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Research Article

Coexistence of Tetracycline Resistance and Virulence Determinants in Klebsiella pneumoniae Isolated from Hospitalized Patients in Pakistan

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ABSTRACT

Background: *Klebsiella pneumoniae* is an important opportunistic pathogen and an increasingly difficult cause of healthcare-associated and community-acquired infections because of its capacity to accumulate antimicrobial-resistance determinants and virulence-associated traits. Data linking phenotypic resistance with specific resistance and virulence genes remain limited in many settings in Pakistan. This study investigated the antimicrobial susceptibility profile of clinical *K. pneumoniae* isolates and characterized selected tetracycline-resistance and virulence-associated genes among tetracycline-resistant isolates.

Methods: A total of 250 clinical specimens, comprising urine, sputum, and blood, were collected from patients with suspected urinary tract, respiratory, or bloodstream infections at Hayatabad Medical Complex. *K. pneumoniae* was identified by colony morphology, Gram staining, and biochemical testing. Antimicrobial susceptibility was determined by the Kirby–Bauer disc diffusion method according to CLSI M100, 35th edition (2025). Genomic DNA was extracted by phenol–chloroform extraction. PCR was used to detect *tetA*, *tetB*, and the selected virulence-associated target *endr* among tetracycline-resistant isolates.

Results: *K. pneumoniae* was recovered from 110/250 specimens (44.0%). The isolates showed marked resistance to ampicillin (96.36%), trimethoprim–sulfamethoxazole (86.36%), ceftriaxone (80.00%), and ciprofloxacin (63.64%). Resistance to tetracycline and doxycycline was 32.73% and 29.09%, respectively, whereas meropenem showed the lowest resistance rate (10.00%). Among the 36 tetracycline-resistant isolates screened by PCR, *tetA* was detected in 27 (75.00%), *tetB* in 22 (61.11%), and *endr* in 30 (83.33%).

Conclusions: The high level of resistance to several commonly used antimicrobials highlights a substantial therapeutic challenge associated with clinical *K. pneumoniae* in this setting. The frequent detection of *tetA* and *tetB* among tetracycline-resistant isolates, together with the high occurrence of the selected *endr* target, indicates a population in which antimicrobial resistance and virulence-associated determinants may coexist. These findings support continued local AMR surveillance, culture-directed therapy, antimicrobial stewardship, and further genomic investigation of resistant *K. pneumoniae* in Pakistan.

Keywords: Klebsiella Pneumonia, Antimicrobial Resistance, Tetracycline Resistance, Teta, Tetb, Virulence Genes.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the most pressing issues in infectious diseases today. diseases, making commonly used antibacterial agents less effective and making the treatment more difficult. Routine and severe infections management. The burden is significant worldwide: bacterial AMR was

Directly associated with an estimated 4.71 million deaths in 2021, of which 1.14 million were direct deaths. caused by resistant infections, and is expected to be significant in the next few decades (1). The problem is particularly concerning for Gram-negative pathogens, for which resistance to Third generation cephalosporins and carbapenems may severely limit therapeutic choices. In its The World Health Organization (WHO) has designated third-generation as a Bacterial Priority Pathogen in the 2024 Bacterial Priority Pathogens List. CPE (carbapenem-resistant and cephalosporin-resistant Enterobacterales) are critical priority pathogens, and Highlighting the importance of ongoing local surveillance and targeted antimicrobial-resistance control (2).

K. pneumoniae is an important member of the Enterobacterales and an established cause of both healthcare- and community-associated infections. It can cause urinary tract The highest incidence of infections, pneumonia, bloodstream infections, and other invasive disease was in the greatest. Clinical impact frequently in hospitalized or other vulnerable patients (3, 4). Its epidemiology is complicated by the presence of classical and hyper virulent phenotypes and by The increasing virulence and antimicrobial resistance. Recent studies have highlighted the ability of *K. pneumoniae* to accumulate resistance determinants while retaining or acquiring virulence-associated traits, creating strains that are increasingly difficult to treat and control (4-6).

The resistance phenotype of *K. pneumoniae* is shaped by several complementary mechanisms, including β-lactamase production, reduced permeability, target modification, and enhanced drug efflux (3, 4). Efflux determinants like tetA and tetB are still found in tetracycline-resistant K. pneumoniae, making tetracycline resistance a clinical and epidemiological interest. Recent molecular studies have revealed that tetracycline-resistance determinants can be found in combination with multidrug-resistance determinants, and contemporary genomic analyses have revealed that tetA-positive *K. pneumoniae* has spread across a variety of lineages and geographical regions (7, 8). These observations highlight the importance of using a combination of phenotypic susceptibility testing and molecular screening, rather than either one or the other.

Resistant *K. pneumoniae* have an additional dimension of clinical significance because of their virulence that contribute in the disease pathogenesis, most importantly the capsular component, adhesins, ability to form biofilm, and the siderophore that sequester iron from the surrounding, collectively all these virulence factors help in the pathogen survival, colonization and evasion from immune response. (5, 6) The emergence of strains with both antimicrobial resistance and enhanced virulence is especially worrisome as it can make treatment more difficult and transmission more likely. This is an important problem in South Asia, where recent studies in Pakistan have reported significant antimicrobial resistance in clinical K. pneumoniae isolates and the presence of virulence-associated determinants in hospital settings (9, 10). Local epidemiological studies are therefore important for defining resistance patterns and identifying molecular targets that may have relevance for surveillance and infection-control strategies.

Despite the increasing availability of molecular data, resistance patterns remain heterogeneous between institutions and geographical settings, and local evidence from tertiary-care hospitals is essential for guiding empirical therapy and antimicrobial stewardship (3, 4, 9). In this context, the present study investigated *K. pneumoniae* recovered from clinical specimens at tertiary care hospitals, Pakistan. The study characterized the antimicrobial susceptibility profile of the isolates and, among tetracycline-resistant *K. pneumoniae*, determined the prevalence of the tetracycline-resistance genes *tetA* and *tetB* together with the selected virulence-associated target *endr*. By linking phenotypic resistance with molecular detection, the study aimed to provide a focused local picture of tetracycline resistance and selected virulence determinants in *K. pneumoniae* and to contribute data relevant to ongoing AMR surveillance in the region.

MATERIALS AND METHODS

Study Design and Setting

This cross-sectional study was conducted over a six-month period of time (May 2025 to November 2025) at the tertiary care hospitals. A total of 240 Clinical specimens were obtained from patients with suspected clinical infection admitted to Hospitals. The sample was transported to microbiology laboratory for identification of bacterial strain. Specimen type

was determined by the patient's clinical presentation. Midstream urine specimens were collected in sterile containers from patients presenting with urinary tract infection, while sputum and blood specimens were collected from patients with suspected *K. pneumoniae* pneumonia, bloodstream infection, or meningitis. All specimens were transported to the microbiology laboratory within 40 minutes of collection to preserve organism viability prior to culture

Sample Inclusion and Exclusion Criteria

A non-probability convenience sampling approach was used to recruit participants. Patients aged 18–30 years presenting with clinically and microbiologically confirmed *K. pneumoniae* infection including urinary tract infection, pneumonia, meningitis, or bloodstream infection, that was resistant to first-line antibiotic therapy, and who had complete accompanying clinical data, were eligible for inclusion. Specimens that were culture-negative for *K. pneumoniae*, or that yielded isolates sensitive to first-line, WHO-recommended antibiotics, were excluded from the study.

Bacterial Isolation and Identification

Specimens were inoculated onto Cystine Lactose Electrolyte-Deficient (CLED) agar using a calibrated 1-µL loop and incubated aerobically at 37°C for 24 h. Growth >100 CFU/mL was considered clinically significant. Presumptive isolates were purified on MacConkey and Eosin Methylene Blue agar. Colony morphology, Gram staining and standard biochemical tests such as indole, urease, triple sugar iron and lactose fermentation were used to identify isolates. Confirmed K. pneumoniae isolates were stored in nutrient broth for short-term storage and in 30% glycerol at −20°C for further analysis.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility was tested using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar following the CLSI M100, 35th edition (2025) (11). A 0.5 McFarland suspension of fresh cultures was uniformly inoculated on Mueller–Hinton agar and antibiotic discs (Oxoid, Turkey) were applied. Plates were incubated at 37°C for 16–18 hours and the inhibition zones were measured and interpreted as susceptible, intermediate, or resistant based on the CLSI breakpoints (11, 12). The antimicrobial panel comprised tetracycline (30 µg), trimethoprim–sulfamethoxazole (1.25/23.75 µg), ampicillin (10 µg), meropenem (10 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), and doxycycline (30 µg), provided by Oxoid Turkey.

Genomic DNA Extraction

Overnight cultures were used for genomic DNA extraction using phenol–chloroform method. In brief, bacterial pellets were lysed in TE buffer with SDS and proteinase K, extracted twice with phenol–chloroform and the aqueous phase precipitated with isopropanol and sodium acetate. The DNA pellet was washed with 70% ethanol, re-suspended in TE buffer and stored at −20°C. DNA concentration and purity were measured using Nano Drop spectrophotometry.

PCR Amplification of Target Genes

The resistance genes tetA and tetB and the virulence-associated gene endr were detected by PCR. The reaction volume was 20 µL, which included 10 µL of 2× PCR master mix, 1 µL of each forward and reverse primer (10 pmol/µL), and 2 µL of template DNA. Each assay had a no-template control. The PCR reaction cycle were comprises of initial denaturation at 94°C for 3 minutes followed by 35cycles 94°C for 45 second, annealing from 52 to 58°C for 45seconds, then 72°C for 60 second, a final extension of 72°C for 10 minutes. List of primer, their sequence, amplicon size and references are mentioned in the Table 1.

Table 1. Primer sequences, amplicon sizes and annealing temperatures for resistance-gene PCR.

Target Gene	Primer Sequence (5′–3′)	Amplicon Size	Annealing Temp.	Reference
tetA	F:GGTTTGCGATCTGGTTTTC R:CGGAAATGGCTCATCACGATC	780 bp	51°C	12
tetB	F: CGAATGCGCACCAG R:TGGTGTTTGGTCGCAAT	250 bp	52°C	12
endr	F: GTTTAACAAAACAACCACC R: R: GGAATAGAATGGCTTAACTCT	560 bp	44°C	12

Agarose Gel Electrophoresis

The PCR products were run on 1% agarose gels in 1× TBE with ethidium bromide. A 100-bp DNA ladder was used for size estimation. Electrophoresis was carried out at 100 V for 45–55 min and amplified products were detected under UV light and recorded with a Bio-Rad gel documentation system.

Statistical Analysis

Microsoft Excel 2017 was used to analyze the data. Categorical variables were presented as The frequencies and percentages were used for continuous variables, while the means ± standard deviation were used for continuous variables.

RESULTS

Isolation and Distribution of K. Pneumoniae

A total of 250 clinical samples (urine, sputum, and blood) were taken from patients who were suspected of having urinary tract, respiratory, or bloodstream infections at Hayatabad Medical Complex. Of these, 110 (44.0%) produced isolates that were identified as Klebsiella pneumoniae by colony morphology, Gram staining and biochemical tests. The distribution of K. pneumoniae isolates according to specimen type is presented in Figure 3.1. The recovered isolates were from urine, sputum and blood, and their frequencies and percentages are indicated in the figure.

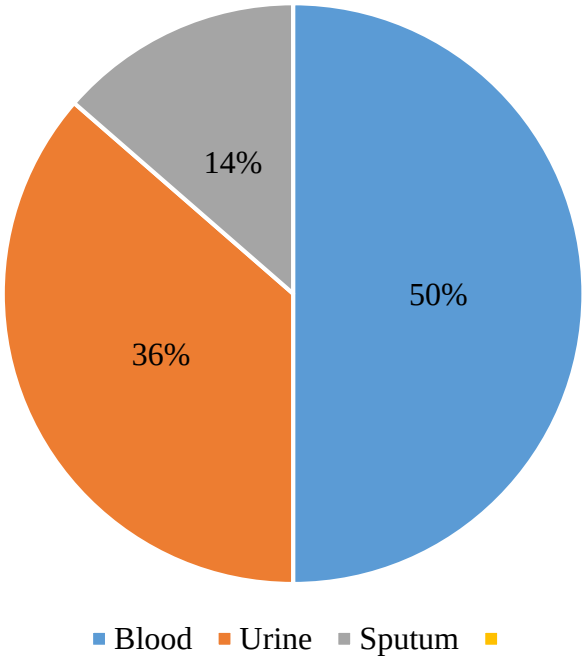

Figure 3.1. Clinical specimen type distribution of K. pneumoniae isolates. The number and percentage of isolates recovered from urine, sputum and blood specimens are shown in a pie chart

3.2 Antimicrobial Resistance Profile

The *K. pneumoniae* in the current study showed variable degree of resistant to the tested antibiotics. The highest resistance was observed for ampicillin (96.36%), followed by trimethoprim–sulfamethoxazole (86.36%) and ceftriaxone (80.00%). Ciprofloxacin resistance was observed in 63.64% of isolates, while resistance to tetracycline and doxycycline was 32.73% and 29.09%, respectively. Meropenem showed the lowest resistance rate (10.00%) among the tested antibiotics.

The overall resistance profile is shown in Figure 3.2, and the corresponding resistance percentages are summarized in Table 3.1.

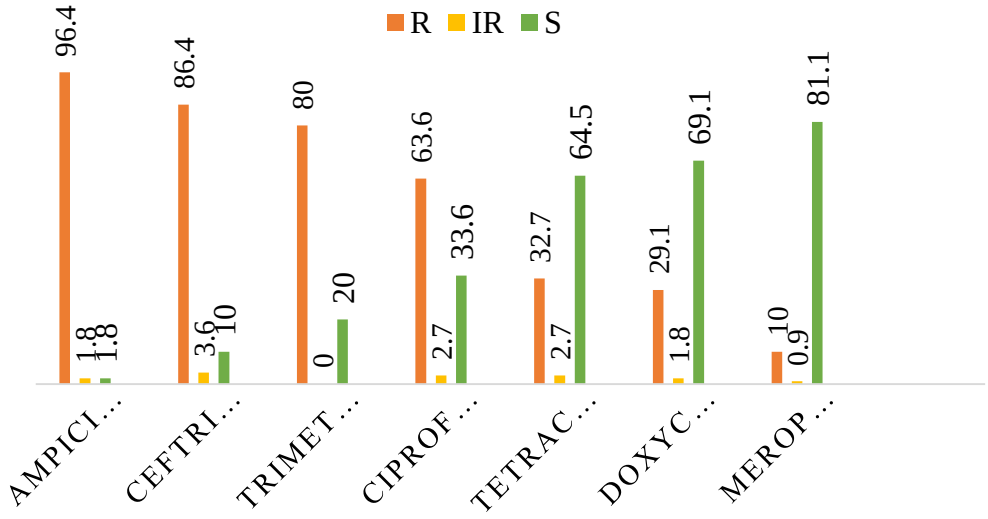

Figure 3.2. Antimicrobial resistance profile of K. pneumoniae isolates

Multiple-bar chart showing the percentage resistance to each tested antimicrobial agent.

Table 3.1. Antimicrobial resistance among *K. pneumoniae* isolates (n=110).

Antibiotic	Number of resistant isolates	Resistance (%)
Ampicillin	106	96.36(%)
Trimethoprim–sulfamethoxazole	95	86.36(%)
Ceftriaxone	88	80.00(%)
Ciprofloxacin	70	63.64(%)
Tetracycline	36	32.73(%)
Doxycycline	32	29.09(%)
Meropenem	11	10.00(%)

Prevalence of Resistance and Virulence Genes

PCR screening was performed among the 36 *K. pneumoniae* isolates exhibiting phenotypic tetracycline resistance. The *tetA* gene was detected in 27/36 (75.00%) isolates, whereas *tetB* was detected in 22/36 (61.11%). The virulence-associated gene *endr* was detected in 30/36 (83.33%) isolates. Among the three targets, *endr* was the most frequently detected gene, followed by *tetA* and *tetB*. The corresponding gene frequencies are presented in Table 3.2, with representative PCR amplification profiles shown in Figure 3.3.

Table 3.2a. Prevalence of resistance and virulence-associated genes among tetracycline-resistant *K. pneumoniae* isolates (n=36).

Gene	Positive-n	Negative-n	Prevalence %
endr	30	6	83.33%
tetA	27	9	75.00%
tetB	22	14	61.11%

Association of Tetracycline Resistance Genes and Virulence Genes among Tetracycline Resistance Isolates (N = 36).

Among the 36 tetracycline-resistant *K. pneumoniae* isolates, the *endr* virulence-associated gene was the most prevalent gene, detected in 30 (83.33%) isolates, followed by *tetA* in 27 (75.00%) and *tetB* in 22 (61.11%) isolates. Co-occurrence analysis revealed a significant association between *tetA* and *endr*. The *endr* gene was detected in 26/27 (96.3%) *tetA*-positive isolates compared with 4/9 (44.4%) *tetA*-negative isolates (Fisher's exact test, p = 0.0018; OR = 32.50, 95% CI: 2.97–355.13). In contrast, *endr* was detected in 18/22 (81.8%) *tetB*-positive isolates and 12/14 (85.7%) *tetB*-negative isolates, and no significant association was observed between

tetB and *endr* (Fisher's exact test, p = 1.000; OR = 0.75, 95% CI: 0.12–4.76).

Table 3.2b. Association between resistance genes and *endr*

Resistance gene	endr + n	endr − n	OR	95% CI	Fisher's exact p-value
tetA +	26	1	32.50	2.97–355.13	0.0018
tetA −	4	5			
tetB +	18	4	0.75	0.12–4.76	1.000
tetB −	12	2			

Abbreviation: OR; odd ratio, Cl; Confidence interval

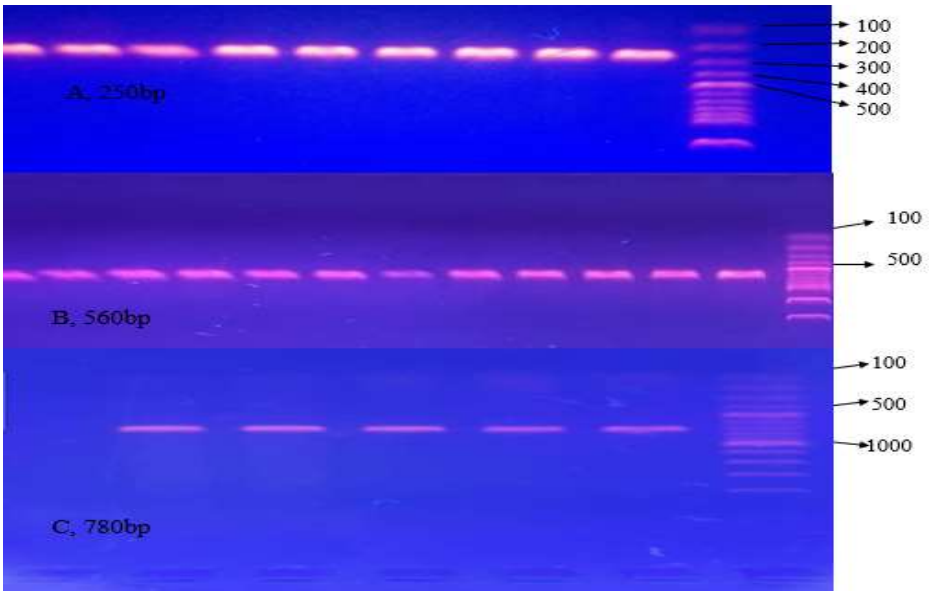

Figure 3.3. PCR amplification profiles of the target genes in *K. pneumoniae* isolates, (A) *tetB,* product size of 250bp, (B) *endr* product size of 560bp, (C), *tetA* with a product size of 780bp, on the right side of each gene there is a 100bp DNA markers.

DISCUSSION

This study demonstrates a substantial burden of antimicrobial resistance among clinical *K. pneumoniae* isolates. Resistance was particularly high to ampicillin, trimethoprim-sulfamethoxazole, ceftriaxone, and ciprofloxacin, whereas meropenem remained active against most isolates. Among tetracycline-resistant isolates, *tetA* and *tetB* were frequently detected, and the selected virulence-associated target *endr* was present in most isolates. Together, these findings highlight the coexistence of clinically important resistance determinants and virulence-associated markers within the local *K. pneumoniae* population.

The high resistance rates observed for commonly used agents are consistent with the broader emergence of *K. pneumoniae* as an important antimicrobial-resistance threat. The particularly high resistance to ceftriaxone is clinically concerning because third-generation cephalosporins are widely used for empirical treatment of serious infections. Although the underlying mechanisms were not characterized in this study, such resistance may reflect the contribution of β-lactamases and other established resistance mechanisms. Similarly, the high resistance to trimethoprim–sulfamethoxazole and ciprofloxacin suggests that these agents may provide unreliable empirical coverage in this setting. Comparable resistance patterns have recently been reported from Peshawar and other parts of Khyber Pakhtunkhwa, supporting the presence of a sustained local AMR burden(9, 10, 12).

The 10% resistance rate to meropenem is comparatively reassuring, but it should be interpreted cautiously. Carbapenems remain important agents for severe infections caused by multidrug-resistant Enterobacterales, and increasing carbapenem resistance can rapidly compromise therapeutic options. The present findings therefore support susceptibility-guided use of carbapenems rather than routine empirical reliance on these agents. Continued local surveillance is essential because resistance patterns may shift rapidly under antimicrobial selection pressure (13, 14).

The molecular findings provide further insight into tetracycline resistance. Among the 36 tetracycline-resistant isolates, *tetA* was detected more frequently than *tetB*. This predominance is consistent with the established role of TetA-mediated efflux in tetracycline resistance among Enterobacterales (15). The relatively high frequencies of both genes likely reflect the deliberate selection of phenotypically resistant isolates for PCR screening and therefore should not be interpreted as prevalence estimates for the entire collection of *K. pneumoniae*. Similar studies have identified *tetA* and *tetB* in multidrug-resistant *K. pneumoniae*, including isolates carrying these determinants in potentially mobile genetic contexts (8, 16, 17).

The presence of tetA and tetB in many of the isolates also demonstrates the importance of using phenotypic susceptibility testing in conjunction with molecular testing. Phenotypic testing determines the expressed resistance phenotype, while PCR detects specific genetic determinants that can be associated with the resistance phenotype. In K. pneumoniae, genes for resistance and virulence may be found on mobile genetic elements, which can be acquired and disseminated (18, 19). The present study, however, cannot determine if the genes detected were genetically linked or if they directly caused the observed clinical phenotype.

83.33% of the tetracycline resistant isolates were found to contain the selected endr target. Although this study reports high prevalence of virulence genes, yet at PCR level genes presence alone dose, not demonstrate genes expression, and its functional activity, hypervirulance, or its association with disease severity. The pathogenicity of *K. pneumoniae* is multifactorial that depends on the combine effects of various virulence factors from capsular polysaccharide to adhesins, iron sequesters siderophore, and biofilm formation all together participate in the pathogenicity, bacterial evasion from immune system, and its colonization, and proliferation (5, 6, 18). Therefore, the simultaneous presence of tetA, tetB and endr should not be interpreted as a direct mechanistic relationship, but rather as co-occurrence in a resistant population.

Our results are similar to recent studies in Pakistan, which reported high levels of antimicrobial resistance and virulence-associated traits in clinical K. pneumoniae (9, 10, 12). While these studies offer valuable regional context, the resistance percentages cannot be directly compared because of the differences in the types of specimens, patient populations, antimicrobial panels, and molecular targets. However, the overall uniformity of the resistance pattern, supported the need for locally generated AMR data and continued surveillance at the molecular level. From a clinical perspective, the resistance profile observed here has important implications for empirical treatment. The resistance to a number of the antibiotics used routinely highlights the need to not assume or rely on past or broad-spectrum susceptibility. Treatment should then be directed by culture and susceptibility testing, if possible, especially in severe infections. At the institutional level, these findings support antimicrobial stewardship, routine AMR surveillance, and infection-prevention measures aimed at limiting the selection and transmission of resistant *K. pneumoniae* (20).

CONCLUSION

This study result demonstrate that antimicrobial resistance is a significant problem in clinical *K. pneumoniae* isolates, especially for ampicillin, trimethoprim–sulfamethoxazole, ceftriaxone, and ciprofloxacin, with meropenem having relatively high activity. The high prevalence of *tetA* and *tetB* suggests that these resistance determinants play an important role in the observed tetracycline phenotype, and the frequent detection of the selected *endr* virulence-associated target suggests that resistance and virulence-associated characteristics may coexist within the same bacterial population. Although the study result demonstrates a significance burden of AMR that effect treatment, and epidemiological concern, yet the detection of resistance genes alone does not establish an expression of the genes or its pathological function. Overall, the findings suggest ongoing local AMR surveillance, judicious antimicrobial stewardship, and culture-based treatment, with enhanced infection-prevention strategies in tertiary-care facilities. Further multicenter studies with detailed resistance and virulence characterization, genomic studies and clinical outcome data are recommended to better understand the transmission and clinical impact of resistant K. pneumoniae in Pakistan.

Limitation of the Study

This study has some limitation especially it is a single-center design and relatively small sample size. Only a few resistance and virulence associated genes were studied and PCR-based detection without sequencing or whole genome analysis did not allow for further genetic characterization of the isolates. Moreover, the cross-sectional design does not allow assessment of changes in resistance patterns over time or establish relationships between resistance and virulence.

Future Recommendation

Larger, multicenter surveillance should be conducted in the future to better define the burden and distribution of antimicrobial resistance in *K. pneumoniae* in Pakistan. More comprehensive molecular and genomic analysis of resistance and virulence determinants would also help to understand the co-existence, transmission and clinical relevance of these determinants. Improving routine AMR surveillance and antimicrobial stewardship could also contribute to prompt, culture-based treatment and reduce the transmission of resistant organisms.

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