.E; formal analysis, L.I.B-E.; investigation, L.I.B-E and P.M.E; resources, L.I.B-E.; data curation, L.I.B-E.; writing—original draft preparation, L.I.B-E writing—review and editing, L.I.B-E and P.M.E; visualization, L.I.B-E and P.M.E; supervision, L.I.B-E.; project administration, L.I.B-E.; funding acquisition, L.I.B-E. All authors have read and agreed to the published version of the manuscript.

---

### ORCID ID

Lorina Badger-Emeka: 0000-0002-9201-6215.  
Promise Madu Emeka: 0000-0003-1943-1874.

## References

- 1) Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol Rev* 2017; 41: 252-275.
- 2) Alsanie WF. Molecular diversity and profile analysis of virulence-associated genes in some *Klebsiella pneumoniae* isolates. *Pract Lab Med* 2020; 19: e00152,
- 3) Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis* 2008; 8: 159-166.
- 4) Effah CY, Sun T, Liu S, Wu Y. *Klebsiella pneumoniae*: an increasing threat to public health. *Ann Clin Microbiol Antimicrob* 2020; 19: 1.
- 5) Infectious Diseases Society of America. Bad bugs, no drugs. Infectious Diseases Society of America, Alexandria, VA 2004.
- 6) Mulani MS, Kamble EE, Kumkar SN, Tawre MS, Pardesi KR. Emerging Strategies to Combat ES-CAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Front Microbiol* 2019; 10: 539.
- 7) Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. Bad bugs, no drugs: no ES-CAPE! *An*

- update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009; 48: 1-12.
- 8) Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol Rev* 2011; 35: 736-755.
  - 9) Candan ED, Aksöz N. *Klebsiella pneumoniae*: characteristics of carbapenem resistance and virulence factors. *Acta Biochim Pol* 2015; 62: 867-874.
  - 10) Cruz-Cordova A, Esteban-Kenel V, Espinosa-Mazariego K, Ochoa SA, Espinosa SM, Elhain AG, Rendón EF, Villegas EOL, Xicohtencatl-Cortes J. Pathogenic determinants of clinical *Klebsiella pneumoniae* strains associated with their persistence in the hospital environment. *Bol Med Hosp Infant Mex* 2014; 71: 15-24.
  - 11) Andriamanantena TS, Ratsima E, Rakotonirina HC, Randrianirina F, Ramparany L, Carod JF, Richard V, Talarmin A. Dissemination of multidrug resistant *Acinetobacter baumannii* in various hospitals of Antananarivo Madagascar. *Ann Clin Microbiol Antimicrob* 2010; 9: 17.
  - 12) Iraz M, Özad Düzgün A, Sandallı C, Doymaz MZ, Akkoyunlu Y, Saral A, Peleg AY, Özgümüş OB, Beriş FŞ, Karaoğlu H.; Çopur Çiçek, A. Distribution of  $\beta$ -lactamase genes among carbapenem-resistant *Klebsiella pneumoniae* strains isolated from patients in Turkey. *Ann Lab Med* 2015; 35: 595-601.
  - 13) Martín-Lozano D, Cisneros JM, Becerril B, Cuberos L, Prados T, Ortíz-Leyba C, Cañas E, Pachón J. Comparison of a repetitive extragenic palindromic sequence-based PCR method and clinical and microbiological methods for determining strain sources in cases of nosocomial *Acinetobacter baumannii* bacteremia. *J Clin Microbiol* 2002; 40: 4571-4575.
  - 14) Badger-Emeka LI, Al-Sultan AA, Bohol MFF, Al-Anazi MR, Al-Qahtani AA. Genetic Analysis, Population Structure, and Characterisation of Multidrug-Resistant *Klebsiella pneumoniae* from the Al-Hofuf Region of Saudi Arabia. *Pathogens* 2021; 10: 1097.
  - 15) Albasha AM, Osman EH, Abd-Alhalim S, Alshaib EF, Al-Hassan L, Altayb HN. Detection of several carbapenems resistant and virulence genes in classical and hyper-virulent strains of *Klebsiella pneumoniae* isolated from hospitalized neonates and adults in Khartoum. *BMC Res Notes* 2020; 13: 312.
  - 16) Yan Q, Zhou M, Zou M, Liu WE. Hypervirulent *Klebsiella pneumoniae* induced ventilator-associated pneumonia in mechanically ventilated patients in China. *Eur J Clin Microbiol Infect Dis* 2016; 35: 387-396.
  - 17) Li J, Ren J, Wang W, Wang G, Gu G, Wu X, Wang Y, Huang M, Li J. Risk factors and clinical outcomes of hypervirulent *Klebsiella pneumoniae* induced bloodstream infections. *Eur J Clin Microbiol Infect Dis* 2018; 37: 679-689.
  - 18) Guo Y, Wang S, Zhan L, Jin Y, Duan J, Hao Z, Lv J, Qi X, Chen L, Kreiswirth BN, Wang L, Yu F. Microbiological and Clinical Characteristics of Hypermucoviscous *Klebsiella pneumoniae* Isolates Associated with Invasive Infections in China. *Front Cell Infect Microbiol* 2017; 7: 24.
  - 19) Amraie H, Shakib P, Rouhi S, Bakhshandeh N, Zamanzad B. Prevalence assessment of *magA* gene and antimicrobial susceptibility of *Klebsiella pneumoniae* isolated from clinical specimens in Shahrekord, Iran. *Iran J Microbiol* 2014; 6: 311-316.
  - 20) Lan P, Jiang Y, Zhou J, Yu Y. A global perspective on the convergence of hypervirulence and carbapenem resistance in *Klebsiella pneumoniae*. *J Glob Antimicrob Resist* 2021; 25: 26-34.
  - 21) Tumbarello M, Viale P, Viscoli C, Trecarichi EM, Tumietto F, Marchese A, Spanu T, Ambretti S, Ginocchio F, Cristini F, Losito AR, Tedeschi S, Cauda R, Bassetti M. Predictors of mortality in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: importance of combination therapy. *Clin Infect Dis* 2012; 55: 943-950.
  - 22) Patel G, Huprikar S, Factor SH, Jenkins SG, Calfee DP. Outcomes of carbapenem-resistant *Klebsiella pneumoniae* infection and the impact of antimicrobial and adjunctive therapies. *Infect Control Hosp Epidemiol* 2008; 29: 1099-1106.
  - 23) Wyres KL, Lam MMC, Holt KE. Population genomics of *Klebsiella pneumoniae*. *Nat Rev Microbiol* 2020; 18: 344-359.
  - 24) Lai YC, Yang SL, Peng HL, Chang HY. Identification of genes present specifically in a virulent strain of *Klebsiella pneumoniae*. *Infect Immun* 2000; 68: 7149-7151.
  - 25) Mirzaie A, Ranjbar R. Antibiotic resistance, virulence-associated genes analysis and molecular typing of *Klebsiella pneumoniae* strains recovered from clinical samples. *AMB Express* 2021; 11: 122.
  - 26) Grisold AJ, Zarfel G, Strenger V, Feierl G, Leitner E, Masoud L, Hoenigl M, Raggam RB, Dosch V, Marth E. Use of automated repetitive-sequence-based PCR for rapid laboratory confirmation of nosocomial outbreaks. *J Infect* 2010; 60: 44-51.
  - 27) Lin ZW, Zheng JX, Bai B, Xu GJ, Lin FJ, Chen Z, Sun X, Qu D, Yu ZJ, Deng QW. Characteristics of Hypervirulent *Klebsiella pneumoniae*: Does Low Expression of *RmpA* Contribute to the Absence of Hypervirulence? *Front Microbiol* 2020; 11: 436.
  - 28) Liu C, Guo J. Hypervirulent *Klebsiella pneumoniae* (hypermucoviscous and aerobactin positive) infection over 6 years in the elderly in China: antimicrobial resistance patterns, molecular epidemiology, and risk factor. *Ann Clin Microbiol Antimicrob* 2019; 18: 4.
  - 29) El-Mahdy R, El-Kannishy G, Salama H. Hypervirulent *Klebsiella pneumoniae* as a hospital-acquired pathogen in the intensive care unit in Mansoura, Egypt. *Germes* 2018; 8: 140-146.
  - 30) Cubero M, Grau I, Tubau F, Pallarés R, Dominguez MA, Liñares J, Ardanuy C. Hypervirulent *Klebsiella pneumoniae* clones causing bacteraemia in adults in a teaching hospital in Barcelona,

- Spain (2007-2013). *Clin Microbiol Infect* 2016; 22: 154-160.
- 31) Liu YM, Li BB, Zhang YY, Zhang W, Shen H, Li H, Cao B. Clinical and molecular characteristics of emerging hypervirulent *Klebsiella pneumoniae* bloodstream infections in mainland China. *Antimicrob Agents Chemother* 2014; 58: 5379-5385.
- 32) Rastegar S, Moradi M, Kalantar-Neyestanaki D, Ali Golabi D, Hosseini-Nave H. Virulence Factors, Capsular Serotypes and Antimicrobial Resistance of Hypervirulent *Klebsiella pneumoniae* and Classical *Klebsiella pneumoniae* in Southeast Iran. *Infect Chemother* 2019; 51: e39.
- 33) Lam MMC, Wyres KL, Duchêne S, Wick RR, Judd LM, Gan YH, Hoh CH, Archuleta S, Molton JS, Kalimuddin S, Koh TH, Passet V, Brisse S, Holt KE. Population genomics of hypervirulent *Klebsiella pneumoniae* clonal-group 23 reveals early emergence and rapid global dissemination. *Nat Commun* 2018; 9: 2703.
- 34) Wu H, Li D, Zhou H, Sun Y, Guo L, Shen D. Bacteremia, and other body site infection caused by hypervirulent and classic *Klebsiella pneumoniae*. *Microb Pathog* 2017; 104: 254-262.
- 35) Russo TA, Olson R, Fang CT, Stoesser N, Miller M, MacDonald U, Hutson A, Barker JH, La Hoz RM, Johnson JR. Identification of Biomarkers for Differentiation of Hypervirulent *Klebsiella pneumoniae* from Classical *K. pneumoniae*. *J Clin Microbiol* 2018; 56: e00776-18.
- 36) Lin YT, Siu LK, Lin JC, Chen TL, Tseng CP, Yeh KM, Chang FY, Fung CP. Seroepidemiology of *Klebsiella pneumoniae* colonizing the intestinal tract of healthy Chinese and overseas Chinese adults in Asian countries. *BMC Microbiol* 2012; 12: 13.
- 37) Ku YH, Chuang YC, Chen CC, Lee MF, Yang YC, Tang HJ, Yu WL. *Klebsiella pneumoniae* Isolates from Meningitis: Epidemiology, Virulence and Antibiotic Resistance. *Sci Rep* 2017; 7: 6634.
- 38) Yeh KM, Kurup A, Siu LK, Koh YL, Fung CP, Lin JC, Chen TL, Chang FY, Koh TH. Capsular serotype K1 or K2, rather than magA and rmpA, is a major virulence determinant for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan. *J Clin Microbiol* 2007; 45: 466-471.
- 39) Enani MA, El-Khizzi NA. Community acquired *Klebsiella pneumoniae*, K1 serotype. Invasive liver abscess with bacteremia and endophthalmitis. *Saudi Med J* 2012; 33: 782-786.
- 40) Melot B, Brisse S, Breurec S, Passet V, Malpote E, Lamaury I, Thiery G, Hoen B. Community-acquired meningitis caused by a CG86 hypervirulent *Klebsiella pneumoniae* strain: first case report in the Caribbean. *BMC Infect Dis* 2016; 16: 736.
- 41) Khaertynov KS, Anokhin VA, Rizvanov AA, Davidyuk YN, Semyenova DR, Lubin SA, Skvortsova NN. Virulence Factors and Antibiotic Resistance of *Klebsiella pneumoniae* Strains Isolated from Neonates with Sepsis. *Front Med (Lausanne)* 2018; 5: 225.
- 42) Remya PA, Shanthi M, Sekar U. Characterisation of virulence genes associated with pathogenicity in *Klebsiella pneumoniae*. *Indian J Med Microbiol* 2019; 37: 210-218.
- 43) Compain F, Babosan A, Brisse S, Genel N, Audo J, Ailloud F, Kassis-Chikhani N, Arlet G, Decré D. Multiplex PCR for detection of seven virulence factors and K1/K2 capsular serotypes of *Klebsiella pneumoniae*. *J Clin Microbiol* 2014; 52: 4377-4380.
- 44) Shankar C, Nabarro LE, Anandan S, Ravi R, Babu P, Munusamy E, Jeyaseelan V, Rupali P, Verghese VP, Veeraraghavan B. Extremely High Mortality Rates in Patients with Carbapenem-resistant, Hypermucoviscous *Klebsiella pneumoniae* Blood Stream Infections. *J Assoc Physicians India* 2018; 66: 13-16.
- 45) Li W, Sun G, Yu Y, Li N, Chen M, Jin R, Jiao Y, Wu H. Increasing occurrence of antimicrobial-resistant hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* isolates in China. *Clin Infect Dis* 2014; 58: 225-232.