Genotyping and Virulence Analysis of Drug Resistant Clinical \textit{Klebsiella pneumoniae} Isolates in Egypt

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Abstract

\textit{Klebsiella pneumoniae} is a highly drug-resistant human pathogen responsible for a variety of serious infections. Integrons, mobile genetic elements capable of integrating antibiotic resistance genes, and the capsule are important virulence factors that increase bacteria resistance to phagocytosis and antimicrobial agents. Molecular typing is an effective tool for identifying the likely etiology of infection. This study aimed to investigate the presence of the \textit{rmpA}, \textit{wcaG}, \textit{intI1}, \textit{intI2}, and \textit{intI3} virulence genes in clinical \textit{Klebsiella pneumoniae} isolates, and explore their molecular genotypes by using ERIC-PCR. Fifty \textit{Klebsiella pneumoniae} strains were isolated from various specimens. Antimicrobial resistance was evaluated by using the disc diffusion method. Five genes were amplified by conventional PCR. Genotyping was performed molecularly by using ERIC-PCR. Forty-seven isolates were multi-drug resistant. In all, 18%, 36%, and 98% of the 50 \textit{K. pneumoniae} isolates were positive for \textit{rmpA}, \textit{wcaG}, and \textit{intI1} genes, respectively; however, all isolates were negative for \textit{intI2} and \textit{intI3} genes. Dendogram analysis of the ERIC-PCR results showed 49 distinct patterns, arranged in five clusters. Our study demonstrates high levels of antibiotic resistance and virulence among clinical isolates of \textit{K. pneumoniae}. Such resistance reflects a growing problem for public health. Further, the presence of integrons increases the horizontal spread of antibiotic resistance and virulence genes among bacterial isolates. The ERIC-PCR technique is an effective method for molecular typing and epidemiological studies of hospital-acquired infections.

Keywords: Egypt, genotyping, integrons, \textit{Klebsiella pneumoniae}, PCR

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INTRODUCTION

Klebsiella pneumoniae (K. pneumoniae) is a Gram-negative rod-shaped highly virulent bacterium, capable of causing serious diseases in humans including, pneumonia, liver abscesses, urinary tract infections, and life-threatening septicemia\textsuperscript{1,3}. The bacterium is a major cause of nosocomial infections in Egypt\textsuperscript{4}. K. pneumoniae currently manifests growing resistance worldwide to several important antimicrobial agents including, beta-lactam antibiotics, fluoroquinolones, and aminoglycosides. This increase in antimicrobial resistance constitutes an arising problem globally\textsuperscript{5}. A primary mechanism for acquisition of resistance of K. pneumoniae is through integrons. These mobile genetic elements integrate and express antibiotic resistance genes. Integrons are carried and transferred by plasmids and transposons; facilitate spread by horizontal gene transfer within bacterial populations\textsuperscript{6}. Five integron classes were defined based on difference in base sequences of integrase enzyme genes (int). Class 1 is the predominant among the five integron classes and is widely detected in Gram-negative bacteria, including K. pneumoniae. Structurally, class 1 integrons consist of two conserved regions: 3’ conserved segment and 5’ conserved segment, in addition to internal gene cassettes coding for antimicrobial resistance genes. Class 2 integrons can be detected occasionally in K. pneumoniae; in contrast, class 3 integrons are rarely documented. More than one hundred and thirty different cassettes that confer resistance against important antibiotic classes e.g.: \beta-lactams, fluoroquinolones, aminoglycosides, and macrolides have been discovered\textsuperscript{7}. The polysaccharide capsule is an important factor for bacterial virulence. The capsule describes bacterial mucoid phenotype and defines resistance to phagocytosis and host defense factors. For example, hypermucoid K. pneumoniae isolates are related to the incidence of invasive syndromes\textsuperscript{8}. The plasmid gene \textit{rmpA} activates transcription of the \textit{wzyKpK1 cps} locus that in turn enhances capsule synthesis in K. pneumoniae\textsuperscript{2}. Moreover, the \textit{wcaG} virulence gene is gene located in transposable chromosomal region. This gene is responsible for synthesis of K. pneumoniae capsules, and is also helps in the conversion of mannose to fucose that may enhance the bacterial resistance to phagocytosis\textsuperscript{9}.

Virulence analysis and molecular typing techniques are robust tools distinguish for identifying the probable source of infection, determining the genetic relationships in nosocomial infection outbreaks, and assisting in management and treatment of MDR \textit{K. pneumoniae} infections\textsuperscript{10}. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) is a molecular technique that is used to estimate genetic diversity among enterobacteriaceae family members. ERIC sequences are 126 bp long, non-coding, and conserved. ERIC sequences occur in variable positions and numbers in bacteria, and the ERIC technique can be used to evaluate genetic differences among bacterial isolates. The use of the ERIC-PCR for investigation of the diversity of bacterial isolates is rapid, sensitive, and consistent\textsuperscript{11,12}.

This study was aimed to investigate the presence of the \textit{rmpA}, \textit{wcaG}, and class 1, 2, and 3 integron virulence genes in clinical K. pneumoniae isolates, and to assess correlations among antibiotic resistance and virulence genes. Moreover, our isolates were molecularly genotyped by using the ERIC-PCR technique. MATERIALS AND METHODS Sample collection, isolation, and identification

Seventy-five different samples were collected from patients at Damanhour General Hospital, El-Behira, Egypt from February to April 2018. The samples were collected from wound swabs, sputum, blood, endotracheal tube (ETT) aspirates, urine, and bedsore swabs. Samples were cultured on MacConkey agar plates for the detection of \textit{K. pneumoniae}. Lactose fermenting mucoid colonies were Gram stained and subjected to several biochemical tests, including triple sugar iron agar, indole, methyl red, Voges Proskauer, citrate, oxidase, and catalase\textsuperscript{13}. The identification of \textit{K. pneumoniae} isolates was confirmed to the species level by using the automated vitek 2 system (Bio-Merieux, l’Etoile, France).

\textbf{Antibiotic susceptibility testing}

The antibiotic resistance was determined by using the standard disc agar diffusion technique according to Bauer et al.\textsuperscript{14}. Fifteen commercially available antibiotic discs representing different antibiotic classes were used to assess resistance to ampicillin (AMP 10µg), amoxicillin/clavulanic acid,
(AMC 30µg), imipenem (IPM 10µg), cefuroxime (CXM 30µg), cephaloridine (CAZ 30µg), ceftriaxone (CRO 30µg), cefoxitin (TE 5µg), chloramphenicol (C 30µg), gentamicin (CN 10µg), aztreonam (ATM 30µg), amikacin (AKN 30µg), ciprofloxacin (CIP 5µg), cotrimoxazole (SXT 25µg), and colistin (CT 10µg) (Oxoid® Ltd, England). Isolates were cultured on Mueller Hinton agar plates with antibiotic discs and incubated at 37°C for 24 hrs. The diameter of inhibition zones was measured in millimeters (mm). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

**DNA extraction**

DNA was extracted directly by using the boiling method with some modifications. Briefly, 4–5 pure bacterial colonies of each isolate cultivated on nutrient agar plates were suspended in 500 μl of sterile water in a sterile Eppendorf tube, and heated at 95°C for 10 min. Bacterial suspensions were cooled on ice for 5 min, then centrifuged at 14,000 rpm for 5 min. The supernatants were transferred to sterile Eppendorf tubes and stored at -20°C.

Molecular detection of *wcaG*, *rmpA*, and class 1, 2, and 3 integrons genes by conventional PCR in our clinical *K. pneumoniae* isolates

All the DNA extracts were tested for five virulence genes (*rmpA*, *wcaG*, *intI1*, *intI2*, and *intI3*) by using a thermal cycler (Veriti, Applied Biosystems, Lincoln Foster City, California, USA) and five pairs of primers (Table 1). PCR amplicons were then resolved on 1.5% agarose gel stained with ethidium bromide, and visualized via ultraviolet illumination. Cycling conditions were:

| Gene      | Primers' sequence                      | Amplicon size (bp) |
|-----------|----------------------------------------|--------------------|
| rmpA      | rmpA-F: ACTGGGGCTACCTCTGCTTCA          | 516 bp             |
|           | rmpA-R: CTTGCATGAGCCATCTTTTCA          |                    |
| wcaG      | wcaG-F: GGTTGGGTCAGCAATCGTA            | 169 bp             |
|           | wcaG-R: ACTATTCCGCCAACTTT TGC         |                    |
| intI1     | intI1-F: CAGTGGACATAAGCCTGTTC          | 160 bp             |
|           | intI1-R: CCCGAGGCATAGACTTA             |                    |
| intI2     | intI2-F: GTACGAAAACGAGTGACGAATAAGT     | 789 bp             |
|           | intI2-R: CACGGATATGCGAACAAAGGT         |                    |
| intI3     | intI3-F: GCCCTCCGCCACGGACCTTTTCA       | 979 bp             |
|           | intI3-R: ACGG ATCTGCCAAACCTGACT        |                    |

**RESULTS**

Fifty (66.7%) of 75 collected clinical samples were initially identified as *Klebsiella* species by their growth appearance on MacConkey agar plates and their morphological and biochemical characteristics. Mucoid lactose fermenting colonies appeared as Gram negative rods upon Gram staining. Biochemically, all isolates were oxidase negative, catalase positive, indole negative...
negative, methyl red negative, Voges Proskauer positive, and citrate positive. On triple sugar iron agar slants, all isolates produced acid butt and slant with gas and no H2 S. These samples were confirmed as *K. pneumoniae* by using the automated vitek 2 system.

Twenty-seven (54%) of the fifty *K. pneumoniae* isolates were obtained from men and 23 (46%) from women. Five (10%) isolates were obtained from wound swabs, 12 (24%) from sputum, 9 (18%) from blood, 6 (12%) from endotracheal tube (ETT) aspirates, 16 (32%) from urine, and 2 (4%) from bedsore swabs.

Forty-seven (94%) were multi-drug resistant (resistant to more than 3 different antibiotic classes). All (100%) isolates were resistant to ampicillin and ceforuxime. Isolates were highly resistant to amoxicillin/clavulanic acid, aztreonam, ceftriaxone, and ceftazidime. Conversely, all (100%) isolates were sensitive to colistin (Fig. 1). Eight resistance patterns were detected among the 50 *K. pneumoniae* isolates (Table 2).

Further, nine (18%) of the 50 *K. pneumoniae* isolates were positive for the *rmpA* gene and 18 (36%) for the *wcaG*. Forty-nine (98%) isolates were positive for the *intI1* gene (Fig. 2 and 3); however, all (100%) *K. pneumoniae* isolates were negative for *intI2* and *intI3* genes. Only one isolate (no. 47) was negative for all genes.

### Table 2. Antibiotic resistance patterns of the 50 *K. pneumoniae* isolates

| The resistance pattern | The antibiotics | The number of isolates N (%) |
|------------------------|-----------------|-----------------------------|
| A1                     | CN-TE-IPM-FEP-ATM-AMP-AMC-CRO-CAZ-CXM-AKN-CIP-SXT | 7 (14%)                     |
| A2                     | IPM-CN-FEP-ATM-AMP-AMC-CRO-CAZ-CXM-AKN-CIP-SXT  | 5 (10%)                     |
| A3                     | C-TE-IPM-FEP-ATM-AMP-AMC-CRO-CAZ-CXM-CIP-SXT    | 4 (8%)                      |
| A4                     | IPM-FEP-ATM-AMP-AMC-CRO-CAZ-CXM-AKN-CIP-SXT    | 11 (22%)                    |
| A5                     | TE-IPM-FEP-ATM-AMP-AMC-CRO-CAZ-CXM- CIP-SXT     | 4 (8%)                      |
| A6                     | C-TE-FEP-ATM-AMP-AMC-CRO-CAZ-CXM-SXT            | 2 (4%)                      |
| A7                     | FEP-ATM-AMP-AMC-CRO-CAZ-CXM-SXT                 | 2 (4%)                      |
| A8                     | AMP-AMC-CXM                                       | 2 (4%)                      |
The remaining forty-nine isolates showed four virulence patterns (Table 3).

Significant correlations were found between the intI1 gene and resistance to aztreonam and ceftriaxone (p-value ≤ 0.05), and between the rmpA gene and resistance to gentamicin and amikacin (p-value ≤ 0.05). No significant correlations were observed between the wcaG gene and resistance to any antibiotic (p-value < 0.05).

The 49 genotypically positive isolates were genotyped by using ERIC-PCR, followed by analysis with TotalLab Quant Analysis software (Version 1.0., TotalLab Ltd. United Kingdom). This analysis used the Dice method for comparison and the UPGMA method for clustering. Dendogram analysis of K. pneumoniae isolates showed forty-nine distinct patterns, arranged in five clusters. These clusters contained 11, 11, 10, 6, and 11 of the 49 K. pneumoniae isolates, respectively (Fig. 4).

A significant correlation was found between the presence of the wcaG gene and ERIC genotypes (p-value ≤ 0.05), however, no such correlation was found for rmpA or intI1 (p-value < 0.05).

**DISCUSSION**

*Klebsiella pneumoniae* is a dangerous pathogen that causes serious wound, urinary tract, respiratory tract infections, meningitis, and bacteremia. *K. pneumoniae* is a common cause of nosocomial infections, and serious outbreaks are reported in hospitals along with increased

**Table 3. Virulence patterns of the 49 K. pneumoniae isolates**

| The virulence pattern | Isolate codes | Number of isolates N (%) | Genes |
|-----------------------|---------------|--------------------------|-------|
| V1                    | 4, 9 and 10   | 3 (6%)                   | positive for rmpA, wcaG and intI1 genes |
| V2                    | 26, 33, 38, 41, 42 and 46 | 6 (12%) | positive for rmpA and intI1 genes |
| V3                    | 1, 3, 5, 6, 7, 8, 12, 16, 17, 18, 19, 21, 22, 24 | 15 (30%) | positive for wcaG and intI1 genes and 31 |
| V4                    | 2, 11, 13, 14, 15, 20, 23, 25, 27, 28, 29, 30, 32, 34, 35, 36, 37, 39, 40, 43, 44, 45, 48, 49 and 50 | 25 (50%) | positive for intI1 gene |

**Fig. 2.** Detection of the rmpA and wcaG genes on 1.5% agarose gel following amplification with PCR. Lanes 1 and 2: two isolates positive for the wcaG gene detected at 169 bp, lane 3: the DNA ladder, lanes 4, 5, 6, and 8: negative isolates and lane 7: one isolate positive for the rmpA gene detected at 516 bp.
morbidity and mortality rates. The uncontrolled use of antibiotics for treating *K. pneumoniae* infections has resulted in increased bacterial resistance and the emergence of MDR strains\(^{12,17}\).

Forty-seven (94%) of our 50 *K. pneumoniae* isolates were MDR. In contrast, lower percentages (54%, 38%, 81.5%, and 90%) of MDR were reported in other studies\(^{17-19}\). The high percentage of MDR in our isolates may be due to overuse of antibiotics in Egypt and the incidence of class 1 integrons in 98% of our isolates. These integrons spread antibiotic-resistant genes by horizontal gene transfer. High resistance percentages ranging from 92% to 100% were exhibited for penicillins and cephalosporins, including ampicillin, ceforuxime, amoxicillin/clavulanic acid, ceftazidime, ceftriaxone, and cefepime, and lower percentages were reported in other studies\(^{17,19,20}\). High level of resistance was also displayed in this study to imipenem, co-trimoxazole, aztreonam, and ciprofloxacin; however, lower percentages were reported by other studies\(^{17,19,20}\). Moderate incidence of resistance (54%, 46%, and 40%) was exhibited for amikacin, tetracycline, and chloramphenicol, respectively, in this study. Such incidence is similar to previous reports\(^{19,20}\). Alternatively, 28% of our isolates were resistant to gentamycin; however, a higher percentage (46%) was reported by Tan et al. in Singapore\(^{7,21,22,24}\).

Finally, forty-nine (98%) out of our 50 *K. pneumoniae* isolates were positive for the *intI* gene. Lower percentages (74%, 69%, and 53%) were reported by Derakhshan et al., Zaki et al., and Liao et al.; in contrast, all (100%) isolates of Firoozeh et al. in Iran were positive for this gene\(^{7,9,22,25}\). Nonetheless, Derakhshan et al. in Iran and Liao et al. reported that 1% and 2% of their isolates, respectively, were positive for class 2 integrons\(^{9,25}\). In addition, Firoozeh et al. in Iran reported that 37% of their isolates were positive for this gene\(^{7}\). Similarly, none of our isolates was positive for the *intI2* gene and resistance to aztreonam and ceftriaxone (p-value ≤ 0.05). In contrast, Derakhshan et al. reported a positive association between the *intI* gene and resistance to ceftaxime, ceftriaxone, ceftazidime, amoxicillin-clavulanic acid, aztreonam, ciprofloxacin, tobramycin, tetracycline, co-trimoxazole, gentamicin, and cefepime. In our study, significant correlations were also found between presence of the *rmpA* gene and resistance to gentamicin and amikacin (p-value ≤ 0.05); however, Derakhshan et al. reported a positive association between this gene and resistance to amoxicillin-clavulanic acid, tobramycin, and gentamicin. In contrast, we observed no significant correlations between

**Fig. 3.** Detection of the *intI* gene on 1.5% agarose gel following amplification with PCR. Lanes 1, 2, 3, 4, 6, and 7: six isolates positive for the *intI* gene detected at 160 bp and lane 5: the DNA ladder.
the wcaG gene and resistance to any antibiotic (p value < 0.05); nevertheless, Derakhshan et al. reported a positive association between the gene and resistance to all antibiotics assessed, except imipenem. Significant correlations confirm that virulence genes rmpA and intI1 in the bacterial genome often co-occur antimicrobial resistance.

The diversity in the ERIC patterns observed in the study may reflect the non-clonal distribution of virulent *K. pneumoniae* strains. Our data support the results of Wasfi et al. as their Dendrogram analysis of ERIC genotyping revealed 21 distinct patterns among 28 isolates divided into three clusters (A–C) containing 12/28, 9/28, and 7/28 of the isolates, respectively. Similarly, Zhang et al. used ERIC-PCR to reveal 60 different distinct patterns among their 62 strains, and Mehr et al. reported 32 different ERIC profiles among their 35 isolates. Such genetic variation among *K. pneumoniae* strains was also reported by other studies in Iran, Taiwan, Russia, and Algeria.

In conclusion, our study demonstrates a high incidence of antibiotic resistance and virulence among *Klebsiella pneumoniae* clinical isolates, reflecting the continuing threat to public health that needs to be followed up continuously. The presence of class 1 integrons increases the risk of spreading antibiotic resistance genes and virulence factors through horizontal gene transfer. Moreover, the ERIC-PCR technique is a powerful tool for the identification and characterization of *K. pneumoniae* strains.

**Fig. 4.** Dendrogram generated with Dice coefficient and the UPGMA clustering method, showing the genetic similarity among *K. pneumoniae* isolates by Enterobacterial Repetitive Intergenic Consensus (ERIC) genotyping.
very effective method for molecular typing and epidemiological studies of nosocomial infections.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTION
Prof. Dr. S designed the experiments. Dr. K. Dr. M and Dr. H performed the experiments. Dr. K and Dr. M analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

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None.

ETHICS STATEMENT
The current research has followed the accepted principles of ethical conduct by the Research Ethics Committee of the Faculty of Pharmacy, Damanhour University, and it has been approved. Informed consent from the parents of young patients was obtained prior to undertaking testing and molecular investigation of their specimens.

DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

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