Abdelhamid et al. | J Pure Appl Microbiol | 14(3):1967-1975 | September 2020
Article 6560 | https://doi.org/10.22207/JPAM.14.3.36

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Genotyping and Virulence Analysis of Drug Resistant Clinical *Klebsiella pneumoniae* Isolates in Egypt

Sarah M. Abdelhamid[1], Hala Mohamed Abd-Elaal[2], Moustafa Osama Matareed[3] and Kholoud Baraka[1]*

[1]Microbiology and Immunology Department, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt.
[2]Clinical Pathology Department, National Medical Institute, Damanhour, Egypt.
[3]Directorate of Health, Damanhour, Egypt.

Abstract

***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 *rmpA, wcaG, intI1, intI2, and intI3* virulence genes in clinical *Klebsiella pneumoniae* isolates, and explore their molecular genotypes by using ERIC-PCR. Fifty *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 *K. pneumoniae* isolates were positive for *rmpA, wcaG,* and *intI1* genes, respectively; however, all isolates were negative for *intI2 and 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 *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, *Klebsiella pneumoniae*, PCR

***Correspondence:** Loody_mri@yahoo.com; +201006878989

(Received: July 31, 2020; accepted: September 10, 2020)

Citation: Abdelhamid SM, Abd-Elaal HM, Matareed MO, Baraka K. Genotyping and Virulence Analysis of Drug Resistant Clinical *Klebsiella pneumoniae* Isolates in Egypt. *J Pure Appl Microbiol.* 2020;14(3):1967-1975. doi: 10.22207/JPAM.14.3.36

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[1-3]. The bacterium is a major cause of nosocomial infections in Egypt[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[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[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. pneumonia*e. 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. pneumonia*e; 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.: ß- lactams, fluoroquinolones, aminoglycosides, and macrolides have been discovered[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[8]. The plasmid gene *rmpA* activates transcription of the *wzyKpK1 cps* locus that in turn enhances capsule synthesis in *K. pneumoniae*[2]. Moreover, the *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[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 *K. pneumoniae* infections[10]. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) is a molecular technique that is used to estimate genetic diversity among enterobacteriacae 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[11,12].

This study was aimed to investigate the presence of the *rmpA*, *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 *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[13]. The identification of *K. pneumoniae* isolates was confirmed to the species level by using the automated vitek 2 system (Bio-Merieux, l'Etoile, France).

Antibiotic susceptibility testing

The antibiotic resistance was determined by using the standard disc agar diffusion technique according to Bauer et al.[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), ceftazidime (CAZ 30µg), ceftriaxone (CRO 30µg), cefepime (FEP 30µg), tetracycline (TE 5µg), chloramphenicol (C 30µg), gentamycin (CN 10µg), aztreonam (ATM 30µg), amikacin (AKN 30µg) ciprofloxacin (CIP 5µg), cotrimoxthazole (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[15].

DNA extraction

DNA was extracted directly by using the boiling method with some modifications[16]. 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: initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min[9].

Molecular genotyping of *K. pneumoniae* isolates by using ERIC-PCR

ERIC-PCR typing was performed by using the primer ERIC2 (5-AAGT AAGTGACTGGGGTGAGCG-3) and with cycling conditions of: initial incubation at 94°C for 15 min., followed by 40 cycles at 94°C for 1 min, 37°C for 1 min, and final elongation at 72°C for 8 min. PCR products were resolved on 1.5% agarose gel stained with ethidium bromide, and visualized *via* ultraviolet illumination[12]. The gel electrophoresis results were analyzed by using the TotalLab Quant Analysis software (Version 1.0., TotalLab Ltd. United Kingdom) following the manufacturer's instructions.

Statistical analysis

Correlations between the antibiotic resistance of the *K. pneumoniae* isolates, virulence genes, and ERIC genotypes were statistically determined by using Chi square test and Monte Carlo method by using the IPM SPSS software package version 20.0 (Armonk, NY:IBM Corp).

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

Table 1. Primers used for the amplification of the five virulence genes[9]

The gene	The primers' sequence	The amplicon size (bp)
rmpA	rmpA-F: ACTGGGCTACCTCTGCTTCA rmpA-R: CTTGCATGAGCCATCTTTCA	516 bp
wcaG	wcaG-F: GGTTGGGTCAGCAATCGTA wcaG-R: ACTATTCCGCCAACTTT TGC	169 bp
intI1	intI1-F: CAGTGGACATAAGCCTGTTC intI1-R: CCCGAGGCATAGACTG TA	160 bp
intI2	intI2-F: GTAGCAAACGAGTGACGAAATG intI2-R: CACGGATATGCGA CAAAAAGGT	789 bp
intI3	intI3-F: GCCTCCGGCAGCGACTTTCAG intI3-R: ACGG ATCTGCCAAACCTGACT	979 bp

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 H_2S. 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. pneumonia*e 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. pneumonia*e 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-C-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%)

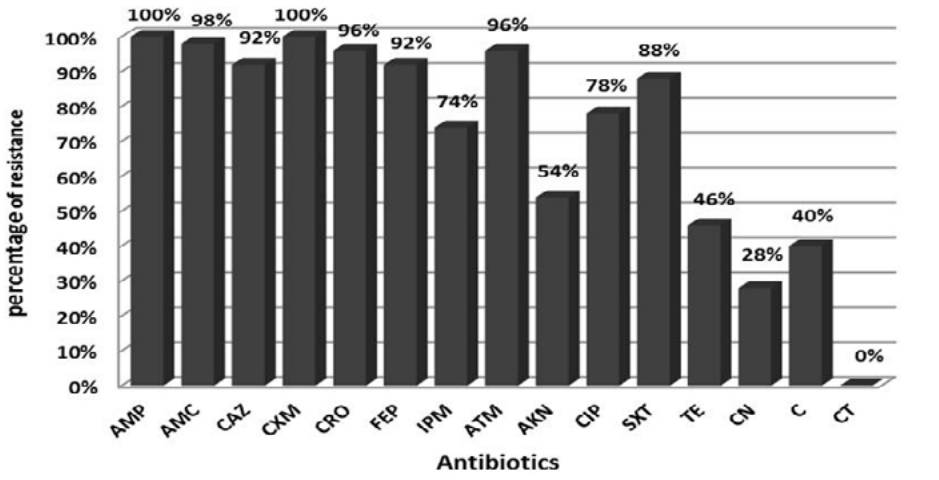

Fig. 1. Antibiotic resistance of the 50 *K. pneumoniae* isolates.

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

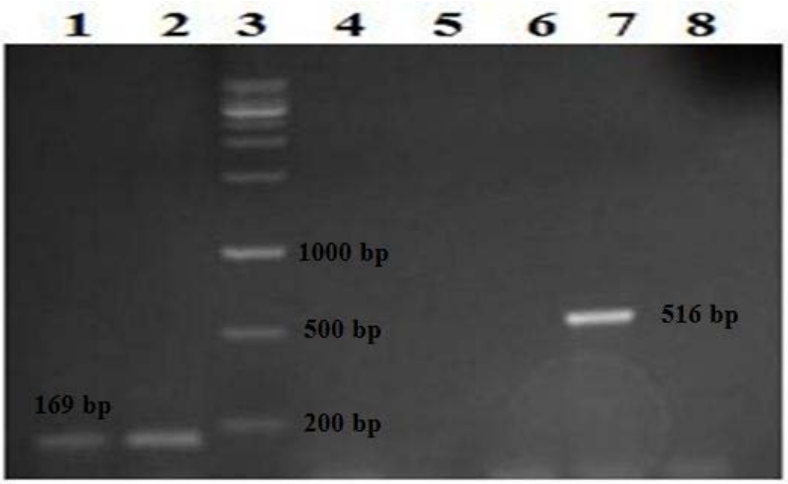

Fig. 2. Detection of the *rmpA* and *wcaG* genes on 1.5% agarose gel following amplification with PCR. Lanes 1 and 2: two isolates positive for the *wcaG* gene detected at 169 bp, lane 3: the DNA ladder, lanes 4, 5, 6, and 8: negative isolates and lane 7: one isolate positive for the *rmpA* gene detected 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 was reported by Farivar et al.[19]. Finally, all isolates in this study were sensitive to colistin; in contrast, Farivar et al.[19] reported a lower percentage (83%). Overall, the incidence of antibiotic resistance among our isolates was high compared to previous studies.

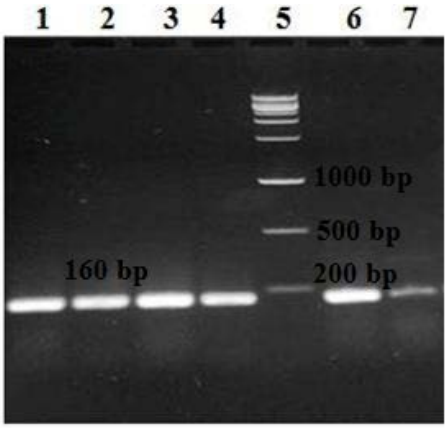

Fig. 3. Detection of the *intI* gene on 1.5% agarose gel following amplification with PCR. Lanes 1, 2, 3, 4, 6, and 7: six isolates positive for the *intI* gene detected at 160 bp and lane 5: the DNA ladder.

We found that 18 (36%) out of isolates were positive for the *wcaG* gene by conventional PCR. Lower percentages (6.5% and 23%) were reported by Zhang et al. in China and by Derakhshan et al. in Iran[9,21]. Nevertheless, Zaki et al., in Egypt (22) reported a higher percentage (50%).

Similarly, we found that 9 (18%) of isolates were positive for the *rmpA* gene. A similar percentage (17%) was reported by Guo et al. in China (23). In contrast, lower percentages (1.6%, 7%, and 10%) were reported by Zhang et al., Derakhshan et al., and Zaki et al., however, a higher percentage (46%) was reported by Tan et al. in Singapore[9,21,22,24].

Finally, forty-nine (98%) out of our 50 *K. pneumonia*e isolates were positive for the *intI1* 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].

None of isolates was positive for *intI2*, consistent with the result reported by Zaki et al. in Egypt[22]. Nonetheless, Derakhshan et al. in Iran and Liao et al. in China 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 *intI3*; this finding matches the results reported by Derakhshan et al., Zaki et al. and Firoozeh et al.[7,9,22].

Significant correlations were found between the *intI1* gene and resistance to aztreonam and ceftriaxone (p-value ≤ 0.05). In contrast, Derakhshan et al. reported a positive association between the *intI1* gene and resistance to cefotaxime, 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

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[9]. 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[12]. 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[21,26]. Such genetic variation among *K. pneumoniae* strains was also reported by other studies in Iran, Taiwan, Russia, and Algeria[27-30].

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

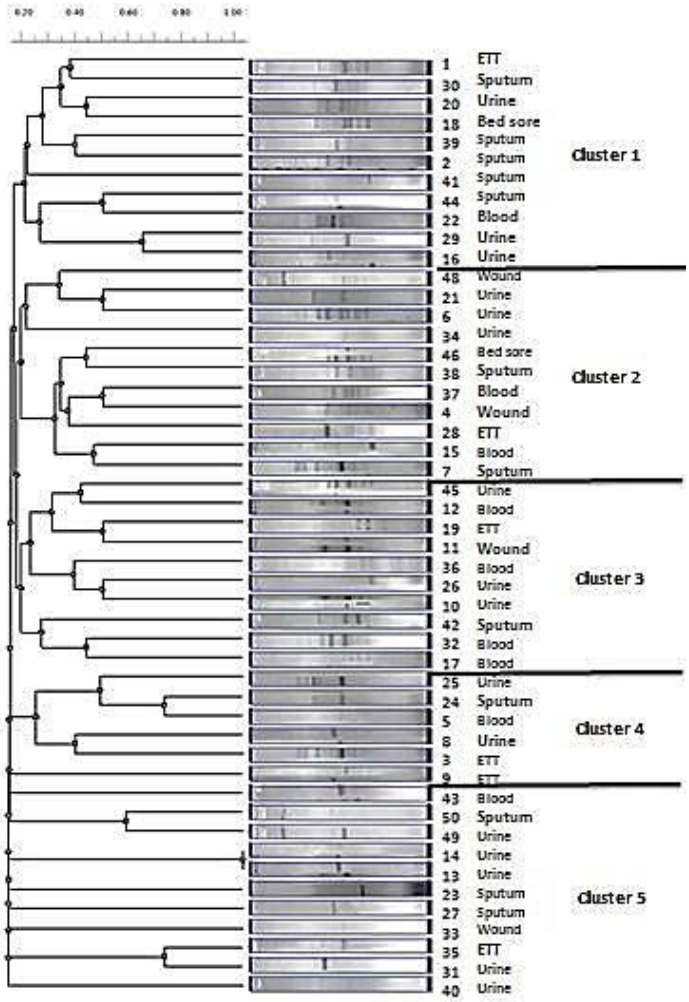

Fig. 4. Dendrogram generated with Dice coefficient and the UPGMA clustering method, showing the genetic similarity among *K. pneumoniae* isolates by Enterobacterial Repetitive Intergenic Consensus (ERIC) genotyping.

very effective method for molecular typing and epidemiological studies of nosocomial infections.

ACKNOWLEDGMENTS

None.

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.

FUNDING

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.

REFERENCES

1. Abbott SL. *Klebsiella, Enterobacter, Citrobacter, Serratia, Plesiomonas*, and Other *Enterobacteriaceae*. In Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (Eds.), *Manual of Clinical Microbiology*. 9th ed. 698-711. Washington, USA. 2007.
2. Vila A, Cassata A, Pagella H, et al. Appearance of *Klebsiella pneumoniae* liver abscess syndrome in Argentina: case report and review of molecular mechanisms of pathogenesis. *Open Microbiol J.* 2011;5:107-113. doi: 10.2174/1874285801105010107
3. Minarini LAR, Gales AC, Palazzo IC, Darini ALC. Prevalence of community-occurring extended spectrum β-lactamase-producing *Enterobacteriaceae* in Brazil. *Curr Microbiol.* 2007;54(5):335-341. doi: 10.1007/s00284-006-0307-z
4. Daef EA, Elsherbiny NM. Clinical and microbiological profile of nosocomial infections in adult intensive care units at Assiut University hospitals, Egypt. *J Am Sci.* 2012;8(12):1239-1250.
5. Ferreira RL, da Silva BCM, Rezende GS, et al. High prevalence of multidrug-resistant *Klebsiella pneumoniae* harboring several virulence and β-Lactamase encoding genes in a brazilian intensive care unit. *Front Microbiol.* 2019;9:3198. doi: 10.3389/fmicb.2018.03198
6. Carattoli A. Importance of integrons in the diffusion of resistance. *Vet Res.* 2001;32(3-4):243-259. doi: 10.1051/vetres:2001122
7. Firoozeh F, Mahluji Z, Khorshidi A, Zibae M. Molecular characterization of class 1, 2 and 3 integrons in clinical multi-drug resistant *Klebsiella pneumoniae* isolates. *Antimicrob Resist Infect Control.* 2019;8:59. doi: 10.1186/s13756-019-0509-3
8. Cheng HY, Chen YS, Wu CY, Chang HY, Lai YC, Peng HL. *rmpA* regulation of capsular polysaccharide biosynthesis in *Klebsiella pneumoniae* CG43. *J Bacteriol.* 2010;192(12):3144-3158. doi: 10.1128/JB.00031-10
9. Derakhshan S, Peerayeh SN, Bakhshi B. Association between presence of virulence genes and antibiotic resistance in clinical *Klebsiella Pneumoniae* isolates. *Lab Med.* 2016;47(4):306-311. doi: 10.1093/labmed/lmw030
10. Sachse S, Bresan S, Erhard M, et al. Comparison of multilocus sequence typing, RAPD, and MALDI-TOF mass spectrometry for typing of β-lactam-resistant *Klebsiella pneumoniae* strains. *Diagn Microbiol Infect Dis.* 2014;80(4):267-271. doi: 10.1016/j.diagmicrobio.2014.09.005
11. Barus T, Hanjaya I, Sadeli J, Lay BW, Suwanto A, Yulandi A. Genetic diversity of *Klebsiella* spp. isolated from tempe based on enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). *Hayati J Biosciences.* 2013;20(4):171-176. doi: 10.4308/hjb.20.4.171
12. Wasfi R, Elkhatib WF, Ashour HM. Molecular typing and virulence analysis of multidrug resistant *Klebsiella pneumoniae* clinical isolates recovered from Egyptian hospitals. *Sci Rep.* 2016;6:38929. doi: 10.1038/srep38929
13. Finegold SM, Baron EJ. Bailey and Scott's Diagnostic Microbiology. The C.V. Mosby company: St. Louis. 1986.
14. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol.* 1966;45(4):493-396. doi: 10.1093/ajcp/45.4_ts.493
15. CLSI. Performance standards for antimicrobial susceptibility testing. 29th ed. CLSI supplement M100. Wayne, PA: Clinical and laboratory standards institute. 2019.
16. Queipo-Ortun MI, Colmenero JDD, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. *Clin Vaccine Immunol.* 2008;15(2):293-296. doi: 10.1128/CVI.00270-07
17. Nirwati H, Sinanjung K, Fahrunissa F, et al. Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a

tertiary care hospital, Klaten, Indonesia. *BMC Proc.* 2019;13(Suppl 11):20. doi: 10.1186/s12919-019-0176-7

18. Cepas V, Lopez Y, Munoz E, et al. Relationship between biofilm formation and antimicrobial resistance in Gram-negative Bacteria. *Microb Drug Resist.* 2019;25(1):72-79. doi: 10.1089/mdr.2018.0027
19. Farivar SA, Nowroozi J, Eslami G, Sabokbar A. RAPD PCR profile, antibiotic resistance, prevalence of *armA* gene, and detection of KPC enzyme in *Klebsiella pneumoniae* isolates. *Can J Infect Dis Med.* 2018;2018:6183162. doi: 10.1155/2018/6183162
20. Manjula NG, Math CG, Nagshetty K, Patil SA, Gaddad SM, Shivannavar CT. Antibiotic susceptibility pattern of ESbetaL producing *Klebsiella pneumoniae* isolated from urine samples of pregnant women in Karnataka. *JCDR.* 2014;8(10):8-11.
21. Zhang S, Yang G, Ye Q, Wu Q, Zhang J, Huang Y. Phenotypic and Genotypic characterization of *Klebsiella pneumoniae* isolated from retail Foods in China. *Front Microbiol.* 2018;9:289. doi: 10.3389/fmicb.2018.00289
22. Bakr AO, Zaki A. Molecular study of *Klebsiella Pneumoniae* virulence genes from patients with hospital acquired sepsis. *Clin Lab.* 2019;65(1). doi: 10.7754/Clin.Lab.2018.180709
23. Guo S, Xu J, Wei Y, Xu J, Li Y, Xue R. Clinical and molecular characteristics of *Klebsiella pneumoniae* ventilator-associated pneumonia in mainland China. *BMC Infect Dis.* 2016;16(1):608. doi: 10.1186/s12879-016-1942-z
24. Tan TY, Ong M, Cheng Y, Ng LSY. Hypermucoviscosity, rmpA, and aerobactin are associated with community-acquired *Klebsiella pneumoniae* bacteremic isolates causing liver abscess in Singapore. *J Microbiol Immunol.* 2019;52(1):30-34. doi: 10.1016/j.jmii.2017.07.003
25. Liao W, Lia D, Liu F, et al. Distribution of integrons and phlogenetic groups among highly virulent serotypes of *Klebsiella pneumoniae* in a chinese tertiary hospital. *J Glob Antimicrob Re.* 2020;21:278-284. doi: 10.1016/j.jgar.2019.11.016
26. Mehr PV, Shokoohizadeh L, Mirzaee M, Savari M. Molecular typing of *Klebsiella pneumoniae* isolates by enterobacterial repetitive intergenic consensus (ERIC)-PCR. *Infection Epidemiology and Microbiology (IEM).* 2017;3(4):112-116.
27. Edelstein M, Pimkin M, Palagin I, Edelstein I, Stratchounski L. Prevalence and molecular epidemiology of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in russian hospitals. Antimicrob. *Agents Chemother.* 2003;47(12):3724-32. doi: 10.1128/AAC.47.12.3724-3732.2003
28. Lagha N, Abdelouahid DE, Hassaine H, Robin FE, Bonnet R. First characterization of CTXM- 15 and DHA-1-lactamases among clinical isolates of *Klebsiella pneumoniae* in Laghouat Hospital, Algeria. *Afr J Microbiol Res.* 20148(11):1221-1227.
29. Seifi K, Kazemian H, Heidari H, et al. Evaluation of biofilm formation among *Klebsiella pneumoniae* isolates and molecular characterization by ERIC-PCR. *Jundishapur J Microbiol.* 2016;9(1):e30682. doi: 10.5812/jjm.30682
30. Yan J-J, Hsueh P-R, Lu J-J, et al. Extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from seven medical centers in Taiwan. *Antimicrob Agents Chemother.* 2006;50(5):1861-1864. doi: 10.1128/AAC.50.5.1861-1864.2006