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Original article

Molecular Epidemiology of Carbapenem-Resistant Klebsiella pneumoniae and its Emerging Patterns of Resistance and Virulence

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Abstract
Carbapenem-resistant Klebsiella pneumoniae (CRKP) posses an urgent public health threat. This study examined antimicrobial resistance, carbapenemase production, and virulence gene profiles in clinical isolates from a tertiary care hospital in southern India. A total of 200 non-duplicate isolates were tested for antimicrobial susceptibility, carbapenemase production using the modified carbapenem inactivation method (mCIM) and extended carbapenem inactivation method (eCIM), and molecular detection of resistance and virulence genes by multiplex PCR. Twenty-seven isolates (13.5%) were identified as carbapenemase producers. The most common resistance determinants were blaOXA-48-like (85%) and blaNDM (57%), while blaKPC, blaVIM, and blaIMP were not detected. Virulence genes were widespread, including entB (85%), mrkD (77%), magA (70%), ybtS (74%), and iutA (44%). Capsular types K1 and K2 were observed; however, hypervirulent CRKP was not identified. These findings highlight the coexistence of carbapenem resistance and virulence traits in clinical isolates and underscore the importance of molecular surveillance, strict infection-control measures, and the exploration of alternative therapeutics.
Keywords: *Klebsiella pneumoniae; carbapenem resistance; antimicrobial resistance; virulence; molecular epidemiology*

Introduction

Klebsiella pneumoniae is a prominent Gram-negative pathogen responsible for hospital- and community-acquired infections such as pneumonia, urinary tract infections, bloodstream infections, and wound infections[1]. Its clinical importance has increased due to the global dissemination of multidrug-resistant strains, particularly carbapenem-resistant *K. pneumoniae* (CRKP), which severely limit treatment options[2].Carbapenems have traditionally been considered last-resort agents for managing infections caused by extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae[8].

India has been identified as a hotspot for CRKP due to extensive antibiotic usage, overcrowded healthcare facilities, and the presence of high-risk plasmids facilitating gene transfer[4]. Previous Indian studies report high prevalence of blaNDM and blaOXA-48-like among CRKP isolates[5], often alongside virulence determinants. Yet, systematic molecular data on the co-occurrence of carbapenemase genes and virulence markers in Indian isolates remain scarce[5,6].

Therefore, this study aimed to (i) evaluate the antimicrobial susceptibility profiles of *K. pneumoniae* isolates from a tertiary care hospital in southern India, (ii) characterize carbapenemase activity and genetic determinants of resistance, and (iii) assess the prevalence of virulence genes associated with pathogenicity. By integrating phenotypic and molecular approaches, this study provides region-specific insights into the epidemiology of CRKP.

Materials and Methods

Two hundred non-duplicate *K. pneumoniae* isolates were collected from urine, blood, respiratory specimens, pus and wound swabs at Meenakshi Medical College Hospital, Tamil Nadu, between January and September 2024. Isolates were identified using standard biochemical tests and confirmed with automated systems[7].Antimicrobial

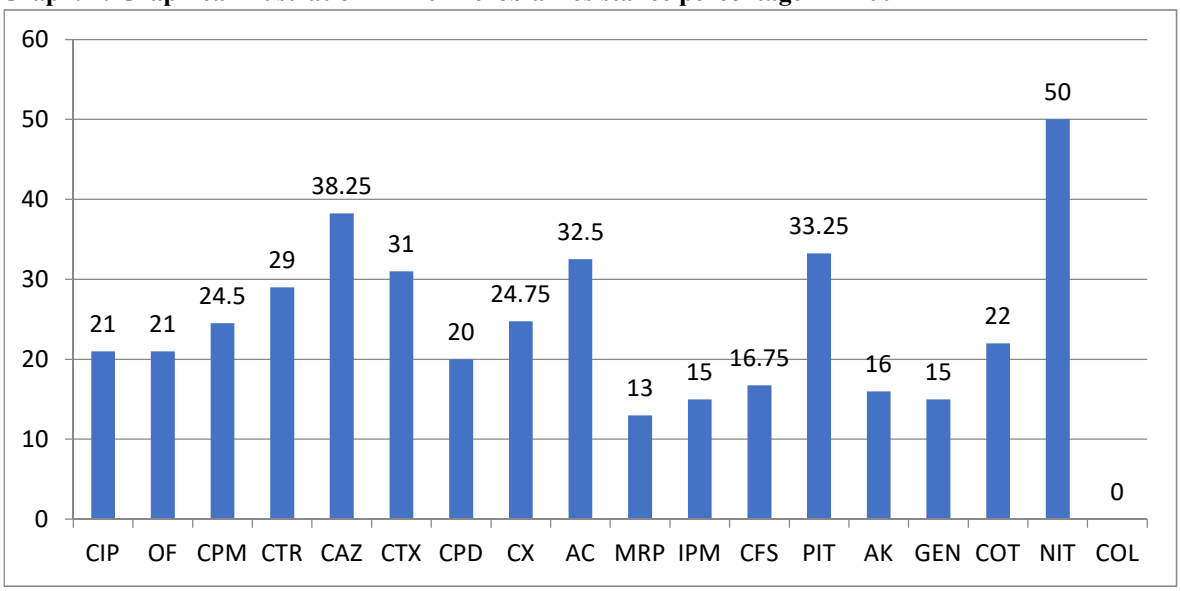
susceptibility was determined using Kirby–Bauer disk diffusion on Mueller–Hinton agar and interpreted according to Clinical and Laboratory Standards Institute (CLSI 2023) guidelines[8,9]

Genomic DNA was extracted using a boiling lysis method[3]. Multiplex PCR assays targeted carbapenemase genes (blaKPC, blaNDM, blaIMP, blaVIM, blaOXA-48-like) and virulence genes (entB, ybtS, iutA, mrkD, rmpA, magA, kfu, allS, K1/K2), following previously published protocols[10] (Table - 1, 2, & 3). PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV transillumination.

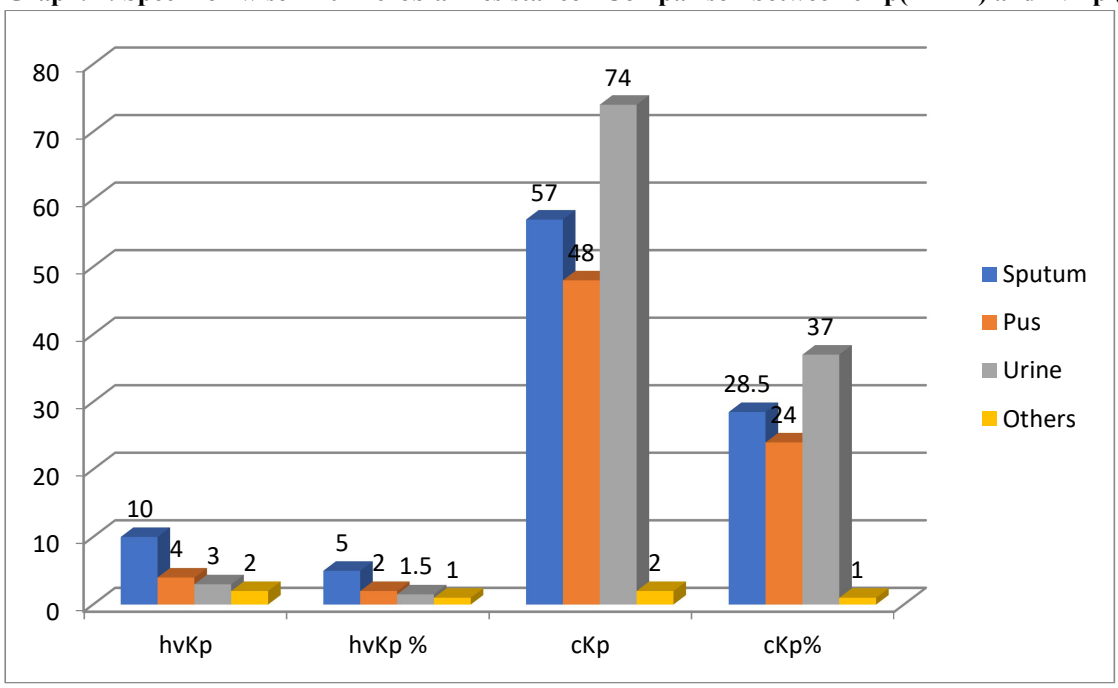
Results

The susceptibility of 200 Klebsiella pneumoniae isolates to antibiotics was tested by disc diffusion method with mixed results of resistance levels. 21% of isolates were resistant to Ciprofloxacin and Ofloxacin, Ceftazidime shown 38% resistance which is higher than Cefotaxime 31%, Ceftriaxone 29%, Cefoxitin 25%, Cefipime 24.5% and Cefpodoxime 20%.Amoxycillin/Clavulanate 32.5%, Piperacillin/Tazobactam 33%, Cefoperazone/Sulbactam 16.5%, Amikacin 16%, Gentamycin 15%, Imipenem 15%, Meropenem 13%, Co-Trimoxazole 22%, were resistant. Isolates from Urine showed 50% resistant to Nitrofurantoin. All isolates were Sensitive to Colistin . Among the Carbapenem Sensitive K.pneumoniae (CSKP) 25% (n=50) were Extended Spectrum Beta Lactamase (ESBL), 17% (n=34) AmpC Producers. ESBL and AmpC co production seen in 11% (n=22) and ESBL Carba in 0.5% (n=1). Resistance to third- and fourth-generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime) exceeded 30%, reflecting widespread β-lactamase activity. Fluoroquinolones also demonstrated reduced efficacy, with resistance rates of 21%. Aminoglycoside resistance was variable 15% of isolates were resistant to gentamicin, while resistance to amikacin was 16%, suggesting that amikacin retains effectiveness. While Carbapenem resistance was confirmed in 27 isolates (13.5%). Colistin retained 100% activity, representing the only reliably effective agent *in vitro against Carbapenem Resistant K.pneumoniae(CRKP)* (Graph. 1). 19 strains (9.5%) detected as hvKp and non of them were Carbapenemase producer[3]. Specimen wise drug resistance shown in Graph. 2.

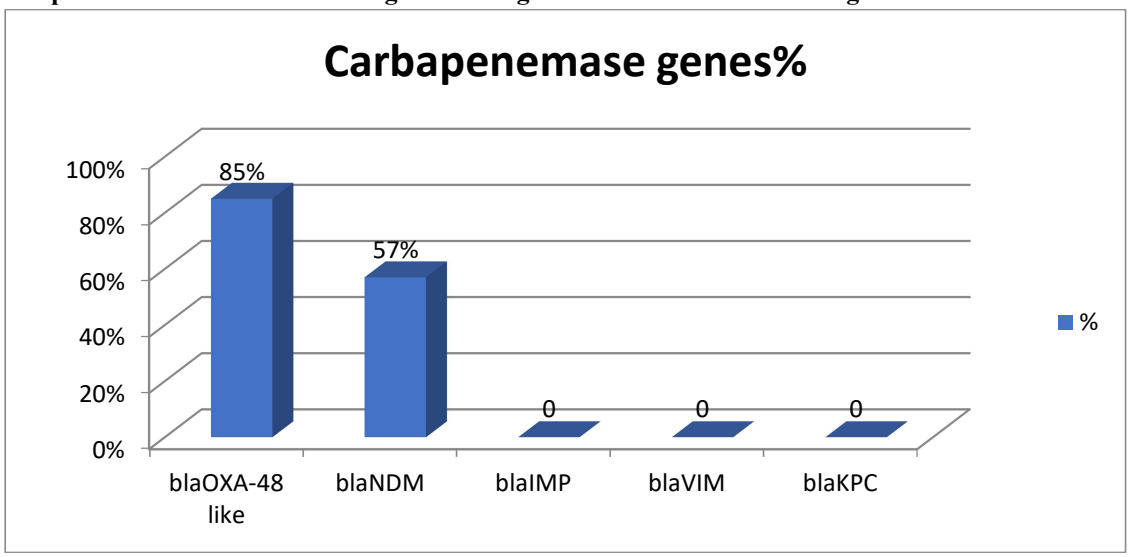
Graph. 1: Graphical illustration – Antimicrobial resistance percentage n= 200



Graph. 2: Specimen wise Antimicrobial Resistance - Comparison between cKp(n=171) and hvKp (n=19).



Graph. 3 - Distribution of Carba genes among 27 CR-KP strains Percentage wise



Graph.4 - Distribution of Virulence genes among 27 CR-KP strains – Percentage wise

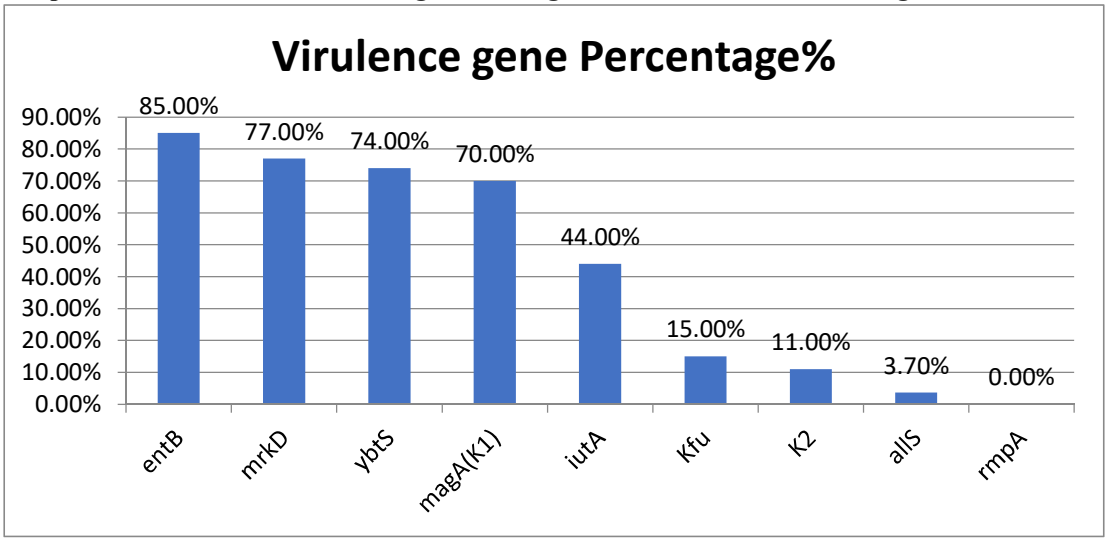


Fig. 5 Gel electrophoresis images

a) Carbapenemase genes

b) Virulence genes

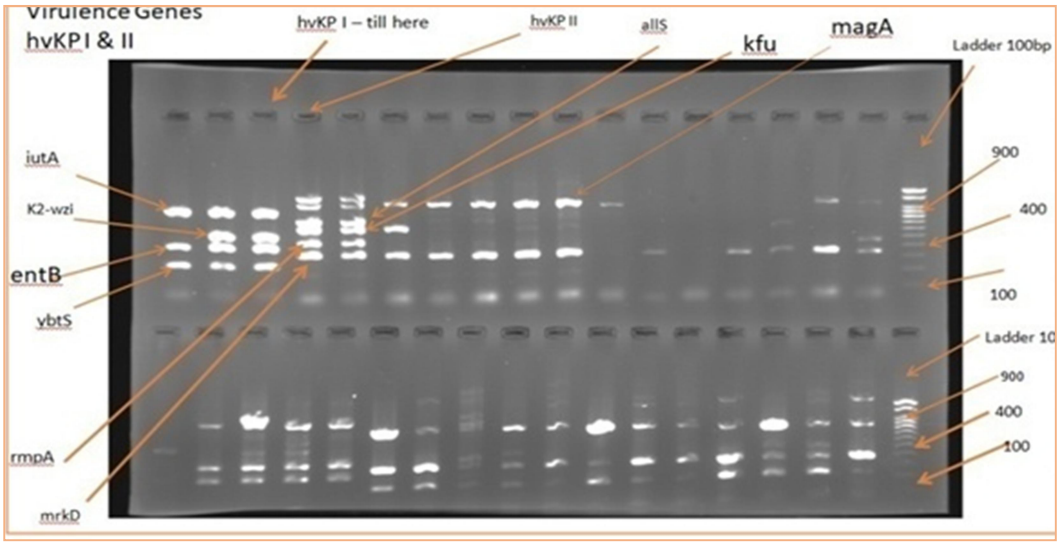


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Table 1 - CR-KP genes - Sequences and PCR cycling conditions.

Genes	Primers	PCR cycling Conditions	Product Size (bp)	Reference
***bla*$_{IMP}$**	GGAATAGAGTGGCTTAAYTCTC	Initial Denaturation 95℃ - 15min Amplification 30 cycles at 94 ℃ for 30 sec Annealing 59℃ for 1.5 Min Initial elongation 72℃ for 1.5 Min Final elongation 72 ̊c for 10 Min	232	Albasha AM et al., 2020 [10]
	GGTTTAAYAAAACAACCACC			
***bla*$_{VIM}$**	GATGGTGTTTGGTCGCATA		390	
	CGAATGCGCAGCACCAG			
***bla*$_{OXA-48\ like}$**	TATATTGCATTAAGCAAGGG		800	
	CACACAAATACGCGCTAACC			
***bla*$_{NDM}$**	CACCTCATGTTTGAATTCGCC		984	
	CTCTGTCACATC GAAATCGC			
***bla*$_{KPC}$**	TGTCACTGTATCGCCCGTC		1011	

Table 2: Multiplex 2 - Virulence Gene Primer Sequences & Cycling Conditions

Genes	Primer sequence (5'-3')	Size (bp)	Cycling Conditions	Reference
ybtS F	GACGGAAACAGCACGGTAAA	242	Initial Denaturation 95℃ – 15 min 30 cycles at 94 ℃ for 30 sec 60 ℃ for 90 sec 72℃ – 1 Min	Albasha AM et al., 2020 [10]
ybtS R	GAGCATAATAAGGCGAAAGA			
entB F	GTCAACTGGGCCTTTGAGCCGTC	400		
entB R	TATGGGCGTAAACGCCGGTGAT			
K2(wzi)-F	CAACCATGGTGGTCGATTAG	531		
K2(wzi)-R	TGGTAGCCATATCCCTTTGG			
iutA F	GGGAAAGGCTTCTCTGCCAT	920		
iutA R	TTATTCGCCACCACGCTCTT			

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			72℃ – 10 Min	

Table 3: Multiplex 3 -Virulence Gene Primer Sequences & Cycling Conditions

Gene	Primer sequence (5'-3')	Size (bp)	Cycling Conditions	Reference
mrkD F	AAGCTATCGCTGTACTTCCGGCA	340	Initial Denaturation 95 ℃ – 15 min 30 cycles at 94 ℃ for 30 sec 60 ℃ for 90 sec 72℃ – 1 Min 72℃ – 10 Min	Albasha AM et al., 2020 [10]
mrkD R	GGCGTTGGCGCTCAGATAGG			
rmpA F	CATAAGAGTATTGGTTGACAG	461		
rmpA R	CTTGCATGAGCCATCTTTCA			
Kfu F	GGCCTTTGTCCAGAGCTACG	638		
Kfu R	GGGTCTGGCGCAGAGTATGC			
Alls F	CATTACGCACCTTTGTCAGC	764		
alls R	GAATGTGTCGGCGATCAGCTT			
magA F	GGTGCTCTTTACATCATTGC	1283		
magA R	GCAATGGCCATTTGCGTTAG			

Table 4: A Comparative virulence gene expression in CSKP and CRKP. Susceptible hvKp virulence genes also detailed.

Virulence gene	Total Virulence (n=121)	Virulence in ESBL producers (n=50)(cKp 42, hvkP 8) CSKP	Virulence in Amp C producers (n=34) (cKp 31, hvKp 3) CSKP	Virulence in Carbapene mase producers (n=27) (cKp 27) CRKP	Virulence in Susceptible strains of (8 hvKP) Total (n=8)
ybtS (siderophore)	**55.1 % (66)**	**48 % (24)**	**44.1 % (15)**	**74 % (20)**	**100 % (9)**
entB (siderophore)	**63.1 % (76)**	**56 % (28)**	**55.8 % (19)**	**85 % (23)**	**50% (4)**
iutA (siderophore)	**48.9% (59)**	**64 % (32)**	**29.4 % (10)**	**44 % (12)**	**37.5 % (3)**

kfu (Iron acquisition))	**17.6% (21)**	**14 %(7)**	**26.5 % (9)**	**15 %(4)**	**37.5 %(3)**
mrkD (Type 3 fimbirial adhesion)	**66.7% (80)**	**62 % (31)**	**44.1 % (15)**	**77 %(21)**	**100 %(8)**
allS (allantoin metabolism)	**11.5% (14)**	**14 % (7)**	**11.7 % (4)**	**3.7% (1)**	**25 % (2)**
rmpA (hypervirulence)	**19.1% (23)**	**14 % (7)**	**11.7%(4)**	**0**	**100% (8)**
magA(K1) (Capsule)	**56.5% (68)**	**57.8% (27)**	**52.9% (18)**	**70.0% (19)**	**62.5% (5)**
K2(wzi) (Capsule)	**20.2% (24)**	**20.0 % (10)**	**14.7 %(5)**	**11 % (3)**	**50 % (4)**

Among the 27 carbapenem-resistant isolates, all demonstrated positive results by the modified carbapenem inactivation method (mCIM), confirming carbapenemase production. Differentiation using the extended carbapenem inactivation method (eCIM) indicated that 15 isolates produced metallo-β-lactamases, while 12 produced non-metallocarbapenemases. This distribution reflects the coexistence of class B and class D enzymes in the study population.

Multiplex PCR revealed that the prevalence of blaOXA-48-like was the most common carbapenemase determinant, detected in 23 of the 27 CRKP isolates (85%). The blaNDM gene was present in 15 isolates (57%). Single blaOXA-48-like gene was seen in 9 strains, Single blaNDM was detected in only 1 strain. Notably, 14 isolates carried both blaOXA-48-like and blaNDM, suggesting co-carriage of resistance plasmids. Neither blaKPC, blaIMP, nor blaVIM was identified in the study cohort(Graph. 3). The highest virulence gene expression seen is entB (85%), ybtS (74%), mrkD(77%) followed by iutA(44%) and majority of them belong to K1 serotype (70%). NDM gene carriers have the highest expression of ybtS than Oxa-48 bearers (Graph. 4)

Analysis of virulence markers revealed widespread gene carriage among the resistant isolates. The siderophore biosynthesis gene entB was present in 23 isolates (85%), while mrkD, associated with fimbrial adhesion, was detected in 21 isolates (77%). The magA gene, linked to the K1 serotype, was identified in 19 isolates (70%). The yersiniabactin synthesis gene ybtS was present in 20 isolates (74%), and the aerobactin receptor gene iutA was identified in 12 isolates (44%). Additional determinants included kfu (4 isolates, 15%), K2 serotype (3 isolates, 11%), allS (1 isolate, 3.7%) and rmpA is absent in all the CRKP tested strains. Capsular serotyping confirmed the presence of K1 in 19 isolates (70%) and K2 in 3 isolates (11%) (Graph. 4). Despite the detection of these serotypes, no isolate met the full criteria for hypervirulent CRKP, as defined by the presence of the complete set of hypervirulence markers (rmpA, rmpA2, iucA, peg-344). Thus, while isolates exhibited partial virulence traits, none were classified as hvKp-CRKP.

The analysis of 9 virulence genes in CRKP showed that 1 strain did not show any virulence gene, Single gene was found in 02 strains, two genes were found in 01 strain, three genes in 07 strains, four genes in 10 strains which is more prevalent, five genes in 4 strains and six genes except K2, allS, kfu, rmpA were found in 2 strains (2 cKp). None of the strains had virulence genes of 7 and above.

It is observed that Multi Drug Resistant (MDR) CSKP (ESBL,AmpC) and CRKP had more virulence genes than the non MDR cKp strains, Whereas there is no significant difference between MDR and Susceptible hvKp

strains carrying virulence genes. More over susceptible hvKp strains carried more number of virulent genes is an alarming indication. The majority of resistant strains CSKP (ESBL, AmpC) and CRKP detected with 3 to 5 virulence genes where as Susceptible cKp strains showed lesser number. The majority of MDR and susceptible strains of hvKp strains are detected with 4 to 7 virulent genes (Fig. 5). A comparative analysis is given in table 4.

Overall, 13.5% of *K. pneumoniae* isolates were carbapenem-resistant, predominantly mediated by blaOXA-48-like and blaNDM. These isolates also carried multiple virulence genes, with frequent detection of siderophore and adhesion factors. The coexistence of carbapenem resistance with virulence determinants, particularly in K1/K2 capsular backgrounds, underscores the potential for future emergence of hvKp-CRKP strains in this setting.

Discussion

This study provides comprehensive insights into the resistance and virulence landscape of CRKP in southern India.Our findings highlight the predominance of blaOXA-48-like and blaNDM genes, consistent with earlier reports from India[12] and neighboring regions[13]. The absence of blaKPC mirrors the regional epidemiology, where blaKPC remains uncommon compared to North America and parts of Europe[15,16]. The detection of isolates carrying both blaOXA-48-like and blaNDM suggests the emergence of high-risk clones capable of disseminating resistance plasmids, which complicates therapeutic decision-making[17].The widespread presence of siderophore-related genes (entB, ybtS, iutA) and adhesion genes (mrkD) which is much higher in CRKP than CSKP indicates that CRKP isolates retain significant pathogenic potential, aligning with studies from China and the Middle East[18],[19]. The detection of K1 and K2 capsular types alongside carbapenemase genes is worrisome, as such genetic convergence could facilitate the emergence of hvKp-CRKP super-strains[20]. The majority of MDR strains CSKP (ESBL, AmpC) and CRKP detected with 3 to 5 virulence genes where as Susceptible cKp strains showed lesser number and the majority of MDR and susceptible strains of hvKp strains are detected with 4 to 7 virulent genes While none of our hvKp isolates met the criteria for CRKP which lacks the rmpA gene expression signifies the less prevalence[14]. The genetic background suggests that continued surveillance is essential. Colistin was the only drug with consistent activity against CRKP in this study, corroborating previous Indian reports[11]. However, colistin use is limited by nephrotoxicity and emerging resistance[12].

Conclusion

The lack of effective alternatives underscores the urgent need for novel antimicrobials, β-lactamase inhibitors, and non-traditional therapies such as bacteriophages and antimicrobial peptides. The findings highlight the importance of antimicrobial stewardship programs, strict infection-control practices, and molecular epidemiology to track transmission dynamics. Whole-genome sequencing should be prioritized in future studies to identify clonal lineages and resistance plasmid structures.This study was limited by the absence of genome sequencing and virulence expression assays. Nevertheless, it provides valuable data on the molecular epidemiology of CRKP in India, complementing existing regional and global surveillance efforts.

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