

Chromosome-Mediated Colistin Resistance in Clinical Isolates of *Klebsiella pneumoniae* and *Escherichia coli*: Mutation Analysis in the Light of Genetic Background

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Purpose: Colistin resistance mechanisms involving mutations in chromosomal genes associated with LPS modification are not completely understood. Mutations in genes coding for the MgrB regulator frequently account for colistin resistance in *Klebsiella pneumoniae*, whereas mutations in genes coding for PhoPQ and PmrAB are frequent in *E. coli*. Our aim was to perform a genetic analysis of chromosomal mutations in colistin-resistant (MIC ≥ 4 $\mu\text{g/mL}$) clinical isolates of *K. pneumoniae* (n = 8) and *E. coli* (n = 7) of different STs.

Methods: Isolates were obtained in a 3-year period in a university hospital in Santiago, Chile. Susceptibility to colistin, aminoglycosides, cephalosporins, carbapenems and ciprofloxacin was determined through broth microdilution. Whole genome sequencing was performed for all isolates and chromosomal gene sequences were compared with sequences of colistin-susceptible isolates of the same sequence types.

Results: None of the isolates carried *mcr* genes. Most of the isolates were susceptible to all the antibiotics analyzed. *E. coli* isolates were ST69, ST127, ST59, ST131 and ST14, and *K. pneumoniae* isolates were ST454, ST45, ST6293, ST380 and ST25. All the isolates had mutations in chromosomal genes analyzed. *K. pneumoniae* had mutations mainly in *mgrB* gene, whereas *E. coli* had mutations in *pmrA*, *pmrB* and *pmrE* genes. Most of the amino acid changes in LPS-modifying enzymes of colistin-resistant isolates were found in colistin-susceptible isolates of the same and/or different ST. Eleven of them were found only in colistin-resistant isolates.

Conclusion: Colistin resistance mechanisms depend on genetic background, and are due to chromosomal mutations, which implies a lower risk of transmission than plasmid-mediated genes. Colistin resistance is not associated with multidrug-resistance, nor to high-risk sequence types.

Keywords: sequence type, LPS-modifying enzymes, polymorphism vs potential mutation, MgrB regulator, PmrA-PmrB and PhoP-PhoQ three-component systems

Introduction

The increasing prevalence of multidrug-resistant Gram-negative bacteria, together with the lack of new antibiotics has led to the reintroduction of disused drugs such as colistin.¹ For many years this antibiotic was used only as a topical treatment in human medicine, because of its nephrotoxicity. In contrast, it was massively used in veterinary medicine for the treatment and prevention of infectious diseases caused by Gram-negative bacteria.² Despite its relatively recent

reintroduction in clinical practice for systemic treatment, reports of colistin-resistant isolates are on the rise worldwide.³ The first reports about colistin resistance were about mutations in chromosomal genes involved in lipopolysaccharide (LPS) synthesis and modification through addition of cationic groups to lipid A that increase the positive charge of LPS leading to reduced interaction of the outer membrane with positively charged colistin.⁴ These LPS modifications are responsible for the acquisition of resistance to this antibiotic in *Enterobacteriaceae*.⁴ The first plasmid-mediated colistin resistance gene, *mcr-1*, was described in *Escherichia coli* in 2015;⁵ Mcr-1 catalyzes the modification of lipid A and captured attention because of its dissemination risk.⁶

Chromosomal genes associated with colistin resistance are of three classes. First, genes coding for enzymes that modify LPS through addition of positively charged groups: *pmrC* that adds phosphoethanolamine (PEtN) to LPS,⁷ *pmrE* and *pmrHFIIJKLM* operon that catalyze the synthesis and union of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to LPS.⁸ Addition of L-Ara4N confers increased levels of positive charge than addition of PEtN.⁹ Second, regulatory genes encoding PhoPQ and PmrAB, two-component systems (TCSs) that in normal conditions detect environmental stimuli and modify the LPS charge accordingly. PmrB is the sensor kinase that activates PmrA, and PmrA activates transcription of *pmrC*, *pmrE* and *pmrHFIIJKLM* operon.¹⁰ Mutations in the *pmrCAB* operon have been described as the most frequent mechanism responsible for acquired resistance to colistin in *E. coli*.¹¹ PhoQ is the sensor kinase that activates PhoP, and PhoP activates *pmrHFIIJKLM* operon, *pmrA* and *pmrD* connector regulatory protein. Mutations in *phoP* and *phoQ* genes are also responsible for the acquired resistance to colistin in *K. pneumoniae* and *E. coli*.¹² Mutated TCSs no longer respond to environmental stimuli and downstream activators become constitutively overexpressed. Third, genes encoding regulators of these TCSs, namely *mgrB* and *crrAB*.¹³ MgrB inhibits the phosphatase activity of PhoQ which consequently decreases PhoP phosphorylation. Alterations in *mgrB* gene lead to derepressed activity of the PhoPQ TCS and are responsible for colistin resistance in *Klebsiella* species.⁴ Nonsense mutations leading to a truncated MgrB protein, amino-acid substitutions, insertions, deletions, or even complete deletions of the *mgrB* locus, are the most frequently reported colistin resistance mechanism in *Klebsiella*,¹⁴ but not in *E. coli*. Other chromosomal genes associated with colistin resistance in *K. pneumoniae* are *kpnE* and *kpnF*,¹⁵ *acrA* and *acrB*,¹⁶ that code for efflux pump systems.

The aim of this study was to perform a retrospective genomic analysis of colistin-resistant clinical isolates of *K. pneumoniae* and *E. coli* obtained in a university hospital in Santiago, Chile. None of the isolates harbored *mcr* genes, therefore the analysis was focused on chromosomal gene mutations and association with their genetic background.

Methods

Strains

Fifteen colistin-resistant *Enterobacteriaceae* isolates were analyzed: 8 *Klebsiella pneumoniae* (named KPN-1, KPN-3, KPN-5, KPN-6, KPN-7, KPN-10, KPN-11, KPN-20) from blood cultures, urine, peritoneal fluid, and oral mucosa, and 7 *Escherichia coli* (EC-4, EC-10, EC-11, EC-13, EC-15, EC-16, EC-19) all from urine (Table 1). They were collected from different outpatients at the Catholic University Hospital in Santiago, Chile between 2015 and 2018. Urine cultures were cultured in 5% sheep blood agar and chromogenic CPS agar. Blood specimens were inoculated in BacT/ALERT blood culture bottles (BioMerieux, France) and then subcultured on MacConkey and 5% sheep blood agar. Oral mucosa swabs and peritoneal fluid were cultured in 5% sheep blood agar, chocolate agar and McConkey agar plates. All agar plates were Biomerieux (Létolile, France). Suspicious bacterial colonies were identified through Matrix Assisted Laser Desorption – Time of Flight Mass Spectrometry (MALDI–TOF) (Bruker-Daltonics, Bremen, Germany). All the isolates were negative for carbapenemase production as determined through CarbaNP test performed according to the guidelines of Clinical and Laboratory Standards Institute 2022.¹⁷ Two colistin-susceptible controls were used, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA-1706. Colistin-susceptible isolates were obtained from the university hospital (BR1-E11, BR1-F11, BR1-C3, BR1-B1) and from the MICROB-R Network of 11 hospitals along Chile (SCL7471, SCL2906, SCL2922). Colistin-susceptible isolates of ST454, ST380 and ST127 types were obtained from PATRIC database and colistin susceptibility was predicted using Kleborate platform (for *K. pneumoniae*)¹⁸ and Resfinder platform (for *E. coli*).¹⁹ Approval from the Ethics Committee of Pontificia Universidad Católica de Chile for the use of strains isolated from human samples was obtained.

Table 1 Antimicrobial Susceptibility, Acquired Resistome and Plasmids Found in *K. pneumoniae* Isolates

| | Specimen | MLST | Resistome | Plasmid | MIC (µg/mL) (Category) | | | | | | | | | | | | |
|--------|------------------|--------|---|-----------------------------|------------------------|----------|----------|----------|------------|-----------|-----------|----------|----------|-----------|----------|--------|------------|
| | | | | | COL | FOT | A/C | CAZ | CZA | ETP | MPN | IMP | AZT | CIP | GEN | AMK | STX |
| KPN-3 | Peritoneal fluid | ST454 | blaLEN-2 | IncFII, IncFIB, IncPI | >8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | ≤0,06 (S) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| KPN-5 | Urine | ST454 | blaLEN-2 | IncFII, IncFIB, IncPI | >8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | ≤0,06 (S) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| KPN-1 | Urine | ST6293 | blaSHV-11 | None | >8 (R) | >8 (R) | 64/2 (R) | >16 (R) | ≤0,5/4 (S) | 0,25 (S) | ≤0,12 (S) | ≤0,5 (S) | >32 (R) | >2 (R) | ≥16 (R) | ≤4 (S) | >8/152 (R) |
| KPN-20 | Urine | ST6293 | blaSHV-11 | none | >8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤1 (S) | >2 (R) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| KPN-7 | Blood | ST45 | blaSHV-1 | None | >8 (R) | >8 (R) | 64/2 (R) | 16 (R) | ≤0,5/4 (S) | >2 (R) | 16 (R) | 4 (R) | >32 (R) | >2 (R) | ≥16 (R) | 32 (I) | >8/152 (R) |
| KPN-11 | Oral mucosa | ST380 | blaSHV-207 | IncFIB | >8 (R) | ≤0,5 (S) | ≤4/2 (S) | 1 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | 0,12 (S) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| KPN-6 | Urine | ST25 | aac(3)-IIa, aac(6')-Ib-cr, aadA, CTXM-15, OXA-1, OXA-10, SHV-11, catB4, cmlA1, dfrA14, qnrB19, sul2, tet(A) | IncFII, IncI1, IncR, IncFIB | >8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | 0,5 (I) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| KPN-10 | Urine | ST25 | aac(3)-IIa, aac(6')-Ib-cr, aadA, strA, strB, CTXM-15, OXA-1, OXA-10, SHV-11, TEM-1, catB4, cmlA1, dfrA14, qnrB1, qnrB19, sul2, tet(A) | IncFIB, IncR | >8 (R) | ≤0,5 (S) | 8/2 (S) | 1 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | 0,12 (S) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |

Antimicrobial Susceptibility Testing

Minimum inhibitory concentration (MIC) of colistin was determined through broth microdilution method using Sensititre Gram-Negative DKMGN Plates according to manufacturer's instructions (ThermoFisher Scientific). Overnight-grown colonies in Mueller Hinton agar plates were resuspended in PBS to obtain a McFarland turbidity standard between 0.50 and 0.55. Plates were inoculated with 10 μ L of adjusted bacterial suspension and incubated for 20 h before reading. Isolates with MIC \geq 4 μ g/mL were considered resistant and those with MIC \leq 2 μ g/mL were classified as intermediately resistant, according to CLSI 2022 guidelines. Susceptibility to amikacin, gentamicin, ertapenem, meropenem, imipenem, aztreonam, amoxicillin/clavulanate, cefotaxime, ceftazidime, ceftazidime/avibactam, trimethoprim/sulfamethoxazole and ciprofloxacin was also determined through the above-mentioned broth microdilution method using the breakpoints suggested by the Clinical Laboratory Standards Institute 2022 guidelines.¹⁷

Whole Genome Sequencing (WGS) Through Illumina Short Reads Method

Isolates were sequenced using short-read WGS (Illumina). A 350 bp insert DNA library was prepared using Illumina DNA Prep Kit (formerly Nextera Flex), following the Hackflex protocol.²⁰ Sequencing was performed in an Illumina Platform PE150, at the University of Technology Sydney's Bioscience Laboratory (Sydney, Australia). The Q30 obtained was $>$ 90% for all isolates. De novo assembly was performed using SPADIS version 3.7 package.²¹ Genomic annotation of the recovered draft genomes was performed with Prokka tool 1.11.²² MLST analysis, plasmid types and resistome were determined using Kleborate platform¹⁸ and Resfinder platform for *K. pneumoniae* and *E. coli* respectively. Final visualization was made using Galaxy-Australia platform (<https://usegalaxy.org.au/>).

Genetic Analysis of Chromosomal Genes Associated with Colistin Resistance

Sequences were compared with wild-type (WT) gene sequences using Sequencher, BLAST, and ClustalW software. The protein functionality was analyzed with SIFT algorithm (Sorting Intolerant From Tolerant) allowing a prediction of functional impact of mutations as "deleterious" (score \leq 0.05) or "neutral" (score $>$ 0.05).²³ Genome sequences described in this paper have been deposited in GenBank database under Bioproject N° PRJNA991619.

Results

Susceptibility of Isolates to Colistin and Other Antimicrobials

Clinical isolates of colistin-resistant *K. pneumoniae* (8 isolates) and *E. coli* (7 isolates) were obtained in a 3-year period in the institutional university hospital. All *K. pneumoniae* isolates had a MIC of colistin $>$ 8 μ g/mL, whereas *E. coli* isolates had a MIC of 4 (1 isolate), 8 (4 isolates) and $>$ 8 μ g/mL (2 isolates) (Table 1 and 2).

Among *K. pneumoniae* isolates, 6/8 were susceptible to all antimicrobials tested (except for isolate KPN-20 that was resistant to ciprofloxacin) and were classified as non-MDR according to Magiorakos criteria (MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories).²⁴ Two isolates were MDR: isolate KPN-7 was resistant to all antimicrobials tested except for ceftazidime/avibactam and intermediately resistant to amikacin, and isolate KPN-1 was resistant to cefotaxime, ceftazidime, amoxicillin/clavulanate, ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole (Table 1). *E. coli* isolates were all susceptible to amikacin, gentamicin, meropenem, imipenem, ertapenem and ceftazidime/avibactam. Among them, 4/7 isolates were non-MDR and 3/7 were MDR. Isolate EC-15 was resistant to cefotaxime, amoxicillin/clavulanate, ceftazidime, aztreonam, ciprofloxacin and trimethoprim/sulfamethoxazole, and isolates EC-4 and EC-19 were resistant to ciprofloxacin and trimethoprim/sulfamethoxazole (Table 2). Overall, 10/15 colistin-resistant isolates were non-MDR.

Genomic Analysis of Colistin-Resistant Isolates: Sequence Types and Phylogenetic Analysis

Among *K. pneumoniae* isolates 2 of them were *K. pneumoniae* subsp. *variicola* and 6 were *K. pneumoniae* subsp. *pneumoniae* according to Kleborate analysis.¹⁸ The STs found were ST25 (2 isolates), ST45 (1 isolate), ST454 (2

Table 2 Antimicrobial Susceptibility, Acquired Resistome and Plasmids Found in *E. coli* Isolates

| Isolate | Specimen | MLST | Resistome | Plasmids | MIC (µg/mL) (Category) | | | | | | | | | | | | |
|---------|----------|-------|---|------------------|------------------------|------------------|-----------------|---------------|------------|-----------|-----------|----------|---------------|------------------|----------|--------|----------------------|
| | | | | | COL | FOT | A/C | CAZ | CZA | ETP | MPN | IMP | AZT | CIP | GEN | AMK | STX |
| EC-4 | Urine | ST69 | aph(3'')-Ib, aph(6)-Id, TEM-1, dfr-A7, sul1, sul2, tet(A) | IncFIA IncQ | 8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | 2 (S) | >2 (R) | ≤0,5 (S) | ≤4 (S) | >8/152 (R) |
| EC-10 | Urine | ST127 | None | None | 8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | ≤0,06 (S) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| EC-11 | Urine | ST127 | None | None | 8 (R) | ≤0,5 (S) | 8/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | >2 (R) | 1 (S) | ≤4 (S) | ≤1/19 (S) |
| EC-13 | Urine | ST59 | None | None | >8 (R) | ≤0,5 (S) | 8/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | ≤0,06 (S) | ≤0,5 (S) | ≤4 (S) | >8/152 (R) |
| EC-15 | Urine | ST131 | None | None | >8 (R) | >8 (R) | 64/2 (R) | 16 (R) | ≤0,5/4 (S) | 0,25 (S) | ≤0,12 (S) | ≤0,5 (S) | 32 (R) | >2 (R) | 1 (S) | 8 (S) | >8/152 (R) |
| EC-16 | Urine | ST131 | TEM-1 | IncFIA IncFII | 8 (R) | ≤0,5 (S) | ≤4/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | ≤0,06 (S) | ≤0,5 (S) | ≤4 (S) | ≤1/19 (S) |
| EC-19 | Urine | ST14 | None | None | 4 (R) | ≤0,5 (S) | 8/2 (S) | ≤0,5 (S) | ≤0,5/4 (S) | ≤0,12 (S) | ≤0,12 (S) | ≤0,5 (S) | ≤0,5 (S) | 0,5 (I) | ≤0,5 (S) | ≤4 (S) | >8/152 (R) |

Notes: MIC for each antimicrobial was determined through broth microdilution method and categories (R: resistant; I: intermediate; S: susceptible) were assigned according to breakpoints of CLSI 2022. MICs categorized as intermediate or resistant are shown in bold letters.

Abbreviations: MLST, multi-locus sequence type; COL, colistin; FOT, cefotaxime; A/C, amoxicillin/clavulanate; CAZ, ceftazidime; CZA, ceftazidime/avibactam; ETP, ertapenem; MPN, meropenem; IMP, imipenem; AZT, aztreonam; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; STX, trimethoprim/sulfamethoxazole.

K. pneumoniae subsp. *variicola* isolates), ST380 (1 isolate) and two isolates were assigned a new ST named ST6293. This new ST is evolutionarily related to ST12. A phylogenetic tree was constructed for each species based on the core genome. Publicly available genomes from PATRIC database of the same STs were included in the analysis. Chilean *K. pneumoniae* isolates clustered together with isolates of the same STs from different countries (Figure 1A). It can be observed that two clusters were formed, one with *K. pneumoniae* subsp. *variicola* isolates and the other one with *K. pneumoniae* subsp. *pneumoniae* isolates (Figure 1A). Isolates belonging to the novel ST6293 formed a cluster close to ST12 isolates. Genomes of ST12 type were included because of their relatedness to ST6293. STs found among *E. coli* isolates were ST131 (2 isolates), ST69 (1 isolate), ST59 (1 isolate), ST14 (1 isolate) and ST127 (2 isolates) according to genomic analysis made using Resfinder in the Galaxy-Australia platform.¹⁹ The phylogenetic tree constructed based on the core genome showed that Chilean *E. coli* isolates clustered together with isolates of the same STs from different countries (Figure 1B).

Genomic Analysis: Resistome and Plasmids

mcr genes were not found in any of the isolates studied here. Analysis of the acquired resistome showed that *K. pneumoniae* subsp. *variicola* isolates had only the *bla*LEN-1 gene coding for the typical endogenous beta-lactamase whereas *K. pneumoniae* subsp. *pneumoniae* isolates had *bla*SHV genes (Table 1). ST25 isolates had additional antimicrobial resistance genes: *aac(3)-IIa*, *aac(6')-Ib-cr*, *aadA*, *CTXM-15*, *OXA-1*, *OXA-10*, *SHV-11*, *catB4*, *cmlA1*, *dfrA14*, *qnrB19*, *sul2*, *tet(A)*. All the *K. pneumoniae* isolates had plasmids belonging to incompatibility groups IncFII, IncFIB, IncP1 and IncR. In contrast, ST6293 and ST45 isolates had no plasmids according to Kleborate analysis.

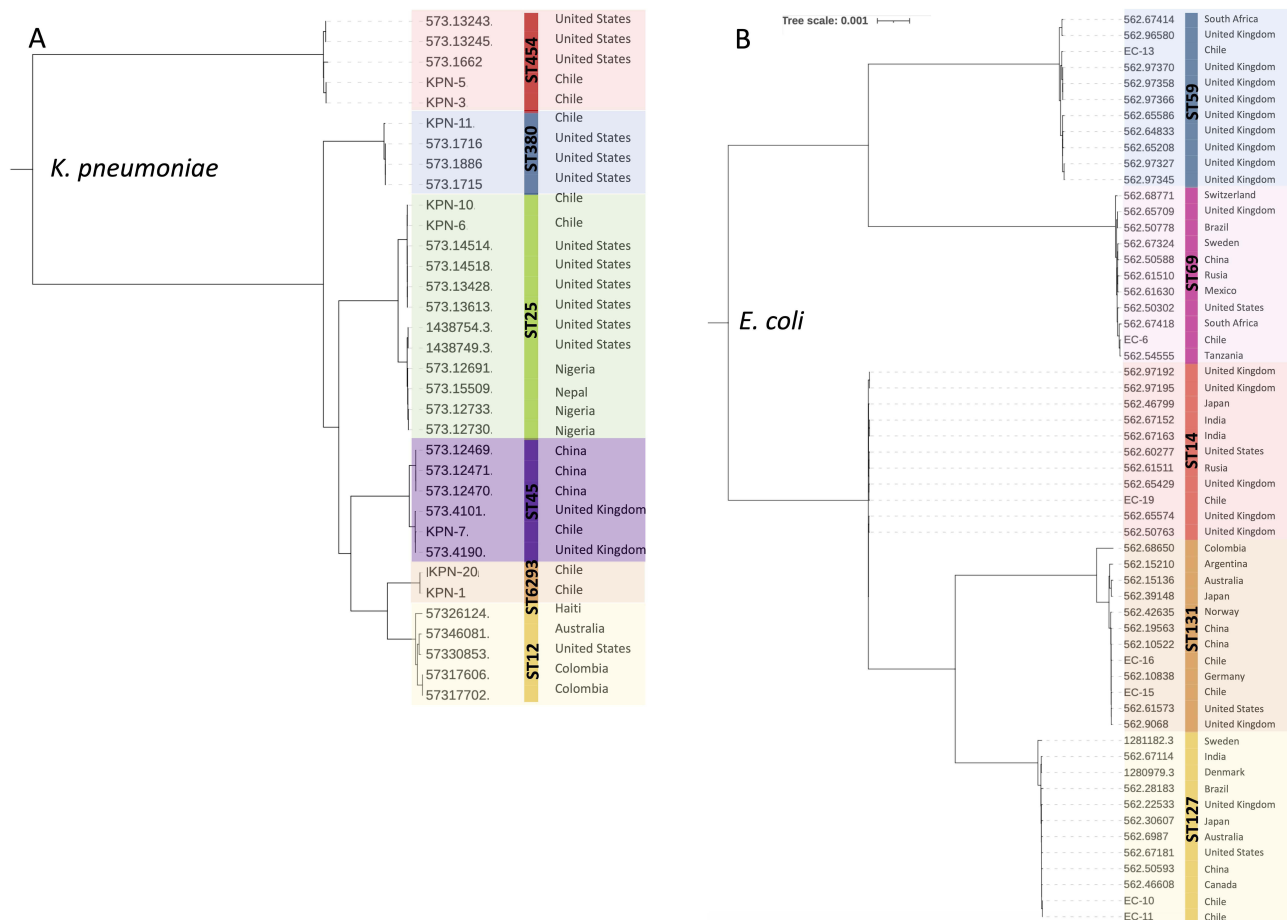


Figure 1 Phylogenetic tree based on the core genome of *K. pneumoniae* (A) and *E. coli* (B) isolates. Genomes of isolates of the same STs found in our work were randomly selected from PATRIC database and included in the phylogenetic tree. The genome ID and country of isolation are shown for each isolate. Multiple sequence alignment was performed using Clustal W and resultant output was generated with MegaX.

Five of the 7 *E. coli* isolates had no resistance genes and no plasmids. Isolate EC-16 had only *bla*_{TEM-1} gene and plasmids IncFIA and IncQ1. Isolate EC-4 had *aph(3'')*-Ib, *aph(6)*-Id, *bla*_{TEM-1B}, *df*rA7, *sul*1, *sul*2 and *tet(A)* and plasmids IncFIA and IncFII (Table 2). There was no evident correlation between resistome and antimicrobial susceptibility, e.g. isolates EC-15 and KPN-7 were MDR but had no resistance genes and only *bla*_{SHV-1} respectively, conversely isolate KPN-6 had several resistance genes including extended-spectrum beta-lactamases but was susceptible to all antimicrobials tested except colistin.

Mutations in Chromosomal Genes Involved in LPS Modification

All the isolates had mutations in chromosomal genes involved in LPS modification with respect to the WT reference strains (*K. pneumoniae* ATCC BAA-1706 and *E. coli* ATCC 25922) (Table 3 and 4). To distinguish between gene polymorphisms associated with a particular ST and potential mutations, colistin-susceptible isolates of the same STs were included in the analysis. It was observed that most of the mutations were present in susceptible isolates of the same ST and different ST as well, meaning that they are most likely gene polymorphisms. Those mutations that were not present in susceptible isolates nor in the reference ATCC strains, are shown in bold underlined letters in Table 3 and 4. *K. pneumoniae* and *E. coli* isolates have a different mutation profile in LPS-modifying enzymes: most of *K. pneumoniae* isolates had mutations in *mgrB*, whereas *E. coli* had mutations in *pmrA*, *pmrB* and *pmrE* genes. Among *K. pneumoniae* isolates, 5/8 had alterations in the *mgrB* gene whereas 3/8 had mutations in *pmr* genes. Isolates KPN-3, KPN-6 and KPN-11 had a premature stop codon in the *mgrB* gene that produced a truncated MgrB protein of 2, 22, and 28 amino acids respectively. The *mgrB* gene was not found in isolate KPN-10, and isolate KPN-5 had a point mutation R33Q predicted as deleterious through SIFT algorithm (Table 3). Among *K. pneumoniae* subsp. *pneumoniae* isolates 11 amino acid changes were found with respect to the reference strain ATCC BAA-1706, but 8 of them were present in susceptible isolates of the same and/or different STs and are considered gene polymorphisms. Only three mutations were not found in susceptible isolates: Q331P in *pmrE* gene, D21A in *pmrB* gene and L716R in *acrB* gene. Isolates KPN-1 and KPN-20 (of the novel ST6293) had the mutation Q331P in their *pmrE* gene that was predicted as neutral through SIFT algorithm. Colistin-susceptible isolate of ST12 type had 21 amino acid changes with respect to ATCC BAA-1706 reference strain, and 20 of them were not present in isolates KPN-1 and KPN-20 and are most likely polymorphisms related to this novel ST6293. Isolate KPN-7 had D21A mutation in *pmrB* gene that was predicted as neutral though SIFT analysis, but also had a deletion of *kpnE* and *pmrD* genes. Isolate KPN-6 having a truncated MgrB protein, had also mutation L716R in its *acrB* gene, predicted as neutral through SIFT analysis. Isolates KPN-3, KPN-5 (*K. pneumoniae* subsp. *variicola*), KPN-7 and KPN-11 did not have *crrA* nor *crrB* genes. The colistin-susceptible isolates of the same STs did not have these genes either, therefore we consider the absence of these genes is not involved in colistin resistance.

K. pneumoniae subsp. *variicola* isolates KPN-3 and KPN-5 had 45 mutations with respect to the reference strain ATCC BAA-1706 (Table 3), but 44 of them were also present in the colistin-susceptible isolate of *K. pneumoniae* subsp. *variicola* of the same ST, meaning that they are most likely polymorphisms associated with this subspecies; only R33Q mutation was not found in susceptible isolates.

Among *E. coli* isolates, all of them (7/7) had a WT *mgrB* gene. All the *E. coli* isolates had mutations in *pmrA*, *pmrB* or *pmrE* genes. Overall, 8 mutations were found in LPS modifying enzymes of *E. coli* isolates and are shown in bold letters in Table 4. Isolates EC-15 and EC-16 each had a point mutation in *pmrA* gene, R81H and G53S respectively. Isolates EC-10 and EC-11 had a mutation in *pmrB*, Y84C. Isolates EC-13 and EC-19 had a point mutation in the *pmrB* gene, P14L and K121E respectively. Isolate EC-4 had two mutations in *pmrE*, K58E and K373R, and L44I in *phoP* gene. Only P14L, R81H and K121E were predicted as deleterious through SIFT analysis, the rest of them were predicted as neutral (Table 3 and 4).

Discussion

The present work corresponds to the first report of chromosomal mutations in colistin-resistant isolates of *K. pneumoniae* and *E. coli* from Chile. Several mutations were found in this work. However, most of them correspond to gene polymorphisms, and only a few of them can account for colistin resistance. MgrB is a crucial regulator and their

Table 3 Amino Acid Changes in Proteins Associated With Colistin Resistance With Respect to *K. pneumoniae* ATCC BAA-1706

| | Isolate | MLST | COL | AcrA | AcrB | CrrA | CrrB | KpnE | KpnF | MgrB | PhoP | PhoQ | PmrB | PmrD | PmrA | PmrE | |
|---|-----------------------|--------|------------------|---|---------------------------------|------|------|------------------------------|----------------------|----------------|------|---|---|---|---------------------------------|--|-------|
| <i>K. pneumoniae</i> subsp. <i>varicola</i> | 244366 [#] | ST454 | (I) [#] | A188T A257S V301I G351D T355S | G23R G648A Q682E S841A | NF | NF | V12I V73L P85L V96I | V14I V36I L96V | WT | WT | K64R K92Q I196V S465G L482Q G488S | S105N S170A V175M T228A V242I S244N I247V A358G R356Q | M45I K46R Q86H K90E Q91R F97Y A102T G113S | T64S D131N E149D H219N | D354N | |
| | KPN-3 | ST454 | >8 (R) | A188T A257S V301I G351D T355S | G23R G648A Q682E S841A | NF | NF | V12I V73L P85L V96I | V14I V36I L96V | 3 stop | WT | K64R K92Q I196V S465G L482Q G488S | S105N S170A V175M T228A V242I S244N I247V A358G R356Q | M45I K46R Q86H K90E Q91R F97Y A102T G113S | T64S D131N E149D H219N | D354N | |
| | KPN-5 | ST454 | >8 (R) | A188T A257S V301I G351D T355S | G23R G648A Q682E S841A | NF | NF | V12I V73L P85L V96I | V14I V36I L96V | R33Q* | WT | K64R K92Q I196V S465G L482Q G488S | S105N S170A V175M T228A V242I S244N I247V A358G R356Q | M45I K46R Q86H K90E Q91R F97Y A102T G113S | T64S D131N E149D H219N | D354N | |
| <i>K. pneumoniae</i> subsp. <i>pneumoniae</i> | SCL747I | ST12 | ≤2 (I) | WT | S173S | WT | WT | Q112K | WT | WT | WT | WT | G256R | T20A | WT | I17V S33A E165A N172D V182D S240N G274A V333I A338V N340Q E342D P348R I350V D354N S360A | |
| | KPN-1 | ST6293 | >8 (R) | WT | G23R | WT | WT | Q112K | WT | WT | WT | WT | G256R | T20A | WT | Q331P D354N | |
| | KPN-20 | ST6293 | >8 (R) | WT | G23R | WT | WT | Q112K | WT | WT | WT | WT | G256R | T20A | WT | Q331P D354N | |
| | BRI-E11 | ST45 | ≤2 (I) | WT | WT | NF | NF | WT | WT | WT | WT | WT | G256R | WT | WT | A354N | |
| | KPN-7 | ST45 | >8 (R) | A188T | G23R | NF | NF | NF | WT | WT | WT | WT | D21A G256R | NF | WT | I17V | |
| | 573.1715 [#] | ST380 | (I) [#] | A188T | G23R | NF | NF | Q112K | WT | WT | WT | WT | Q120K T246A | WT | WT | T270N D354N | |
| | KPN-11 | ST380 | >8 (R) | A188T | G23R | NF | NF | Q112K | WT | 29 stop | WT | WT | WT | Q120K T246A | WT | WT | D354N |
| | BRI-F11 | ST25 | ≤2 (I) | WT | G23R | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT | WT |
| | KPN-6 | ST25 | >8 (R) | WT | G23R L716R | WT | WT | WT | WT | 21 stop | WT | WT | WT | WT | WT | WT | WT |
| KPN-10 | ST25 | >8 (R) | WT | G23R | WT | WT | WT | WT | NF | WT | WT | WT | WT | WT | WT | WT | |

Notes: Amino acid changes are shown as wild type amino acid, position, mutated amino acid. Mutations that are present only in colistin-resistant isolates are shown in bold letters. Stop: the position of the termination codon in MgrB is shown. WT, Wild Type sequence (respect to *K. pneumoniae* ATCC BAA-1706); NF, not found. COL: colistin MIC expressed in µg/mL (category: R: resistant or I: intermediate). *SIFT scores ≤0.05 are predicted as deleterious for protein function and SIFT scores >0.05 are predicted as neutral. SIFT scores were: R33Q: 0.00. Colistin-resistant isolates analyzed in this study are shaded light gray. Colistin-susceptible isolates used as reference sequence for each ST are shaded dark gray. [#]Genome sequences obtained from PATRIC database (Genome ID is shown); their colistin susceptibility was predicted using Kleborate platform¹⁸ for *K. pneumoniae* isolates.

Table 4 Amino Acid Changes in Proteins Associated With Colistin-Resistance With Respect to *E. coli* ATCC 25922

| Isolate | MLST | COL | MgrB | PhoP | PhoQ | PmrA | PmrB | PmrE |
|------------------------|-------|------------------|------|-------------|------|--------------------------|--------------------------------|-------------------------|
| BRI-C3 | ST69 | ≤2 (I) | V8A | WT | R6H | T3IS I128N G144S | E123D N138S V351I | V362I |
| EC-4 | ST69 | 8 (R) | V8A | I44L | R6H | T3IS I128N G144S | E123D N138S V351I | E58K R373K V362I |
| 562.46608 [#] | ST127 | (I) [#] | WT | WT | WT | T3IS I128N G144S | E123D V351I V360A | WT |
| EC-10 | ST127 | 8 (R) | WT | WT | WT | T3IS I128N G144S | Y84C E123D V351I V360A | WT |
| EC-11 | ST127 | 8 (R) | WT | WT | WT | T3IS I128N G144S | Y84C E123D V351I V360A | WT |
| SCL2906 | ST59 | ≤2 (I) | V8A | WT | R6H | T3IS I128N G144S | E123D V351I V360A | T178A |
| EC-13 | ST59 | >8 (R) | V8A | WT | R6H | I128N G144S | P14L* E123D V351I V360A | T178A |
| BRI-BI | ST131 | ≤2 (I) | WT | WT | R6H | I128N G144S | WT | T178A V362I |
| EC-15 | ST131 | >8 (R) | WT | WT | R6H | H81R* I128N G144S | WT | T178A V362I |
| EC-16 | ST131 | 8 (R) | WT | WT | R6H | S53G I128N G144S | WT | T178A V362I |
| SCL2922 | ST14 | ≤2 (I) | WT | WT | R6H | I128N G144S | WT | V362I |
| EC-19 | ST14 | 4 (R) | WT | WT | R6H | I128N G144S | K121E* | V362I |

Notes: Amino acid changes are shown as wild type amino acid, position, mutated amino acid. Mutations that are present only in colistin-resistant isolates are shown in bold letters. WT, Wild Type sequence (respect to *E. coli* ATCC 25922). COL, colistin MIC expressed in µg/mL (category: R: resistant or I: intermediate). *SIFT scores ≤ 0.05 are predicted as deleterious for protein function and SIFT scores >0.05 are predicted as neutral. SIFT scores were: P14L:0.00; K121E: 0.03; H81R: 0.00. Colistin-resistant isolates analyzed in this study are shaded light gray. Colistin-susceptible isolates used as reference sequence for each ST are shaded dark gray. [#]Genome sequences obtained from PATRIC database (Genome ID is shown); their colistin susceptibility was predicted using Resfinder platform¹⁹ for *E. coli* isolates.

mutations have been described as sufficient to increase the colistin MIC in *K. pneumoniae*.¹⁴ Hence, truncated or absent MgrB protein in 5/8 *K. pneumoniae* isolates most likely accounts for their colistin resistance; mutation R33Q in MgrB of isolate KPN-5 has been previously described in a colistin-resistant *K. pneumoniae* isolate.^{25,26} Isolates with WT *mgrB* gene have mutations or loss of *pmr* genes that may account for colistin resistance: Q331P in *pmrE* gene of KPN-1 and KPN-20, D21A in *pmrB* gene together with loss of *pmrD* gene in KPN-7. To our knowledge, these mutations have not been reported to date in colistin-resistant clinical isolates. It must be noted that mutation D21A in *pmrB* gene of KPN-5 is accompanied by loss of *kpnE* and *pmrD* genes. KpnE together with KpnF form an efflux-pump associated with resistance to several antibiotics including colistin: mutant *K. pneumoniae* lacking *kpnEF* genes reduced its MIC to colistin two-fold.¹⁵ PmrD is a transcriptional activator of the PmrAB TCS, and this activation is required for LPS modification. Although LPS modification can also be activated independently of PmrD,⁴ further experiments should be made to understand how the lack of these genes, which a priori contribute to LPS modification, affects colistin resistance. Isolate KPN-6 besides a truncated MgrB protein has the amino acid change L716R in *acrB* gene, that codes for an efflux-pump. Mutation Q331P in PmrE was the only mutation in isolates KPN-1 and KPN-20 that was not present in susceptible isolates. PmrE is the first enzyme in L-Ara4N synthesis and is required for LPS modification. Q331P is located in the binding site of NAD⁺ of PmrE enzyme and it could increase efficiency of PmrE enzyme.¹⁰ In *K. pneumoniae* genes *crrA* and *crrB* code for a regulator protein and a sensor kinase respectively.⁴ Although its role is not completely understood, inactivation of CrrB leads to overexpression of PmrAB operon and addition of positive charges to LPS.²⁷ These genes were not found in *K. pneumoniae* subsp. *variicola* isolates, nor in the colistin-susceptible isolate. The same was observed in isolates of ST45 and ST380 types. It is known that all isolates of the ST258 type have *crrAB* genes, whereas other STs do not have these genes.²⁷ It is possible that other, yet unknown genes, are involved in colistin resistance in isolates that lack *crrAB* genes.

The two-component system PmrAB is commonly the most affected in colistin-resistant *E. coli* isolates.^{4,9} Mutations H81R and S53G in PmrA were not reported to date. However, similar mutations have been described in colistin-resistant *Salmonella*, C81R and E53G,²⁸ and in *K. pneumoniae*, G81R,²⁹ and S81R.³⁰ Mutations H81R and S53G are located

close to D51 in the active site of phosphate receiver domain and mutations in these positions were shown to increase colistin MIC.²⁸ Amino acid changes P14L and K121E in PmrB were not reported to date, however, similar mutations, S14L, F14L and A121E were described in colistin-resistant *Salmonella* isolates and were genetically confirmed to be sufficient to confer colistin resistance.²⁸ These mutations are in trans-membrane-1 and linker domains of PmrB, and mutation analysis with PmrB homologs in *Salmonella* showed that amino acid changes in these domains increase kinase activity of PmrB.²⁸

Most of the amino acid changes found in this work are considered polymorphisms owing to their presence in colistin-susceptible isolates. Some of these polymorphisms, such as G256R were already reported in colistin-susceptible isolates,³¹ and in colistin-resistant isolates.³² Moreover, in a recent report, 6 of the 21 polymorphisms that we found here in PmrE were reported to be associated with colistin resistance.³³ These data highlight the importance of performing point mutation analysis of resistant isolates together with colistin-susceptible isolates of the same ST, to distinguish between gene polymorphisms and potential mutations.

The association of ST with diverse colistin resistance mechanisms in *K. pneumoniae* was previously reported.^{26,27} Azam and coworkers reported mutations in MDR and PDR isolates obtained from hospitalized patients that received colistin antibiotic therapy,²⁶ in contrast to isolates reported in this work that are mostly non-MDR and were obtained from outpatients. None of the mutations found by Azam and coworkers were found in our work and vice versa, and isolates belonged to STs other than those found here. A similar finding was observed with STs and mutations reported by Wright and coworkers.²⁷ The results found here further support the association of ST with a colistin-resistance mechanism. To understand and clarify this association a higher number of isolates should be analyzed, and site-directed mutagenesis should be performed to confirm the contribution of each novel mutation to the resistant phenotype.

Colistin resistance is rising worldwide³ and in our setting too (unpublished data of our laboratory). Nevertheless, we can be optimistic about three aspects of the results found here. First, colistin resistance is associated with chromosomal mutations rather than plasmid-mediated *mcr* genes, lowering the risk of widespread dissemination through horizontal gene transfer. Second, colistin resistance is not associated with high-risk clones of *E. coli* and *K. pneumoniae*. Successful *E. coli* clones associated with community and hospital-acquired infections are ST131, ST410, ST38, ST73, ST405 and ST648.³⁴ Among *E. coli* isolates studied here 3/7 belong to high-risk clones (ST131 and ST69), and none of the *K. pneumoniae* isolates belong to the globally distributed high-risk clones, namely ST258, ST11, ST512, ST14 and ST15 among others.³⁵ Third, colistin resistance is not associated with multidrug-resistance. Most of the isolates reported here (10/15) are non-MDR and are susceptible to all the antibiotics tested, except for colistin. In contrast to the frequently reported carbapenem+colistin resistant clinical isolates for which few or no treatment options exist,^{26,33,36} antibiotic therapies are still available for isolates like the ones reported here.

Conclusion

We report here several mutations in genes coding for LPS-modifying enzymes, mgrB, pmrAB, phoPQ, and in other genes such as *pmrE*, *pmrD*, *acrB* and *kpnE*, whose actual contribution to colistin resistance requires analysis with isogenic mutants. Genomic analysis and results previously reported support the idea that colistin resistance mechanisms depend on genetic background. Colistin resistance in our setting is due to chromosomal mutations, which implies a lower risk of transmission than plasmid-mediated genes. Additionally, colistin resistance is not associated with multidrug-resistance since most of the isolates were susceptible to all antibiotics tested, and nor is it associated with high-risk STs.

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Disclosure

The authors declare no conflicts of interest in this work.

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