Carbapenem-Resistant *Klebsiella pneumoniae*: Diversity, Virulence, and Antimicrobial Resistance

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**Background:** *Klebsiella pneumoniae* (*K. pneumoniae*) is one of the most important pathogens in nosocomial infections. It has resistance to most antibiotics, even carbapenem, resulting in restricted therapeutic options.

**Purpose:** We tried to assess the antimicrobial resistance and virulence fitness of carbapenem-resistant *K. pneumoniae* (CRKP) in addition to their phenotypic and genotypic diversity.

**Materials and Methods:** The conventional methods, automated Vitek-32 system, and antimicrobial susceptibility pattern were used to detect CRKP isolates. Virulence and resistance genes profiles were created by using PCR technique. The correlation analysis was done by using R-program.

**Results:** The antimicrobial resistance profile for all our *K. pneumoniae* isolates was shocking as the MDR and CRKP were the most prominent phenotypes. Unfortunately, high degrees of heterogeneity among our CRKP isolates were recorded, as 97.5% of them were differentiated into different clusters. We found a negative correlation between the existence of virulence and antimicrobial resistance genes. In contrast to sputum and urine CRKP isolates, the blood isolates showed high antimicrobial resistance and low virulence fitness. Finally, *K. pneumoniae* creates several outbreaks and crises in Egypt owing to the highly heterogeneity and the wide spread of multidrug-resistant (MDR) and multi-virulent CRKP phenotypes.

**Conclusion:** Our results are significant and alarming to health organizations throughout the world for the severity and heterogeneity of *K. pneumoniae* infections. Therefore, the traditional method for treatment of CRKP infections must be renewed. Additionally, the treatment protocols must be well correlated with the site of infections, phenotypes, and genotypes of CRKP strains.

**Keywords:** *K. pneumoniae*, virulence genes, heterogeneity, multidrug resistant, MDR, carbapenem resistance *K. pneumoniae*, CRKP

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**Introduction**

The wide spread of antimicrobial resistance among both bacteria and fungi creates many worldwide crises and leads to treatment failure. 1–3 This issue is common among the infected persons with *Klebsiella pneumoniae* (*K. pneumoniae*). The *K. pneumoniae* is an opportunistic, Gram-negative pathogen that is often associated with various hospital-related infections. 4 It can cause many nosocomial infections including bloodstream infections (BSIs). The increasing prevalence of antimicrobial drug resistance is an overstated problem, especially in intensive care units (ICUs) by increasing vast amounts of resistance mechanisms, leading to high mortality and morbidity rates. 5,6 Unfortunately, BSI with multi-drug resistance (MDR) and carbapenem-resistant *K. pneumoniae* (CRKP) cause limitation of treatment options. 7,8 One of the most important crises is the worldwide spread of the carbapenem-resistant *K. pneumoniae* (CRKP) during the last decade. 9
On the other hand, several studies have reported that carbapenem-resistant hypervirulent *K. pneumoniae* (hvKP) isolates have emerged in the healthcare setting and can cause severe infections.\(^{10,11}\) The HvKP strains can spread to unusual sites causing severe conditions including meningitis, endophthalmitis, and pyogenic liver abscesses.\(^{12}\) Several mechanisms of *K. pneumoniae* antimicrobial resistance have been announced, and one of the most important ones is antibiotic efflux pumps.\(^{13,14}\) Efflux of the antimicrobial agent leads to a decrease in its intracellular concentration, which can augment bacterial survival.\(^{15}\) The multidrug efflux pump system (*AcrAB*) was significantly correlated with the presence of extensive resistance in *K. pneumoniae* isolates.\(^{16,17}\) The *K. pneumoniae* produces two classic trimeric porins (*OmpK35* and *OmpK36*) which allow the passage of small hydrophilic molecules such as antibiotics through the outer cell membrane. Therefore, loss of porins (*OmpK35* and *OmpK36*) led to an increase in carbapenem, chloramphenicol, and ciprofloxacin resistance.\(^{18}\)

Specification of bacterial virulence may require the existence of a single and sometimes multiple virulence factors. Accordingly, several factors are associated to hyper-virulence and pathogenicity of *K. pneumoniae* such as capsule serotype. The capsule production can be triggered by regulator genes (*rmpA, wabG, and uge*) and mucoviscosity-associated gene A (*magA*).\(^{19}\) Other virulence-encoded genes include *acrAB, ompK*, and *mdtK* for efflux pump system and *fimH-1* for adhesion and biofilm formation.\(^{20,21}\) The *K. pneumoniae* can attach to biotic as well as abiotic surfaces through Type 3 pilus (T3P). The T3P is one of the most important *K. pneumoniae* virulence arrays which is associated with *mrk* operon.\(^{22–24}\) The aim of this study was to highlight the increase in prevalence and heterogeneity of multidrug-resistant (MDR) and multi-virulent CRKP strains and the correlation analysis between antimicrobial resistance and virulence profiles in addition to the sample types.

**Materials and Methods**

**Ethical Statement**
The study was conducted according to the guidelines of the World Medical Association Helsinki Declaration for studies on human subjects. It was approved by the Institutional Review Board (IRB) (No. 202009376) of Al-Azhar University, and written informed consents were obtained from the patients.

**Sample Collection**
This report is a retrospective study, which was conducted on three hundred blood, sputum, and urine samples (100 for each without duplications). All our samples were collected from random critical patients with respiratory problems, who were admitted to the ICU of the internal medicine department, Al-Zahraa University Hospital, Cairo, Egypt, over a period of six months from March 2019 to August 2019. Out of 300 patients, 175 of them were males and 125 were females, and their age ranged from 25 to 78 years. All patients had variable clinical diagnosis, but most cases were community-acquired pneumonia (34%) and 51% of all cases presented with septic shock.

**Phenotypic Identifications of *Klebsiella pneumoniae***
The brain heart infusion broth (Oxoid, UK) was used as enrichment medium for all our collected samples to enhance the growth of the bacterial isolates. After 24 h of incubation, one loopful from each tube was inoculated into blood agar media (Oxoid, UK), and then the plates were incubated at 37 °C for 24 h. Based on culture and other biochemical characteristics such as catalase, oxidase, gelatin liquefaction, urease, IMViC, TSI, and O/F test,\(^{25,26}\) all *K. pneumoniae* suspected isolates were collected for further investigation. All *K. pneumoniae* isolates were confirmed by using API 20E strips (BioMérieux, Mary l’Etoile, France) and Vitek-32 System (Bio Merieux- France).

**Antimicrobial Susceptibility Testing**
Antimicrobial susceptibility testing was done for all common 14 prescribed antimicrobial drugs (Oxoid, UK), namely amoxicillin/clavulanic acid (AMC; 20/10 µg), gentamicin (CN; 10 µg), amikacin (Ak; 30 µg), ciprofloxacin (CIP; 5 µg), levofloxacin (LEV; 5 µg), cefuroxime (CXM; 30 µg), ceftiraxone (CTX; 30 µg), aztreonam (ATM; 30 µg) ceftazidime (CAZ; 30 µg), colistin (CO; 10 µg), piperacillin/tazobactam (TZP; 110 µg), imipenem (IPM; 10 µg), meropenem
(MEM; 10 µg), and ertapenem (ETP; 10 µg), by disc diffusion method as described in Clinical and Laboratory Standards Institute (CLSI guidelines, 2018), and confirmed using an automated system (Vitek-32 System, Bio Merieux, France).

Detection of Extended Spectrum β-Lactamase (ESBL)-Producing Isolates
All K. pneumoniae strains were tested for β-lactamase production according to CLSI, 2018. The production of extended spectrum β-lactamase (ESBL) was assessed on all positive β-lactamase isolates by using a modified double disc synergy test (MDDST). In this test, amoxicillin-clavulanate disc was used alongside 3 cephalosporins from third generation (cefotaxime, ceftriaxone, cefpodoxime) and one from fourth generation (cefepine). The potentiation or augmentation ≥5 mm of an inhibition zone of the cephalosporin discs towards the amoxicillin-clavulanate discs was considered positive for ESBL production.

Detection of Carbapenemase Activity
All tested isolates were subjected to modified Hodge test using a meropenem disc (10 µg) (Oxoid, Basingstoke, UK). The clover leaf-like appearance between the test streaks near the disc was taken as positive for carbapenemase production.

Molecular Characterization
DNA extraction from the isolates was performed using the QIAmp DNA extraction minikit (QIAGEN Hilden, Germany) as per the manufacturer’s instructions.

Antimicrobial Resistance Gene Detections
The blaKPC, blaNDM, and blaOXA-48 genes were detected using multiplex PCR technique; meanwhile the uniplex PCR was used to detect the blaCTX-M gene. The reaction mixture consisted of 5 µL of the extracted DNA, and 2 µL from the primers forward and reverse; 13 µL of distilled water was added to the GoTaq® Green Master 2× Ready Mix (Promega, USA). The primers and the size of amplicons were collected in Table 1.

Virulence Gene Detections
The virulence gene profiles were detected by using both uniplex and multiplex PCR techniques. The reaction mixture composition and PCR programs for amplification were performed according to the previous studies. The specific primer and the amplicon sizes were listed in Table 1. The positive and negative controls were included in each run. The amplicons were separated in 1.5% agarose gel and purified with the use of the Gene JET Gel Extraction Kit (Thermo Scientific, USA) according to the manufacturer’s recommendations. Electrophoresis gel (1.5% agarose stained with 0.5 µg/mL of ethidium bromide) was used to separate the amplified PCR products. The ultraviolet transilluminator (Spectroline, USA) was used to visualize the amplicons and then photographed.

Statistical Analysis
The R package corrplot, heatmaply, hmisc, and ggpur and GraphPad Prism (version 6; GraphPad Software Inc.; San Diego, CA, USA) were used to construct all dendrograms and heatmaps and to assess all correlation and statistical analyses between antimicrobial resistance and virulence profile in this study. The cutoff point for the significant values were considered at p-values <0.05.

Results
Characterization of Klebsiella pneumoniae Isolates
Based on the culture, biochemical, API, and Vitek-32 characterization methods, 90 confirmed K. pneumoniae isolates were recovered from 300 samples with a prevalence rate equal to 30%. With observation of case clinical outcome, the mortality rate was 61%, and 39% had clinical improvement. The mean duration of ICU admission was 21 days (Supplementary Table 1).
Antimicrobial Susceptibility Testing of K. pneumoniae Isolates

High resistance rates were observed for all tested antimicrobial drugs. The fluoroquinolone-resistant isolates were recorded with high prevalence rate (84.4%, 76/90); meanwhile, the lowest resistance was found against imipenem (34.4%, 31/90) as shown in Figure 1. Fortunately, all our isolates were sensitive to colistin as shown in Figure 1. Out of 90 K. pneumoniae isolates, 41 (45.5%) of them were carbapenem-resistant K. pneumoniae (CRKP), which showed resistance to one agent belonging to carbapenem class. Of note, all CRKP isolates were multidrug-resistant (MDR) isolates.

Detection of Carbapenemase and ESBL Producing Isolates

Screening of ESBL production revealed that 34.4% (31/90) of the isolates were positive for phenotypic identification of ESBL; 35.5% (32/90), of our isolates were carbapenemase-producing isolates according to the phenotypic detection of carbapenemase using MHT test.

Genotypic Detection of Antimicrobial Resistance and Virulence Genes

The blaCTX-M gene was detected in high prevalence followed by blaOXA (75.6% and 46.3%, respectively); meanwhile, the lowest prevalence antimicrobial resistance genes were blaNDM and blaKPC (34.1% and 9.8%, respectively) as shown in Figure 2. Regarding the existence of virulence genes among all CRKP isolates as shown in Supplementary Figure 1, 97.56% of the CRKP isolates were positive for ompK35 gene, which is responsible for porin loss. Of note, 34.15% of the tested isolates harbored ompK36 gene. Interestingly all our isolates harbored the acrAB gene; meanwhile, the mdtK gene could not be detected in any of our CRKP isolates. The ability of CRKP to form a capsule was assessed by examining the prevalence of capsule-associated genes (rmpA, wabG, and uge), and these genes were commonly distributed among tested isolates and ranged from 43% to 61%. Additionally, the fimH1 gene was amplified in 85.37% of the CRKP isolates as shown in Figure 2.

### Table 1 Nucleotide Sequences and Amplicon Size of Oligonucleotides Primers

| Gene   | Sequences                                      | Annealing Temperature | Amplicon Size (bp) | References |
|--------|------------------------------------------------|-----------------------|--------------------|------------|
| blCTX-M | F: ATGGTTAAAAAAATCACTGCGYC R: TTCAAAACCGTCGGTG | 51°C                  | 876                | [26]       |
| blOXA   | F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTTCAACCACCC | 45°C                  | 438                | [27]       |
| blKPC   | F: CGTCTAGTTCTGGTGTTTG T: CTGTGATCCCTTGGTTAGC   | 45°C                  | 798                | [27]       |
| blNDM   | F: GGTGTTGCGATCTGTTTTTC R: CGGAATGCGATCACTGAC  | 45°C                  | 621                | [27]       |
| RmpA    | F: ACTGGGTCTACCTTGCTTCA R: CCATGAGCCCATTTCTTCA  | 53°C                  | 535                | [28]       |
| WabG    | F: ACCATCGGCGCCATTAGA R: CGGACTGCAGATCCATATC   | 58°C, 683             |                    |            |
| uge     | F: TCTTCACGCGTCTTCCTCACT R: GATCATCCGTCCTCCCTGTA | 58°C, 534             |                    |            |
| fimH1   | F: GCAAAGCGCTAGGTAAACCTGG R: ATATTCGACGCTGCGTAAA | 43°C                  | 180                | [20]       |
| AcrAB   | F: ATCGACGCGCGAGTGTGAAA R: CGGGTTCGGGAAATAGCGCG | 53°C                  | 312                | [20]       |
| mdtK    | F: GCGCTTTACCTGCACTCAG T: GATGATAAATCCACCCAGAA | 43°C                  | 453                | [20]       |
| OmpK35  | F: GCCAGCTCCTAAACGTAGCC R: GGTCCTGAACGCGTGATGG  | 51°C                  | 241                | [20]       |
| OmpK36  | F: GAATTATACAAAGACGCGG R: GACGTTACGTCGTATACCTAG | 43°C                  | 305                | [20]       |
Virulence Factor Coexistence Among CRKP Isolates

All our CRKP isolates showed multi-virulence profiles. The most common coexistence profile showed the presence of seven virulence genes (rmpA, rap, uge, fimh-1, acrAB, ompK35, ompK36) in 34.15% of the CRKP isolates as shown in Figure 2.

Heterogeneity of CRKP Based on the Discriminatory Power of All Typing Methods

Regarding the phenotypic and genotypic characterization of all CRKP isolates and based on the resistance profiles and the existence of both antimicrobial resistance and virulence genes as shown in Supplementary Figure 1, all our isolates were differentiated into different lineages with the exception of two urine isolates (code U1 and U7). According to our results the combined typing methods in this study showed high discriminatory power (D-value = 0.998), which reflects the heterogeneity of our isolates as shown in Figures 2 and 3.

Correlation Analysis of CRKP Isolates

The blood and urine meropenem-resistant isolates were more common in contrast to sputum meropenem-resistant isolates. This is shown in Figure 4, with resistance to meropenem negatively correlated with sputum isolates (r-value = −0.4); meanwhile, weak positive correlation with blood and urine isolates was seen (r-value = 0.2 for each). In contrast to blood isolates, the urine and sputum isolates showed higher resistance to amikacin, amoxicillin/clavulanic acid, and gentamicin. This could be illustrated by the weak positive correlation between resistance to amikacin, amoxicillin/clavulanic acid, and gentamicin and both urine and sputum isolates (r-value = −0.1) and the weak negative correlation between blood isolates and resistance to amikacin, amoxicillin/clavulanic acid, and gentamicin (r-value = 0.1) as shown in Figure 4. For that the amikacin.

As was expected, there is a cross-resistance between ETP, IPM, and MEM, which was confirmed by their positive correlation in Figure 4 (r-value = −0.3: 0.6). Of note, the blood isolates showed positive correlation with the presence of antimicrobial resistance genes blaOXA, blaCTX, blaNDM, and blaKPC (r-value ≥ 0.1) and negative correlation with the existence of virulence genes ompK36, wap, and uge (r-value ≤ −0.1). On the other hand, the sputum isolates were negatively correlated with antimicrobial resistances (blaOXA, blaCTX, and blaKPC) and positively correlated with
Figure 2 Hierarchical clustering of CRKP isolates, according to the generated antimicrobial resistance patterns, virulence, and resistance genes profiles. Blue and red colors indicate the absence and presence of genes or resistance to antimicrobials, respectively. The code numbers on the right side of the heat map denote the CRKP from urine (U), blood (B), and sputum (S).
virulence genes (ompk35, fimH, wap, and uge). Regarding the urine isolates, there was a negative correlation between them and the occurrence of antimicrobial resistances genes (blaOXA, blaCTX) and a positive correlation with the presence of virulence genes (ompk35, rmp, fim, ompk36, wap, and uge). Therefore the blood isolates showed high antimicrobial resistance and low virulence fitness in contrast to sputum and urine isolates which were more virulent and in which less resistance was seen, as shown in Figure 4.

Generally, with few exceptions, a negative correlation was seen between the existence of both antimicrobial resistance genes (blaOXA, blaCTX, and blaNDM) and virulence genes (ompk35, rmp, fimH, ompk36, wap, and uge). Interestingly, there was a strong positive correlation between the existence of uge and wap (r-value =1) as shown in Figure 4.

**Discussion**

Over the past decades, the wide spread of MDR side by side with multi-virulent pathogens has caused several public panics.33–35 Regarding the pathogenic *K. pneumoniae* strain, it can cause a wide variation of hospital-acquired infections,36 particularly bloodstream infections.37 The extensive use of antimicrobial agents especially against hypervirulent *K. pneumoniae* in the hospital settings has led to high incidence of resistance.37 The crises of the infections with *K. pneumoniae* especially CRKP were announced in several reports.38,39 High level of resistance among *K. pneumoniae* specifically bloodstream infections was described in this study as well as several other studies in Egypt.36,40 A positive correlation between the existence of antimicrobial resistance genes and blood *K. pneumoniae* isolates was announced. This is due to lack of strict antimicrobial stewardship policy in Egypt. The emergence of antibiotic resistance has been caused by many factors, including the achievement of resistance genes, transfer of antibiotic resistance genes, immunosuppressed
states, healthcare exposure, limited diagnostic facilities, use of indwelling medical devices, lack of effective and reliable surveillance systems, lack of new antimicrobial therapeutics, as well as inappropriate and excessive antibiotic use in health care, food-producing animals, and agriculture.\textsuperscript{41–43} Surprisingly, CRKP isolates were detected with high prevalence among our isolates. Therefore, the last therapeutic option (carbapenems) for the treatment of MDR Gram-negative bacteria became inefficient especially for bloodstream infections. The emergence of CRKP which showed resistance to colistin was recorded in several reports.\textsuperscript{44,45} The bright spot in our results was the absence of colistin-resistant phenotypes, which have high lethality. Alarmingly, an extensive variability was recorded among our isolates. In accordance to this result, \textit{K. pneumoniae} strains in United States and in Taiwan exhibit high heterogeneity.\textsuperscript{46,47} This heterogeneity creates several troublesome problems in tracking of the infections sources, therefore there is an urgent need to modify the infection control guideline, as well as to discover new alternative therapies.\textsuperscript{47–49}

The relation between antimicrobial resistances and the virulence factors has gained attention of several clinicians. This correlation depends on several factors including the mechanisms of resistance and virulence, host, type of pathogens, and the ecological niche.\textsuperscript{50} A negative correlation between the existence of antimicrobial resistance genes and virulence genes was recorded in this study. The blood isolates showed high antimicrobial resistance and low virulence fitness in contrast to sputum and urine isolates which were more virulent and less resistant. Several reports announced that the strains with high virulence arrays showed a sensitivity pattern to most antimicrobial drugs and vice versa.\textsuperscript{50} This hypothesis was explained depending on the genetic capacity of the bacterial genome, as the acquisition of resistance gene is done at the expense of the virulence gene.\textsuperscript{2,51}
The morbidity and mortality rates of CRKP infections can be reduced by avoiding the treatment failure through the proper selection of antimicrobial therapies. Several factors can affect the treatment protocols, which can be divided into therapy-related factors (side effect, route of administration, and other physical factors); patient-related factors (age, gender, and health states); and disease factors (severity and site of infections). The CRKP causes several outbreaks and can infect several sites such as urinary tract, surgical site, bloodstream, and respiratory system. In our reports, meropenem and ertapenem were the most effective drugs for treating pulmonary and urinary CRKP infections; meanwhile, amikacin, amoxicillin/clavulanic acid, and gentamicin were the best choices for treating the CRKP bloodstream infections. Therefore, we did not recommend the use of meropenem and ertapenem for treating bloodstream CRKP infections. Confirmatory to our recommendations, meropenem-resistant phenotypes were common among bloodstream CRKP infections, in contrast to respiratory infections. In another study, ceftazidime–avibactam was a promising therapeutic option for the treatment of CRKP-induced pneumonia. This variation in the susceptibility patterns may be attributed to the difference in the prescribed antibiotics from certain geographic areas to others and heterogeneity of CRKP strains side by side with the strain variability according to the clinical sites.

Conclusions
The wide spread of CRKP strains and the high genotypic and phenotypic heterogeneity of these strains create compounded threats especially in the health care setting. The traditional method for treatment of CRKP infections must be renewed. We recommended the using of meropenem and ertapenem for treating CRKP pulmonary and urinary infections, respectively; meanwhile, amikacin, amoxicillin/clavulanic acid, and gentamicin were the most suitable drugs for blood CRKP infections. Of note, new therapeutic options and more and advanced restricted infection control guidelines are urgently needed due to the high resistance and heterogeneity of CRKP strains.

Institutional Review Board Statement
Not applicable for this study. Informed consent statement were obtained from all patients.

Data Sharing Statement
All generated data in this report are available in the submitted manuscript.

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Disclosure
There is no conflict of interest with any authors.

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