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Chromosomal Mechanisms of Colistin Resistance in Clinical Isolates of Carbapenem-Resistant *Klebsiella pneumoniae* from a Tunisian Tertiary-Care Hospital

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Abstract

Background/Objectives: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a major nosocomial pathogen. Although newer agents have reduced colistin use in high-income countries, this polymyxin remains important in many low- and middle-income settings. Colistin resistance in *K. pneumoniae* is mainly driven by chromosomal changes in the MgrB–PhoPQ pathway or plasmid-mediated *mcr* genes. This study aimed to investigate chromosomally mediated colistin resistance in CRKP clinical isolates from a Tunisian tertiary hospital. **Methods:** Between 2010 and 2015, 317 non-duplicate CRKP isolates were collected at Charles Nicolle Hospital, Tunis. Colistin MICs were determined by broth microdilution. Phenotypic tests and PCR characterized carbapenemases, extended-spectrum β-lactamases, AmpC, plasmid-mediated quinolone resistance, *mcr* and virulence genes. Porins (OmpK35/OmpK36) and the *mgrB, phoP* and *phoQ* loci were analyzed by SDS-PAGE and sequencing. Clonal relatedness was assessed by ERIC-PCR and multilocus sequence typing. **Results:** Five isolates (1.6%) were colistin-resistant. All were multidrug-resistant, produced OXA-48 and two also carried NDM-1. The isolates belonged to five distinct sequence types, including high-risk clones (ST11, ST101, ST147). No *mcr* genes were detected. Four isolates carried disruptive mutations in *mgrB*, and the remaining strain harbored inactivating mutations in both *phoP* and *phoQ* with an intact *mgrB*. Truncating alterations in PhoP/PhoQ and frequent loss or truncation of OmpK35/OmpK36 were observed. **Conclusions:** In Tunisian CRKP, colistin resistance was mediated by chromosomal alterations, primarily disruption of the MgrB–PhoPQ pathway, in the absence of *mcr* genes. These mechanisms in both high-risk and emerging sequence types underscore the adaptability of CRKP and the need for surveillance where colistin remains an important therapeutic option.

Keywords: colistin resistance; *mgrB*; *phoP*; *phoQ*; carbapenemase; porins; multidrug resistance; virulence factors

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1. Introduction

Antimicrobial resistance (AMR) is a global public-health crisis, and carbapenem-resistant *Klebsiella pneumoniae* (CRKP) epitomizes the problem [1]. Over the past two decades, CRKP has spread throughout hospitals, aided by acquisition of carbapenemase genes and loss or alteration of major porins that impede β-lactam entry. Patients with CRKP infections often have few therapeutic options, so polymyxins such as colistin (polymyxin E) are used as last-resort drugs [2].

Colistin's bactericidal action involves binding to the negatively charged lipid A component of lipopolysaccharide (LPS), displacing Mg^{2+}/Ca^{2+} ions and disrupting the outer membrane [3].

For many years, colistin has been used as a last-resort drug against multidrug-resistant Gram-negative pathogens, including carbapenem-resistant *K. pneumoniae* (CRKP). More recently, however, several new treatment options have become available for CRKP, such as β-lactam/β-lactamase inhibitor combinations and cefiderocol, which have displaced colistin as a first-line option in many high-income settings [4]. Nevertheless, in many low- and middle-income countries, including North African hospitals, access to these newer agents remains limited and colistin is still widely used in routine practice.

Chromosomal colistin resistance results from mutations that dysregulate lipid A modification pathways. In *K. pneumoniae*, the PhoPQ and PmrAB two-component systems (TCSs) sense environmental signals and control expression of the pmrCAB and arnBCADTEF (pmrHFIJKLM) operons responsible for adding 4-amino-4-deoxy-L-arabinose (L-Ara4N) or PEtN to lipid A. Disruptive mutations in the *mgrB* gene (a 47-amino acid negative regulator of PhoPQ) remove feedback inhibition, leading to constitutive activation of PhoPQ and PmrAB and up-regulation of LPS-modifying enzymes. Similarly, activating mutations in *phoP*/*phoQ*, *pmrA*/*pmrB*,or *crrA*/*crrB* can up-regulate these operons; the net effect is lipid A modification and reduced colistin binding. These chromosomal changes are stable and cannot be transferred horizontally, but they can accumulate within clonal lineages [3].

Beyond colistin, CRKP often carries multiple β-lactamases, aminoglycoside-modifying enzymes and plasmid-borne quinolone resistance genes, contributing to multidrug-resistance profiles. The combination of carbapenemase production, porin loss, and colistin-resistance mechanisms produces extensively or pan drug-resistant strains that are difficult to treat.

Tunisia has reported both plasmid-mediated *mcr* genes [5,6] and chromosomal *mgrB* mutations [7,8] among clinical *K. pneumoniae* isolates, highlighting the local public-health importance of this resistance.

This study aimed to investigate the molecular and phenotypic basis of colistin resistance in CRKP clinical isolates recovered from a Tunisian university hospital. We focused on chromosomal mechanisms involving the mgrB–PhoPQ regulatory pathway, while acknowledging that other two-component systems (such as PmrAB and CrrAB) and genome-wide changes may also contribute to colistin resistance but were not explored here due to resource constraints. In addition, because colistin resistance in CRKP usually emerges on a background of broad β-lactam resistance, we also characterized outer membrane porin alterations to better define the multidrug-resistant profile of these isolates.

2. Materials and Methods

2.1. Study Design

Between 2010 and 2015, all clinical CRKP strains were collected from various specimens at the Microbiology Laboratory of Charles Nicolle Hospital in Tunis, Tunisia. Among the collected CRKP isolates, colistin resistance was assessed by determining the minimum inhibitory concentration (MIC) using the broth microdilution method, following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [9]. All subsequent phenotypic assays, molecular characterizations, and genetic analyses were performed on the colistin-resistant CRKP strains.

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2.2. Species Identification and Antimicrobial Susceptibility Testing

Bacterial species were identified using the API20E system (BioMérieux, Marcy-l'Etoile, France) and confirmed by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) (Bruker Daltonics GmbH, Bremen, Germany).

Initially, susceptibility to amoxicillin, amoxicillin–clavulanic acid, cefoxitin, ceftazidime, cefotaxime, cephalotin, cefepime, ertapenem, imipenem, aztreonam, amikacin, tobramycin, netilmicin, nalidixic acid, ofloxacin, ciprofloxacin, fosfomycin, tetracycline, minocycline, and tigecycline was determined by disk diffusion using Mueller-Hinton agar (Bio-Rad), according to EUCAST guidelines [9].

MICs of ertapenem, imipenem, and meropenem were determined using E-test strips (BioMérieux, France), while MICs of amoxicillin–clavulanic acid, cefepime, cefotaxime, ceftazidime, and tigecycline were assessed using the Vitek 2 system (BioMérieux). All results were interpreted according to EUCAST breakpoints and guidelines [9].

2.3. Phenotypic Assays

The modified Hodge test (MHT) was performed according to CLSI guidelines [10] using an ertapenem disk (10 μg). In addition, the Carbapenem Inactivation Method (CIM) was used to detect carbapenemase activity in all isolates, as previously described [11].

Screening for class A carbapenemases was performed using the phenylboronic acid (PBA) method. Inhibition zones obtained with imipenem disks with or without PBA (0.05 M solution) were compared after overnight incubation; an increase of ≥5 mm in the presence of PBA was interpreted as positive for class A carbapenemase production [12].

Screening for class B carbapenemases was performed by comparing inhibition zones around imipenem disks with or without ethylenediaminetetraacetic acid (EDTA, 10 μL of a 0.5 M solution) [13]. An increase in the inhibition zone of >7 mm in the presence of EDTA was considered indicative of metallo-β-lactamase production.

Phenotypic detection of extended-spectrum β-lactamases (ESBLs) was carried out by the double-disk synergy test (DDST) according to CLSI recommendations [10]. AmpC producers were defined as isolates showing a negative ESBL phenotype while being resistant to both cefoxitin and amoxicillin–clavulanic acid.

2.4. Chromosomal Modifications in LPS Biosynthesis

A targeted molecular analysis was conducted to investigate chromosomal alterations in the LPS biosynthesis pathway, focusing on the *mgrB*, *phoP*, and *phoQ* genes. These genes were amplified by polymerase chain reaction (PCR) using gene-specific primers under optimized thermal cycling conditions. The resulting PCR products were purified and subjected to Sanger sequencing.

DNA and deduced amino acid sequences were analyzed using the VECTOR NTI (Invitrogen, California, USA) and compared with the reference genome of the colistin-susceptible *K. pneumoniae* ATCC 13883 strain (GenBank accession number NZ_JOOW00000000).

2.5. Molecular Detection of Resistance Genes

Multiplex PCR assays were conducted to identify the most commonly encountered carbapenemase genes, including class A (*bla*$_{KPC}$ and *bla*$_{GES}$), class B (*bla*$_{IMP}$ and *bla*$_{VIM}$), and class D (*bla*$_{OXA\text{-}48\text{-}like}$), following previously established protocols [14]. The detection of the *bla*$_{NDM}$ gene was carried out as described in other studies [15].

In addition, screening was performed for ESBL genes (*bla*$_{CTX\text{-}M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{VEB}$, *bla*$_{GES}$, and *bla*$_{PER}$) and plasmid-mediated AmpC β-lactamase genes (*bla*$_{CIT}$, *bla*$_{MOX}$, *bla*$_{FOX}$, *bla*$_{EBC}$, *bla*$_{DHA}$, and *bla*$_{ACC}$) [14]. Furthermore, plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *oqxAB*, and *aac(6′)-Ib-cr*) were investigated [16], together with aminoglycoside resistance genes (*acc(6′)-Ib*, *aac(3′)-Ia*, *aac(3′)-IIa*, *aac(3′)-IVa*, *aph(3′)-Ia*, *aph(3′)-IIa*, *aph(3′)-VIa*, *ant(2′′)-Ia*) [17].

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The presence of plasmid-mediated colistin resistance was assessed through PCR amplification of *mcr* genes using specific primers for each target [18].

The PCR products were subsequently purified and sequenced, and the resulting DNA sequences were compared with reference nucleotide sequences available in the GenBank database.

2.6. Detection of Virulence-Associated Genes in K. pneumoniae

The virulence profile was analyzed by PCR to check for the presence of nine genes linked to virulence in *K. pneumoniae*, namely capsular serotype K1 and hypermucoviscosity phenotype (*magA*), allantoin metabolism (*allS*), regulator of mucoid phenotype A (*rmpA*), iron system capture (*iroN*), capsular serotype K2 and hypermucoviscosity phenotype (*cps*), adhesion type 3 fimbriae (*mrkD*), iron transport and phosphotransferase function (*kfu*), siderophore (*entB*), and siderophore yersiniabactin (*ybtS*) [19].

2.7. Molecular Epidemiology and Phylogenetic Analysis

Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR was used as described previously [20] to assess the genetic relatedness of the *K. pneumoniae* strains. This method targets repetitive DNA sequences within the bacterial genome, providing insight into clonal diversity.

Multilocus sequence typing (MLST) was performed using a previously standardized MLST protocol. The scheme used the following seven housekeeping genes: *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* [21]. The allelic profile was summarized by assigning a sequence type (ST) via a web database (www.pasteur.fr/recherche/genopole/PF8/mlstKpneumoniae.html).

The phylogenetic relationship among colistin-resistant CRKP isolates was inferred from the concatenated nucleotide sequences of the seven MLST housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB, and tonB) using the neighbor-joining method implemented in MEGA (version 11.0.13). Prior to tree construction, nucleotide sequences were aligned with the ClustalW algorithm. Evolutionary distances were computed using the Tamura–Nei model, and branch lengths represent the number of base substitutions per site. The tree with the optimal topology was selected automatically, and all positions containing gaps and missing data were eliminated from the final dataset (Figure 1).

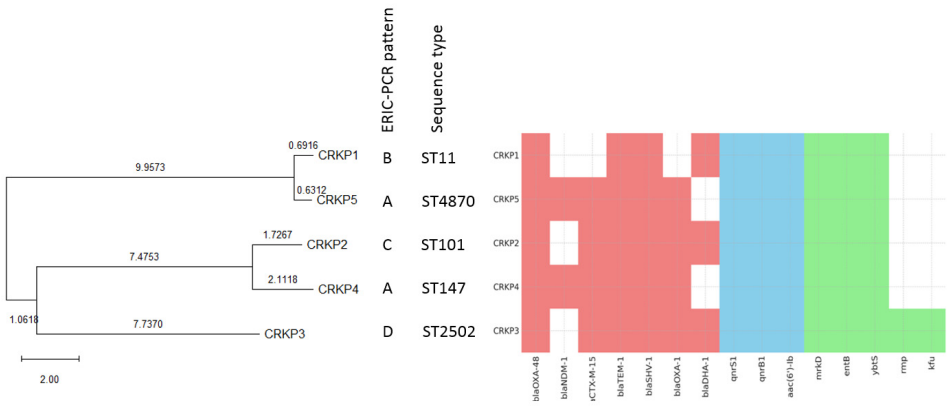

Figure 1. Phylogenetic tree of colistin-resistant CRKP isolates constructed using the neighbor-joining method in MEGA (v11.0.13) after multiple sequence alignment with ClustalW. Evolutionary distances were estimated according to the Tamura–Nei model, and branch lengths indicate the number of nucleotide substitutions per site. The dendrogram illustrates the genetic relatedness among the five CRKP isolates (CRKP1–CRKP5) and is annotated with the corresponding ERIC-PCR patterns and sequence types (STs). The heatmap aligned with the tree depicts the distribution of acquired β-lactamase genes (red color), plasmid-mediated quinolone resistance (PMQR) genes (blue color), and virulence-associated genes (green color).

2.8. Characterization of Outer Membrane Proteins and Sequencing of ompK35 and ompK36 Genes

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Outer membrane protein preparations were obtained by sonication of bacterial cells cultured in Mueller-Hinton broth, followed by selective solubilization of the cytoplasmic components with 2% sodium lauroyl-sarcosynate and ultracentrifugation. The samples were then boiled, loaded onto 11.0% sodium dodecyl sulfate-polyacrylamide gels, and stained with Coomassie blue [22]. The *ompK35* and *ompK36* genes were amplified, sequenced, and compared with the *ompK* gene sequences of *K. pneumoniae* KCTC2242, which produces OmpK35 and lacks OmpK36 (NCBI accession number CP002910), and *K. pneumoniae* NTUH-K2044, which produces OmpK36 and lacks OmpK35 (NCBI accession number AP006725), using VECTOR NTI (Invitrogen, California, USA) [23].

3. Results

3.1. Prevalence of Colistin Resistance in CRKP Isolates and Antimicrobial Resistance Profile

A total of 317 nonredundant clinical CRKP strains were collected from various wards of Charles Nicolle Hospital between 2010 and 2015. Of these, 5 isolates exhibited resistance to colistin, accounting for 1.6% of the total isolates. The colistin-resistant isolates were primarily recovered from the Intensive Care Unit (ICU) (n=4), with one isolate from the Orthopedics ward. They were recovered from different specimen types: pulmonary (n=2), catheter (n=2), and wound (n=1).

The patients' ages ranged from 24 to 84 years, with 4 of the 5 patients being male. They were admitted either for respiratory distress or polytrauma. Three of these patients were treated during hospitalization with a combination of colistin and imipenem. Besides, combinations of different antibiotic families, namely aminoglycosides (such as amikacin or gentamicin) and fluoroquinolones (mainly ciprofloxacin), in association with imipenem, were used. Despite antibiotic treatment, 1 patient died during hospitalization (Table 1).

Table 1. Clinical characteristics, treatment, and outcomes of patients infected with colistin-resistant CRKP.

Patient	Strain ID	Ward	Specimen Type	Isolation Date	Age (Years)/Gender	Underlying Disease	Antibiotic Treatment	Outcome
1	CRKP 1	ICU[1]	Pulmonary	2013	27/M[2]	Respiratory distress	Tigecycline, Gentamicin, Ceftazidime	Improved
2	CRKP 2	Orthopedics	Catheter	2015	84/F[3]	Polytraumatism	Amoxicillin+clavulanate	Improved
3	CRKP 3	ICU[1]	Catheter	2015	75/M[2]	Respiratory distress	Vancomycin, Imipenem, Rifampin, Amikacin, Colistin, Fosfomycin	Died
4	CRKP 4	ICU[1]	Pulmonary	2015	32/M[2]	Polytraumatism	Gentamicin, Imipenem, Colistin, Vancomycin, Ciprofloxacin	Improved
5	CRKP 5	ICU	Wound	2015	24/M[2]	Polytraumatism	Amoxicillin+clavulanate, Gentamicin, Imipenem, Fosfomycin, Colistin, Ciprofloxacin	Improved

[1] ICU, Intensive Care Unit; [2] M, Male; [3] F, Female; **A.** Nucleotide alignment; **A1.** *mgrB* gene.

All isolates were confirmed as *K. pneumoniae* by both API20E and MALDI-TOF. Antimicrobial susceptibility testing by disk diffusion revealed resistance to all tested penicillins and cephalosporins,

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as well as fluoroquinolones and gentamicin; two isolates were non-susceptible to tigecycline based on MICs obtained with the VITEK2 system (Table 2).

E-test results confirmed resistance to ertapenem for all five isolates, with MICs ranging from 2 to >32 mg/L. Two isolates were resistant to imipenem (MICs 2–24 mg/L), and three were resistant to meropenem (MICs 1–24 mg/L). All isolates exhibited colistin resistance, with MICs ranging from 8 to 32 mg/L (Table 2). Furthermore, all isolates tested positive for carbapenemase production using both MHT and CIM. EDTA-based synergy testing was positive for the two *bla*$_{NDM-1}$-producing isolates and negative for the remaining strains, whereas no isolate showed a ≥5-mm increase in imipenem inhibition zone in the presence of phenylboronic acid, in keeping with the absence of KPC-type carbapenemases (Table 2).

3.2. Characterization of Antimicrobial Resistance Genes

Molecular fingerprints grouped the 5 isolates into 4 distinct patterns: A (2 strains), B (1 strain), C (1 strain), and D (1 strain). Furthermore, the MLST results revealed the presence of 5 different sequence types (STs): ST11, ST101, ST2502, ST147, and ST4870 (Figure 1).

The phylogenetic analysis of the five colistin-resistant CRKP isolates revealed two main clusters. CRKP1 and CRKP5 grouped closely together, whereas CRKP2 and CRKP4 formed a separate sub-cluster, and CRKP3 appeared more distant from the other isolates (Figure 1).

All strains carried the *bla*$_{OXA-48}$ gene, and *bla*$_{NDM-1}$ was additionally detected in two strains (CRKP4 and CRKP5). The extended-spectrum β-lactamase (ESBL) *bla*$_{CTX-M-15}$ was present in all strains except for CRKP1, which harbored the AmpC β-lactamase *bla*$_{DHA-1}$. Other β-lactamase genes identified included *bla*$_{TEM-1}$, *bla*$_{SHV-1}$, and *bla*$_{OXA-1}$, along with plasmid-mediated quinolone resistance genes *qnrS1*, *qnrB1*, and *aac(6')-Ib-cr* in certain strains. No aminoglycoside resistance genes were detected. No plasmid-mediated colistin resistance genes (*mcr*) were detected. Three virulence-associated genes, *mrkD*, *entB*, and *ybtS*, were present in all strains, while *rmp* and *kfu* were exclusively found in CRKP3 (Figure 1).

3.3. Porin Expression and ompk35/ompk36 Alterations

SDS-PAGE analysis showed that all strains expressed a ~32 kDa protein corresponding to the structural protein OmpA but failed to express a full complement of porins compared to the positive controls *K. pneumoniae* KCTC2242 (expressing OmpK35 but lacking OmpK36) and *K. pneumoniae* NTUH-K2044 (expressing OmpK36 but lacking OmpK35) (NCBI accession numbers CP002910 and AP006725, respectively).

Notably, three isolates, CRKP1, CRKP2, and CRKP3 (belonging to pulsotypes B/ST11, C/ST101 and D/ST2502, respectively), lacked the ~39 kDa band corresponding to the major porin OmpK35.

Sequence analysis of *ompK35* in CRKP1 revealed the insertion of an ISKpn14 element (780 bp) located 852 nucleotides upstream of the start codon. This IS1-family insertion, positioned within the putative promoter region, is likely responsible for the absence of detectable OmpK35 among outer membrane proteins (Table 3).

In CRKP2 (pulsotype C, ST101) and CRKP3 (pulsotype D, ST2502), sequencing of *ompK35* followed by alignment with the reference strain *K. pneumoniae* KCTC2242 identified multiple alterations. These included point mutations (C156T in CRKP3), deletions [a six-nucleotide deletion (5'-CACCAA-3') at position 137 in CRKP2 and a single-nucleotide deletion at position 184 in CRKP3], and a single-nucleotide insertion at position 166 in CRKP2. Collectively, these changes introduced premature stop codons, leading to truncated OmpK35 proteins of 314 amino acids in CRKP2 and only 62 amino acids in CRKP3, compared with the full-length 359-amino acid protein in the reference strain (Table 3).

In the CRKP4 strain (pulsotype A, ST147), sequence analysis of the *ompK36* gene revealed several point mutations as well as the insertion of a nine-nucleotide sequence [5'-CTGTCTCCT-3'] located at position 550 upstream of the start codon. These genetic alterations result in a protein sequence that

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diverges from that of the reference strain *K. pneumoniae* NTUH-K2044. Consistent with these findings, no detectable OmpK36 band was observed in SDS-PAGE profiles(Table 3).

In the CRKP5 strain (pulsotype B, ST4870), two major mutations were identified: the insertion of a guanine at position 65 and a thymine at position 129, both upstream of the start codon. These frameshift events introduce a premature stop codon, leading to the production of a truncated protein of only 22 amino acids instead of the full-length 365-residue OmpK36 (Table 3).

3.4. Chromosome-Mediated Colistin Resistance: Mutations in mgrB, phoP and phoQ

Sequencing of colistin resistance-associated genes (*mgrB*, *phoP*, and *phoQ*) revealed multiple alterations across the five CRKP isolates (Table 4; Figure 2).

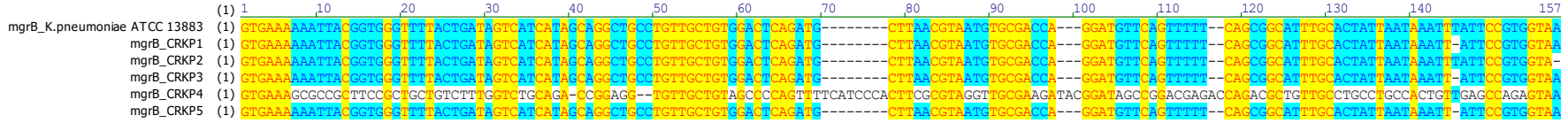
Analysis of the *mgrB* gene revealed distinct alterations among the CRKP isolates. Three isolates (CRKP1, CRKP3, and CRKP5) carried a deletion at nucleotide 132, resulting in frameshift events and altered protein sequences. CRKP4 exhibited multiple point mutations and nucleotide deletions (ΔA42, ΔCC48–49), leading to a premature stop codon at amino acid 19. In contrast, CRKP2 showed no detectable alterations, maintaining an intact *mgrB* sequence. Phylogenetic analysis supported these observations, with CRKP1, CRKP2, CRKP3, and CRKP5 clustering closely with the reference *K. pneumoniae* ATCC 13883. CRKP4 was the most divergent isolate, forming a separate branch due to its unique disruptive mutations and premature stop codon.

Comparative analysis of the *phoP* gene revealed heterogeneous mutational profiles among the five CRKP isolates. CRKP1 carried three nucleotide changes (C29T, C31A, C363T) leading to amino acid substitutions. CRKP2 showed mutations (G57C, T457G, G554C), also resulting in amino acid substitutions without evidence of truncation. CRKP3 harbored three substitutions (A471T, C510A, and C537T) predicted to alter the amino acid sequence. In contrast, CRKP4 exhibited a complex mutation pattern (insertion of nucleotide A at position 7, C32T, C47A, C112A, insertion of nucleotide A at position 143), generating a premature stop codon at amino acid 7, consistent with a truncated non-functional protein. Finally, CRKP5 displayed two substitutions (T17A and C31G) affecting the N-terminal region.

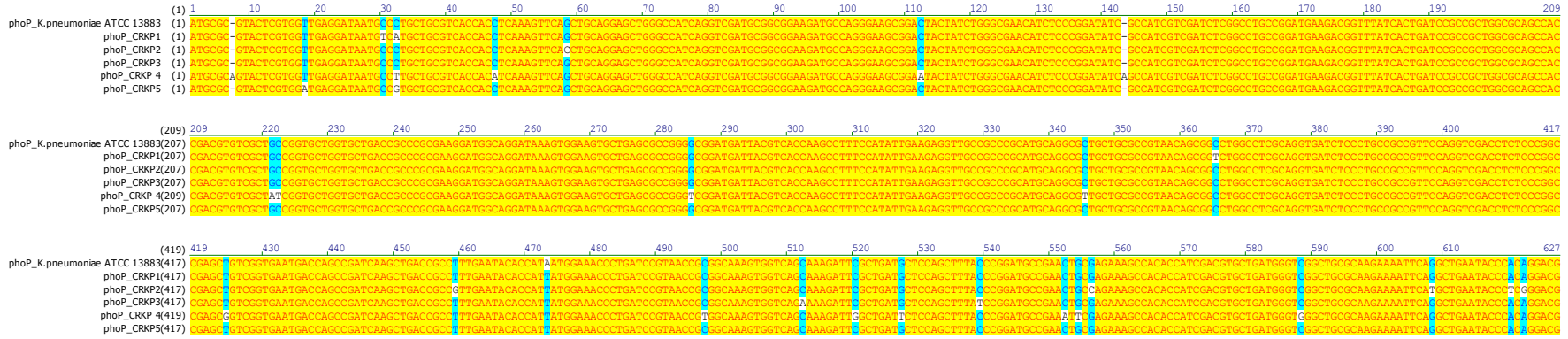
Phylogenetic reconstruction supported these findings: CRKP1 and CRKP5 clustered closely, reflecting their related mutation profiles, while CRKP2 grouped separately but remained relatively close to the reference strain**.** CRKP3 branched independently, consistent with its distinct substitutions. In contrast, CRKP4 was the most divergent isolate, clearly separated from both the reference and the other strains, in line with its severely truncated protein sequence.

Analysis of the *phoQ* gene revealed multiple disruptive events across the studied CRKP isolates. CRKP1 carried combined mutations (C219T, InsA486, InsGC498-499), which introduced a premature stop codon at amino acid 175. CRKP2 harbored an extensive set of substitutions and indels (T32G, C35G, A36C, T37A, T38G, ΔC48, InsA58, InsAA65-66, ΔC91) that generated a premature stop codon at amino acid 41, predicting a severely truncated PhoQ protein. CRKP3 exhibited frameshift-inducing mutations (ΔA5, G29C, T130A, ΔC611), leading to an altered protein sequence. The most severe alterations were found in CRKP4, with a cluster of substitutions and deletions (C10T, T11G, G12A, T14C, G15T, G17T, C19T, A20T, ΔT34, ΔGCC105-106-107) that produced a premature stop codon at amino acid 4, consistent with complete loss of function. Finally, CRKP5 carried multiple substitutions and indels (C219T, C512A, C517A, ΔG527, InsT538), resulting in a premature stop codon at amino acid 207.

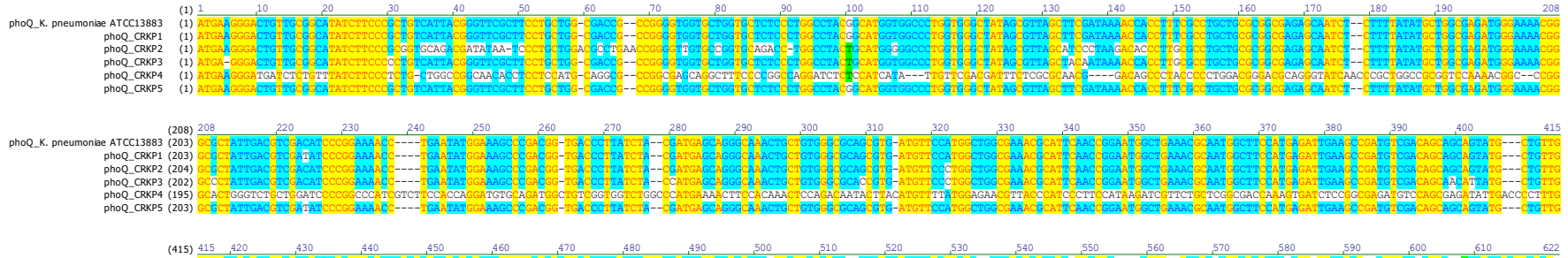
Phylogenetic reconstruction further highlighted these differences: CRKP1, CRKP2, and CRKP3 formed distinct but relatively close clusters. CRKP5 remained closer to the reference strain but branched separately, consistent with its partial truncation. In contrast, CRKP4 was the most divergent isolate, clearly separated from all other strains and the reference, in agreement with its early stop codon at position 4.

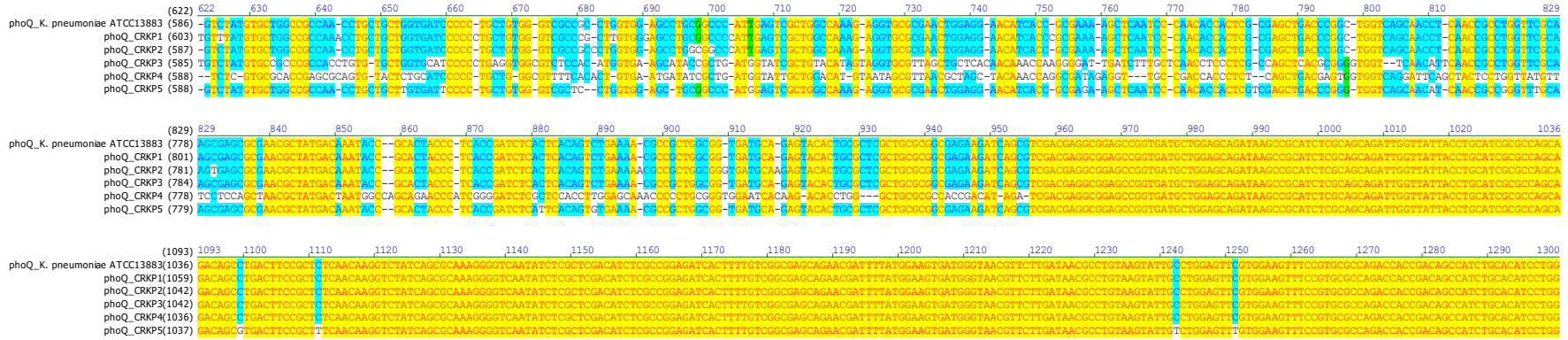


A2. *phoP* gene



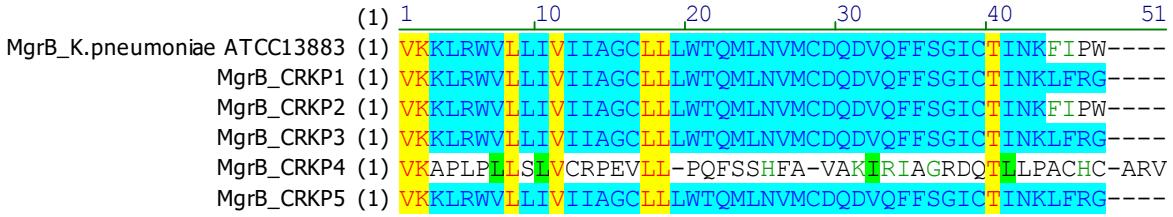
A3. *phoQ* gene



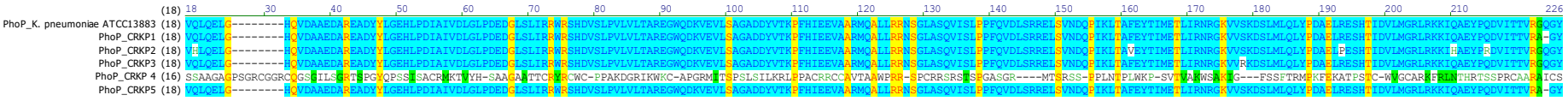


B. Amino acid alignment

B1. MgrB



B2. PhoP



B3. PhoQ

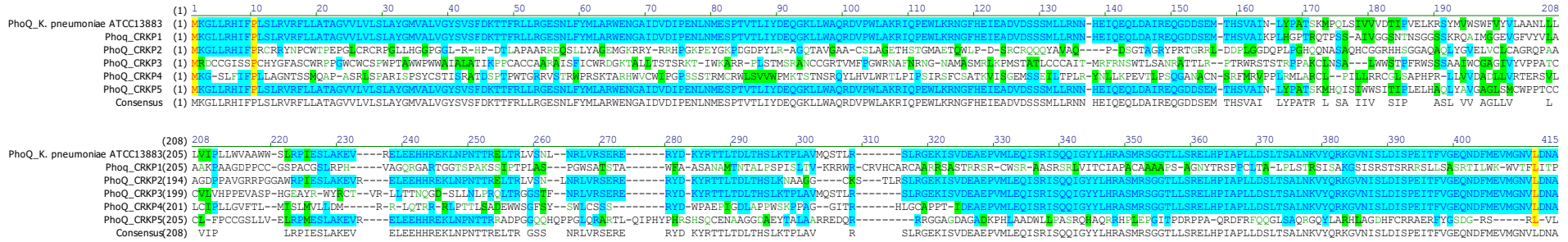


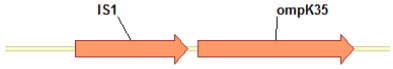
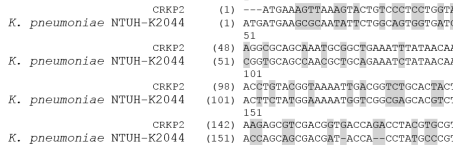
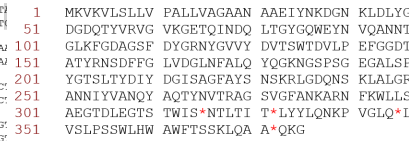
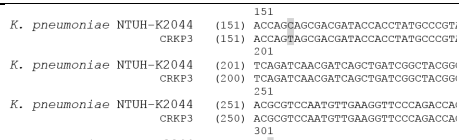
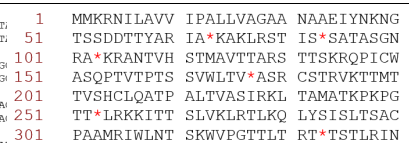
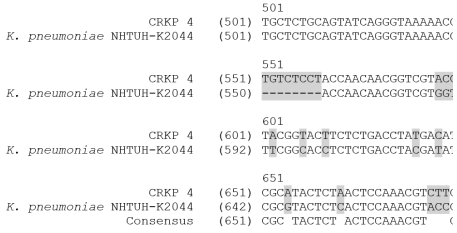
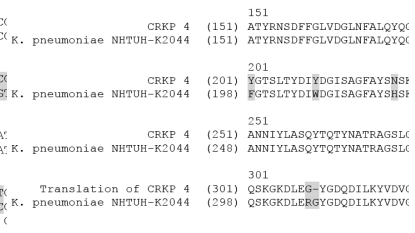
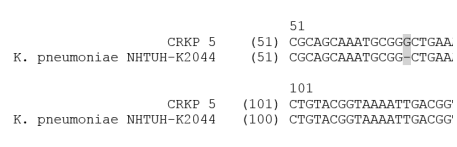
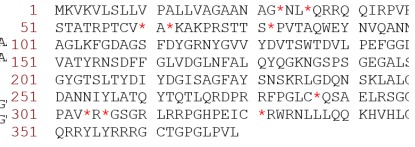
Figure 2. Multiple sequence alignments of *mgrB*, *phoP*, and *phoQ* genes and their encoded proteins in colistin-resistant CRKP strains. **A**. Nucleotide sequence alignments of **A1** *mgrB*, **A2** *phoP*, and **A3** *phoQ* genes in colistin-resistant CRKP isolates compared to the *K. pneumoniae* ATCC 13883 reference strain. **B.** Corresponding amino acid sequence alignments of **B1** MgrB, **B2** PhoP, and **B3** PhoQ proteins. Conserved regions are indicated by colored boxes, while mismatches, insertions, and deletions highlight genetic variations. Yellow indicates conserved positions across all isolates, blue highlights variable sites (at least one sequence with a differen**ce)**, and dashes represent insertions/deletions.

Table 2. Antibiotic resistance profiles of colistin-CRKP isolates.

Strain	Resistance Patterns	Phenotypic Assays				MICs*																
		MHT[1]	CIM[2]	EDTA[3]	PBA[4]	E-Test Strips			VITEK													Broth Microdilution Method
						ETP[20]	IMP[21]	MEM[22]	AMP[5]	AMC[6]	TZP[7]	CXM[8]	FOX[9]	CTX[10]	CAZ[11]	FEP[13]	AMK[14][19]	GM	NAL[15]	CIP[16]	TGC[18]	SXT[17]	COL[23]
CRKP 1	AMP[5], AMC[6], TZP[7], CXM[8], FOX[9], CTX, [10] CAZ[11], CRO[12], FEP[13], AMK[14], NAL[15], [16]CIP, SXT[17][17]	+	+	-	-	>32	24	24	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≤1	16	≤1	≥32	≥4	1	≥320	32
CRKP 2	AMP[5], AMC[6], TZP[7], CXM[8], FOX[9], CTX[10], CAZ[11], CRO[12], FEP[13], AMK[14], GM[19], NAL[15], [16]CIP, SXT[17]	+	+	-	-	>32	4	12	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	16	≥16	≥32	≥4	2	40	16
CRKP 3	AMP[5], AMC[6], TZP[7], CXM[8], FOX[9], CTX[10], CAZ[11], CRO[12], FEP[13], AMK[14], GM[19], NAL[15], [16]CIP, TGC[18], SXT[17]	+	+	-	-	>32	4	6	≥32	≥32	≥128	≥64	≥64	≥64	16	≥64	≥64	≥16	≥32	≥4	2	≥320	8
CRKP 4	AMP[5], AMC[6], TZP[7], CXM[8], FOX[9], CTX[10], CAZ[11], CRO[12], FEP[13], GM, NAL[15], [16]CIP, TGC[18], SXT[17]	+	+	+	-	32	12	16	≥32	≥32	≥128	≥64	≥64	≥64	≥64	16	8	≥16	≥32	≥4	4	≥320	8
CRKP 5	AMP[5], AMC[6], TZP[7], CXM[8], FOX[9], CTX[10], CAZ[11], CRO[12], FEP[13], GM, NAL[15], [16]CIP, TGC[18], SXT[17]	+	+	+	-	2	2	1	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	8	≥16	≥32	≥4	4	≥320	8

[1] MHT, Modified Hodge Test ; [2] CIM, Carbapenem Inactivation Method ; [3] EDTA, Ethylene Diamine Tetraacetic Acid ; [4] PBA, Phenyl Boronic Acid ; [5] AMP, Ampicillin ; [6] AMC, Amoxicillin-clavulanic acid ; [7] TZP, piperacillin–tazobactam ; [8] CXM, cefuroxim ; [9] FOX, cefoxitin ; [10] CTX, cefotaxime ; [11] CAZ, ceftazidime ; [12] CRO, ceftriaxone ; [13] FEP, cefepime ; [14] AMK, Amikacin ; [15] NAL, nalidixic acid ; [16] CIP, ciprofloxacin ; [17] SXT, sulfamethoxazole/trimethoprim ; [18] TGC, tigecycline ; [19] GM : Gentamicin ; [20] ETP, Ertapenem ; [21] IMP, Imipenem ; [22] MEM, Meropenem ; [23] COL, Colistin ; *MICs were performed according to EUCAST guidelines (mg/L).

Table 3. Porin alterations, nucleotide and protein mutations in *ompK35* and *ompK36* among colistin-resistant CRKP.

Strain	Porin Characteristics				Nucleotide Mutations	Protein Mutations
	ompK35		*ompK36*			
	SDS-PAGE result	Modification	SDS-PAGE result	Modification		
CRKP 1	-	Disruption by *IS1* at position (-852) upstream of the A of the start codon	+	NA [1]		NA
CRKP 2	-	Premature codon stop at position (+317) downstream of the A of the start codon	+	NA[1]		
CRKP 3	-	Premature codon stop at position (+63) downstream of the A of the start codon	+	NA[1]		
CRKP 4	+	NA[1]	-	Frameshift mutation at position (+178) downstream of the A of the start codon		
CRKP 5	+	NA[1]	-	Frameshift mutation at position (+178) downstream of the A of the start codon		

Mutations are highlighted in grey; premature stop codons are indicated by red asterisks; [1] NA, Not Applicable.

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Table 4. Chromosome-mediated colistin resistance: mutations in *mgrB*, *phoP* and *phoQ*.

Strain	mgrB Mutations	Predicted Effect	phoP Mutations	Predicted Effect	phoQ Mutations	Predicted Effect
CRKP1	ΔT132	Frameshift →Altered protein sequence	C29T; C31A; C363T	AA substitutions	C219T; InsA486; InsGC, 498-499	Premature stop codon at AA 175
CRKP2	None	Intact MgrB	G57C; T457G; G554C	AA substitutions	T32G; C35G; A36C; T37A; T38G; ΔC48; InsA58; InsAA65-66; ΔC91	Premature stop codon at AA 41
CRKP3	ΔT132	Frameshift → altered protein sequence	A471T; C510A; C537T	AA substitutions	ΔA5; G29C; T130A; ΔC611	Frameshift → altered protein sequence
CRKP4	Multiple point mutations; ΔA42, ΔCC48-49	Premature stop at AA19	InsA7; C32T; C47A; C112A; InsA143	Premature stop codon at AA7	C10T; T11G; G12A; T14C; G15T; G17T; C19T; A20T ; ΔT34 ; ΔGCC105-106-107	Premature stop codon at AA 4
CRKP5	ΔT132	Frameshift → altered protein sequence	T17A; C31G	AA substitutions	C219T; C512A; C517A; ΔG527; InsT538	Premature stop codon at AA 207

Δ, nucleotide deletion; Ins, nucleotide insertion; AA, amino acid.

4. Discussion

In this study, five colistin-resistant CRKP isolates were identified among 317 non-duplicate clinical strains collected over a five-year period in a Tunisian hospital.

These colistin-resistant isolates accounted for 1.6% of all CRKP strains and were mostly recovered from intensive care unit (ICU) patients. Although this prevalence is relatively low, it remains clinically concerning. Reported colistin resistance rates vary globally, with higher values in some regions, such as 39.1% in Nigeria, 22.5% in Kenya, and 19.2% in parts of Asia, while many European and North American countries report rates below 5% [24–26]. Our findings align more closely with these lower global estimates. However, since our isolates were collected between 2010 and 2015, direct comparison with more recent prevalence data should be interpreted cautiously.

Colistin resistance is primarily driven by the burden of healthcare-associated infections and extensive use of polymyxins, particularly in ICUs [27]. Resistance may also emerge spontaneously through chromosomal mutations or be acquired via horizontal gene transfer, even in the absence of prior colistin exposure [8]. In our study, patients infected with colistin-resistant CRKP ranged in age from 24 to 84 years, were predominantly male, and were admitted for severe conditions such as respiratory distress and polytrauma, clinical scenarios often requiring broad-spectrum antibiotics and invasive procedures, both risk factors for acquiring MDR infections.

Combination therapies incorporating colistin and imipenem, along with aminoglycosides and fluoroquinolones, were commonly used in our cohort, reflecting current clinical practices aimed at enhancing treatment efficacy against CRKP [28]. Although in vitro and observational studies support the synergistic potential of colistin-imipenem combinations [29], therapeutic success remains variable. In our series, only one patient died during hospitalization, suggesting a possible benefit of combination therapy, though clinical outcomes remain unpredictable in critically ill patients.

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Colistin-resistant isolates in our study exhibited resistance to multiple other clinically relevant antimicrobials, consistent with many reports documenting extensive multidrug resistance in CRKP worldwide (32, 33). Moreover, literature indicates that colistin MICs tend to be higher in isolates harboring chromosomal mutations affecting regulatory genes compared to isolates with plasmid-mediated resistance mechanisms (*mcr* genes) [32], highlighting distinct resistance dynamics between genomic and plasmid sources.

All the colistin-resistant CRKP isolates belonged to diverse sequence types (ST11, ST101, ST147, ST2502, and ST4870), reflecting substantial genomic plasticity. All carried the *bla*$_{\text{OXA-48}}$ carbapenemase gene, while most also harbored ESBL genes such as *bla*$_{\text{CTX-M-15}}$; additionally, two strains carried *bla*$_{\text{NDM-1}}$. This resistance gene profile aligns with patterns observed in other studies, where high-risk clones like ST11 and ST147 are key vectors of *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$, and the regional distribution of these enzymes can vary significantly [33–35]. Co-occurrence of OXA-48 with ESBLs, particularly *bla*$_{\text{CTX-M-15}}$, is also commonly reported [36]. Moreover, the detection of plasmid-mediated quinolone resistance genes (*qnrS1*, *qnrB1*, and *aac(6')-Ib-cr*) is consistent with previous studies showing their frequent association with ESBL- and carbapenemase-producing *K. pneumoniae*, especially in NDM- and KPC-positive strains [37]. Importantly, none of our isolates carried plasmid-mediated *mcr* genes, in line with surveillance data indicating that *mcr*-driven colistin resistance remains relatively uncommon in *K. pneumoniae* (39, 40).

Although porin loss primarily contributes to reduced susceptibility to β-lactams rather than to polymyxins, we included OmpK35/OmpK36 characterization to provide a more complete picture of the multidrug-resistant background in which chromosomal colistin resistance arises. We did not investigate other potential contributors to resistance such as efflux pumps or penicillin-binding proteins, which represent an additional limitation of our study.

Carbapenem resistance in *K. pneumoniae* is frequently associated with loss or functional alteration of the major porins OmpK35 and OmpK36, often caused by insertion sequences or mutations leading to truncated, non-functional proteins, as demonstrated in this study. These findings are consistent with Tunisian reports that highlight porin loss, in combination with carbapenemase production, as a key mechanism of resistance (25, 35). These data underscore the multifactorial nature of CRKP resistance, where both enzymatic degradation and impaired antibiotic influx through porin disruption synergistically reduce treatment efficacy.

The virulence gene profile, dominated by *entB*, *ybtS*, and *mrkD*, with *rmpA* and *kfu* detected only in one isolate, suggests that colistin-resistant CRKP typically retain baseline virulence traits but only occasionally acquire hypervirulence markers.

The absence of plasmid-mediated *mcr* genes in our isolates is consistent with prior studies suggesting that chromosomal mutations are the dominant mechanism of colistin resistance in clinical *K. pneumoniae* strains [3]. All isolates harbored disruptive mutations in the *mgrB-phoPQ* regulatory system, including insertions, deletions, or substitutions causing premature stop codons or frameshifts. One isolate (CRKP2) lacked *mgrB* alterations but presented inactivating mutations in both *phoP* and *phoQ*, suggesting that colistin resistance can emerge independently of *mgrB* disruption. Importantly, although our study did not detect insertion sequences in *mgrB*, previous studies have reported that *IS* elements are the most frequent mechanism of *mgrB* inactivation globally (41, 42). These sequences, such as ISKpn14 or IS5-like elements, often insert into the coding region or promoter of *mgrB*, thereby silencing its expression and conferring resistance [42]. Our findings support previous reports indicating that while *mgrB* inactivation is a predominant mechanism, mutations in other components of the PhoPQ system can also confer resistance (43, 44).

In our isolates, the predicted impact of the observed mutations in PhoP and PhoQ ranged from non-synonymous substitutions to very early truncations. For example, several strains carried multiple amino acid changes in PhoP or PhoQ without protein truncation, for which the functional effect on signalling and lipid A modification cannot be inferred from sequence data alone. In contrast, the premature stop codon at position 7 in PhoP in CRKP4 and the extremely early stop codon at position 4 in PhoQ in the same strain almost certainly abolish protein function. Together with the

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disruptive mutations observed in mgrB, these findings strongly suggest profound dysregulation of the PhoPQ regulatory cascade in some isolates. However, we were not able to perform functional studies such as complementation assays, lipid A profiling or gene expression analysis, so we cannot definitively distinguish between allelic variation with limited impact and mutations that directly drive the colistin-resistant phenotype.

Our study confirms that chromosomal mechanisms remain the primary drivers of colistin resistance in Tunisian CRKP and that these occur in diverse clonal lineages, both globally disseminated and locally emerging. These observations underline the importance of genomic surveillance to monitor the evolution and spread of resistance in critical-care settings.

Limitations

This study has several limitations. First, only five colistin-resistant CRKP isolates were investigated, all recovered from a single tertiary-care hospital, which limits the generalizability of our findings to other Tunisian or regional healthcare settings. Second, the isolates were collected between 2010 and 2015; they therefore reflect an earlier therapeutic context in which colistin was more frequently used against CRKP and may not fully capture the current epidemiology in the era of newer agents such as β-lactam/β-lactamase inhibitor combinations and cefiderocol. Third, our molecular analysis focused on a limited set of chromosomal loci (*mgrB*, *phoP*, *phoQ*), and we did not explore other pathways that may contribute to colistin or carbapenem resistance, such as PmrAB/CrrAB two-component systems, efflux pumps or penicillin-binding proteins. Fourth, we did not have access to whole-genome sequencing for these isolates, which would have provided a more comprehensive view of resistance determinants and mobile genetic elements. Finally, we did not perform functional assays to validate the impact of the identified mutations on lipid A modification or colistin susceptibility; as a result, the causal role of individual amino acid changes in PhoP/PhoQ remains inferred rather than experimentally demonstrated.

5. Conclusions

Our findings show that chromosomally mediated colistin resistance in CRKP isolates from a Tunisian hospital is driven primarily by inactivating mutations in the mgrB–PhoPQ pathway, in the absence of mcr genes. These mutations occur in both globally disseminated and locally emerging sequence types, underscoring the diversity and adaptability of CRKP in clinical environments. Although newer therapeutic options for CRKP have become available in recent years, colistin remains an important component of treatment in many settings, particularly where access to these agents is limited. Continuous surveillance and molecular characterization are therefore essential to detect the emergence of resistance and to inform infection control and treatment strategies.

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Institutional Review Board Statement: Ethical review and approval were not required for this study because it involved only retrospective analysis of anonymized bacterial isolates, with no identifiable human data, in accordance with institutional and national guidelines.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial resistance
CIM	Carbapenem Inactivation Method
CLSI	Clinical and Laboratory Standards Institute
CRKP	Carbapenem-resistant *Klebsiella pneumoniae*
DDST	Double Disk Synergy Test
EDTA	Ethylenediaminetetraacetic acid
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ICU	Intensive Care Unit
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization–Time of Flight
MDR	Multidrug-resistant
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
NDM	New Delhi Metallo-β-lactamase
OMP	Outer membrane protein
PBA	Phenylboronic acid
PMQR	Plasmid-mediated quinolone resistance
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ST	Sequence type
TCS	Two-component system

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