ORIGINAL ARTICLE

Molecular Determination of some Virulence Genes Associated with *Klebsiella pneumonia*e Clinical Isolates

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ABSTRACT

Background: *K. pneumoniae is an opportunistic emerging pathogen responsible for severe hospital-acquired as well as community-acquired infections, often with multidrug resistance and multiple virulence factors, posing a significant clinical challenge due to its ability to cause hard-to-treat infections.* ***Aims****: The objective of this study was to identify the phenotypic virulence factors, antibiotic resistance, and molecular identification of dominant virulence genes among K. pneumoniae isolates.* ***Methodology:*** *Twenty clinical isolates were screened. Phenotypic screening included capsule production, biofilm formation, proteinase, gelatinase, hemolysin, and hyaluronidase. Antibiotic susceptibility testing was conducted to determine resistance profiles. Molecular detection targeted virulence genes such as mrkD, T1 fim, magA, and T1fim.* ***Results:*** *Capsule production was detected in 100% isolates, biofilm formation in 75%, proteinase in 80%, gelatinase in 55%, hemolysin in 10%, and hyaluronidase in 15%. Antibiotic susceptibility testing showed high resistance to penicillin (100%), ampicillin (95%), and cephalosporins, while carbapenems demonstrated high effectiveness (imipenem 85% sensitive, meropenem 90% sensitive). Molecular analysis revealed mrkD in 100%, T1 fim in 90%, magA in 65% of isolates, The coexistence of strong virulence determinants and multidrug resistance in K. pneumoniae highlights its elevated pathogenic potential.* ***Conclusion:*** *Molecular characterization of virulence genes provides essential insights for clinical management, therapeutic planning, and infection control strategies.*

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen of the Enterobacteriaceae family, being Gram-negative and encapsulated, and it became a relevant global health threat by virtue of its dual capacity for the development of multidrug resistance and the production of a vast array of virulence determinants, being thereby responsible for severe hospital- and community-acquired infections such as pneumonia, bacteremia, urinary tract infections, liver abscesses, meningitis, and wound infections [1, 2].

The organism's pathogenicity is mediated to a great extent by its polysaccharide capsule, lipopolysaccharides, fimbrial adhesins, siderophores, and secreted enzymes that facilitate immune evasion, adhesion, iron acquisition, tissue invasion, and survival in the host. Some of the most clinically relevant virulence genes are *rmpA* and *rmpA2*, regulating hypermucoviscosity and capsule overproduction; *wzi*, *wzc*, and *magA*, responsible for capsule biosynthesis; *fimH* and *mrkD*, coding for fimbrial adhesins with roles in host cell attachment and biofilm formation; siderophore-associated genes *entB* (enterobactin), *iucA/iutA* (aerobactin), and *iroB/iroN* (salmochelin), which are strongly linked with hypervirulent strains; and other accessory genes *wabG*, *uge*, and *kfu*, involved in endotoxin structure, immune evasion, and iron uptake. The coexistence of hypervirulence and multidrug resistance among carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKp) strains has provoked clinical concern, as these strains are the cause of high mortality, limited treatment, and nosocomial outbreaks [2, 3].

Molecular techniques such as polymerase chain reaction (PCR), multiplex PCR, real-time PCR, multilocus sequence typing (MLST), and whole-genome sequencing (WGS) have become indispensable for rapid detection of virulence genes, resistance genes, and clonal lineages with increased sensitivity and specificity compared to conventional phenotypic techniques and making epidemiological surveillance of outbreaks possible. Recent studies have indicated high prevalence of *fimH*, *mrkD*, and *entB* in clinical isolates and the *rmpA* and aerobactin genes being found overwhelmingly together in highly virulent lineages of ST23, ST11, and ST147, indicating the ongoing pandemic spread of high-risk clones [4, 5].

Additionally, the detection of extended-spectrum β-lactamase (ESBL) and carbapenemase genes together with virulence determinants emphasizes the necessity for molecular surveillance to underpin infection control strategies and treatment practice. *K. pneumoniae* is a classic example of the concerning overlap of resistance and virulence, and molecular typing of its virulence genes not only allows for early diagnosis and risk stratification but also its evolving epidemiology, informing clinical management as well as public health response [6-8]. The aim of the present study is to investigate molecular detection of *K. pneumoniae* isolates with special focus on distribution of the key virulence genes to further clarify their role in pathogenicity, track the spread of high-risk clones, and to present data that can enable improved therapeutic methods and infection-control practices in hospitals.

METHODOLOGY

The current study involved 100 patients who underwent variable surgeries and had surgical wound infection. Wound swabs was obtained from all patients at Baghdad Medical City Hospitals during January 2025 to May 2025.

Microbial isolation

All the samples are submitted to routine culture on blood and MacConkey agar. Microbes are isolated were further identified by biochemical tests in addition to confirmation by DL microbiology diagnostic system (DL biotech. China). Kirby Baurer disc diffusion method was employed on isolates to establish the sensitivity of bacteria towards various antimicrobial agents according to[9, 10] Phenotypic expressions of some of the common virulence factors of the isolated bacteria was accomplished by culturing the isolates in culture media specific to each virulence factor like blood agar for detection of hemolysis pattern, Congo red agar, India ink stain, Gelatinase agar, Proteinase agar and Hyaluronidase agar.

Genomic DNA extraction

K. pneumoniae cultivated in brain heart infusion agar and incubated overnight at 37 °C. The extraction protocol was applied as directed by the manufacturer company (Genomic DNA extraction kit, Promega, USA).

Primers

The primers implied in the target genes amplification were designated by via through NCBI online primer designing tools for magA-F: 3' GGTGATTCAAGCACTATACC'5 and magA-R: 5' CGGACTGGCCATATTGCTCC'3 product size: 360bp, mrkD-F: 3'GGGAGAGCGGCGGTAACCCG'5 and mrkD-R: 5'CACCGGCGAGGTGGAGTCGC'3 product size: 477 bps, and bps, T1fim-F: 3'GCAAAACGGCCACCGGGGCG'5 and T1fim-R: 5'CCGCCGGTGGGGACCACCAC3' product Size: 472 bps.

PCR protocol

DNA recovered from all isolates was subjected to various runs to amplify the genes responsible for mucoviscosity (*magA*), fimbrial adhesion lectin (*mrkD*), and type1 fimbrae (*T1 fim*). G2 Green Master mix (Promega, USA) was employed for all the PCR run in the following concentration: 10µl G2 Green Master mix, 1µl of 10 pmol of forward and reverse primers, 5µl of DNA temples and brought up to 25µl with nuclease free water. The PCR reaction mixture was filled in the thermocycler machine (G storm gradient thermocycler, USA) and amplification protocols were carried out as an initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 15 seconds, annealing 58°C for 30 seconds and extension at 72 °C for 1 min with 35 cycles for the last three steps. The PCR products were separated by 1.5% agarose gel (Promega, USA) for 1h at 80 volts. The Gels were viewed under UV transilluminator (ClearView, UK) to see the amplified bands.

Ethical approval

The study gained ethical approval from Ethical Approval Committee at College of Science/University of Tikrit (issue number 3/7/5607 at 30/11/2024). A written consent form was provided to all participants prior to commencing sample collection.

Statistical analysis

The data generated by the research was analyzed and as descriptive tables and comparison was made with Qi square and tTest wherever required using Graph Pad Prisim version.

RESULTS

In terms of the biometric characteristics of the study population, as presented in Table 1, showed that male was a slightly higher in number (55.37%) compared to females (44.63%), indicating a slight male preponderance in *K. pneumoniae* infections.

Table 1: Biometric data of the study group

Patients' biometrics	**Males**		**Females**	
	N0.	**%**	**N0.**	**%**
	67	55.37	54	44.63
Residency	**Urban**		**Rural**	
	22	22.00	78	78.00
Mean age	**Males**		**Females**	
	Mean	±SD	Mean	±SD
	46.27	18.22	34.52	16.81

Number of virulence factor detected in the isolated bacteria are shown in Table 2.

Table 2: Phenotypic expression of virulence factors of the isolated bacteria

Virulence factors	*K. pneumonia* (n=20)	
	N0	%
Biofilm production	15	75.00
Capsule	20	100.00
Hemolysin	2	10.00
Gelatinase	11	55.00
Hyaluronidase	3	15.00
Proteinase	16	80.00

The antimicrobial susceptibility profile of *K. pneumoniae* is showed in Table (3) high levels of resistance to some commonly used antibiotics, resonating with the increasing global issue of multidrug resistance (MDR). Resistance to third-generation cephalosporins was also elevated, wherein 75% were resistant to cefixime and 65% to cefotaxime, while 60% were resistant to ceftriaxone.

Table 3: Antibiotic susceptibility test for *K. pneumonia*

Antibiotics	*K. pneumonia* (n=20)			
	R		S	
	No.	%	No.	%
Penicillin	20	100.00	0	0.00
Ampicillin	19	95.00	1	5.00
Tetracycline	6	30.00	14	70.00
Cefixime	15	75.00	5	25.00
Cefotaxime	13	65.00	7	35.00
Cefepime	8	40.00	12	60.00
Ceftriaxone	12	60.00	8	40.00
Imipenem	3	15.00	17	85.00
Meropenem	2	10.00	18	90.00
Ciprofloxacin	11	55.00	9	45.00
Levofloxacin	8	40.00	12	60.00
Gentamycin	7	35.00	13	65.00
Amikacin	5	25.00	15	75.00
Trimethoprim	16	80.00	4	20.00

The molecular detection of virulence genes of *K. pneumoniae* is as demonstrated in Table (4) and (Fig 1).

The distribution of major virulence genes among *K. pneumoniae* is illustrated by Table 4.

Table 4: Virulence gene for *K. Pneumonia*

***K. pneumoniae* virulence Genes (n=20)**	**Positive**		**Negative**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**
MagA	13	65.00	7	35.00	20	100.00
mrkD	20	100.00	0	0.00	20	100.00
T1 fim	18	90.00	2	10.00	20	102.00

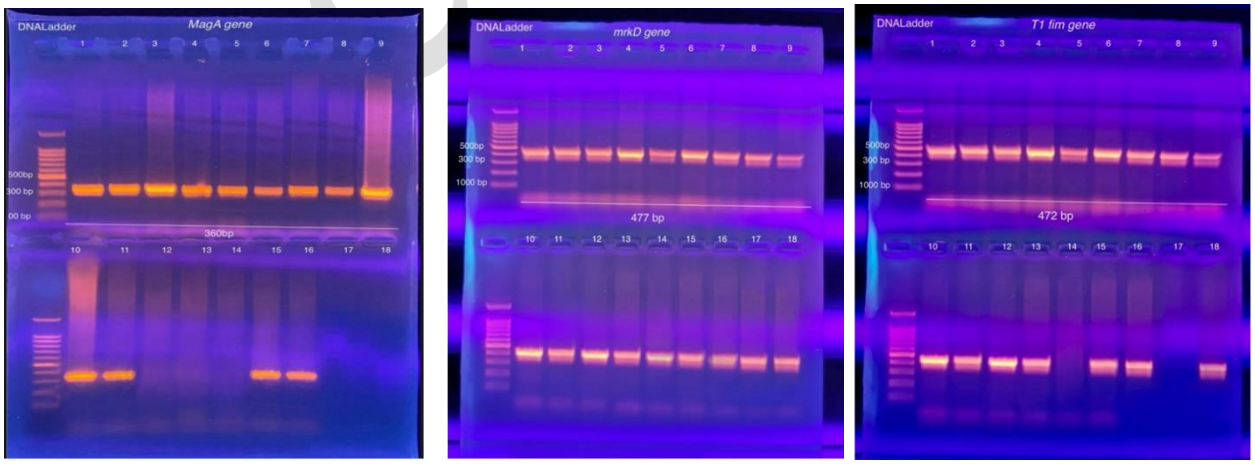

Fig. 1: PCR amplification of *magA* gene (top left), *mrkD* gene (top right), *t1fim* gene (bottom). All gels were run on 1.5% agarose gel at 80V for 1h. The 1st lane on left represents 100bo DNA ladder.

DISCUSSION

Predominance of male infection with *K. pneumoniae*, presumably may be due to increased occupational and environmental risk factors exposure, smoking prevalence, or comorbidity with conditions such as chronic lung disease and diabetes. This is consistent with an earlier study that showed male have a higher prevalence of Gram-negative infection. Residence-wise, the majority of patients (78%) were from rural areas compared with only 22% from urban areas, suggesting that rural communities are more vulnerable, perhaps due to lower access to healthcare facilities, inappropriate use of antibiotics, poor-level hygiene, and higher exposure to resistant organisms in the environment. These findings have also been observed in other recent epidemiological reports, where higher burden of antimicrobial-resistant it has been noted. *K. pneumoniae* in rural and semi-urban towns were high compared to towns and cities where infection-control is generally well practiced in town[11]. The average age results indicated that males under study were older (46.27 years) compared to females (34.52 years). This mean that males are likely to be co-present with *K. pneumoniae* infection at older ages, perhaps reflecting cumulative exposure, age-related comorbidities, or health care-associated risk factors, whereas women were overrepresented in young age groups as would be anticipated from the reproductive and working population. The same trends were seen in a Chinese multicenter study, where hypervirulent *K. pneumoniae* infection was more common among young females and old men with classical multidrug-resistant isolates[12]. Yet another study from the Middle East brought out age and gender disparity, older men being more susceptible to infection by virulent and resistant strains of *K. pneumoniae* in concurrence with the present findings[13, 14]. These demographic findings are pertinent to understanding virulence gene prevalence since acquisition and clinical effectiveness of hypervirulent and drug-resistant isolates of *K. pneumoniae* may be impacted by age, gender, and residency factors and consequently, such enzymes while playing a secondary role in virulence are nevertheless part of pathogenic variation. Collectively, these results identify the *K. pneumoniae* multifactor virulence factors of which capsule and biofilm formation are most common characteristics[13].

Biofilm formation was observed in 75% of the isolates, which significantly enhances bacterial persistence on medical devices and results in multidrug resistance, as reported recently linking biofilm-associated infection with poor clinical outcomes[15]. Extremely high frequency of protease activity (80%) also testifies to its involvement in host tissue invasion as well as the uptake of nutrients. Gelatinase production was observed among 55% of the isolates, marking its contribution towards extracellular matrix destruction and tissue lysis, in agreement with[16].

The entire isolates (100%) were resistant to penicillin, and a vast majority (95%) to ampicillin, confirming the intrinsic resistance of *K. pneumoniae* to β-lactam antibiotics due to rampant production of extended-spectrum β-lactamases (ESBLs), which degrade penicillin's and cephalosporins.

These results validate previous reports implicating it is virulence gene clusters—e.g., *magA*, *rmpA*, *fimH*, and siderophore genes (*iucA*, *entB*)—with improved survival, immune evasion, and transmission of clinical infections[17-18]. The resulting high rate of these virulence factors in this study confirms the strong need for molecular monitoring and focused treatment interventions to reverse the tide of hypervirulent and resistant *K. pneumoniae* the results are consistent with other reports of worldwide studies that ESBL-producing *K. pneumoniae* is a serious cause of hospital-acquired infections[19]. Carbapenems, represented by imipenem (85% susceptible) and meropenem (90% susceptible), respectively, however, did have the highest potency, lending validity to them being resorted to as rescue antibiotics; yet, finding resistance in 10–15% of isolates are concerning because it can be indicative of the dissemination of carbapenemase-producing strains, which have been rising globally in their reporting[17]. Among aminoglycosides, amikacin was the most active (75% susceptible), followed by gentamicin (65%), which is consistent with earlier observations regarding the activity of amikacin against MDR *K. pneumoniae*[20, 21]. Moderate resistance was observed among fluoroquinolones, 55% to ciprofloxacin and 40% to levofloxacin, suggesting plasmid-mediated quinolone resistance mechanisms. High trimethoprim resistance (80%) limited its clinical applicability. Overall, these findings show that *K. pneumoniae* in this group is very resistant to β-lactams and trimethoprim, while the only drugs that have continued to be highly effective are carbapenems and aminoglycosides. The link between resistance and carriage of virulence genes, as established by recent molecular studies, underscores the twofold threat of hypervirulent MDR strains, to render therapy more challenging and clinical outcomes less gratifying[22]. In line with these findings[13] the multi-factorial mode of its pathogenicity. The *mrkD* gene, encoding for fimbrial adhesins required for biofilm formation and colonization of host tissue, was detected in all the isolates (100%), confirming its critical role in colonization and persistence. This global dissemination is in accordance with previous research that had designated *mrkD* as a highly conserved virulence determinant strongly associated with biofilm-associated disease and device-associated bacteremia[23]. The *magA* gene linked to hypermucoviscosity and capsule

expression was detected in 65% of the isolates, and this reflects a high prevalence of hypervirulent strains within this population.

also similarly recorded such evidence, identifying *magA* as a marker that was especially relevant in invasive *K. pneumoniae* infections such as liver abscess. *T1 fim* gene for encoding type 1 fimbriae was present in 90% of the isolates, consistent with its role in epithelial cell adhesion and colonization of the urinary tract, based on studies showing its prevalence among clinical isolates[24]. Overall, all these findings together depict the predominance of adhesin and capsule-related genes over toxin-related genes which sustains the importance of biofilm formation as well as evasion of immunity in *K. pneumoniae* pathogenesis. These results are in agreement with worldwide reports that indicate hypervirulent strains tend to carry *magA*, *rmpA*, *mrkD*, and *fimbrial* genes that contribute to enhanced survival and spread, resulting in difficult-to-treat infections, particularly if multidrug resistant [16].

CONCLUSION

The molecular characterization of virulence determinants in *Klebsiella pneumoniae* clinical isolates reveals a widespread occurrence of important determinants such as *mrkD*, *MagA*, and *T1 fim* that drive strong biofilm formation, capsular synthesis, and virulence. This highlights the role of molecular diagnosis in the identification of virulent strains, guiding specific antimicrobial treatment, and supporting effective infection control practices against multi-drug-resistant *K. pneumoniae*.

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Conflict of Interest: The authors do not have financial competing interests.

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