New Microbiologica, 45, 3, 199-209, 2022, ISSN 1121-7138

FULL PAPER

Identification of a novel mutation involved in colistin resistance in *Klebsiella pneumoniae* through Next-Generation Sequencing (NGS) based approaches

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SUMMARY

The spread of multidrug-resistant (MDR) *K. pneumoniae* carbapenemase-producing bacteria (KPC) is one of the most serious threats to global public health. Due to the limited antibiotic options, colistin often represents a therapeutic choice.
In this study, we performed Whole-Genome Sequencing (WGS) by Illumina and Nanopore platforms on four colistin-resistant *K. pneumoniae* isolates (CoRKp) to explore the resistance profile and the mutations involved in colistin resistance. Mapping reads with reference sequence of the most common genes involved in colistin resistance did not show the presence of mobile colistin resistance (*mcr*) genes in all CoRKp. Complete or partial deletions of *mgrB* gene were observed in three out of four CoRKp, while in one CoRKp the mutation V24G on *phoQ* was identified. Complementation assay with proper wild type genes restored colistin susceptibility, validating the role of the amino acid substitution V24G and, as already described in the literature, confirming the key role of *mgrB* alterations in colistin resistance.
In conclusion, this study allowed the identification of the novel mutation on *phoQ* gene involved in colistin resistance phenotype.

Received April 24, 2022 *Accepted* June 12, 2022

INTRODUCTION

The widespread emergence of multidrug-resistant (MDR) pathogens has become one of the most serious threats to global public health (Amann *et al.*, 2019; WHO, 2017). Infections caused by MDR pathogens are associated with high mortality rates, longer hospitalization and high healthcare costs (Lambert *et al.*, 2011; Martin-Loeches *et al.*, 2015; Neidell *et al.*, 2012). In 2014, the World Health Organization (WHO) identified *Klebsiella pneumoniae* (Kp) as one of the primary pathogens involved in antibiotic resistance ((GLASS) Global Antimicrobial Resistance Surveillance System, 2018; Tacconelli *et al.*, 2014; WHO, 2017). KPC carbapenemase-producing Kp strains (KPC-Kp) proved capable of rapid epidemic diffusion in healthcare settings, causing large outbreaks in many countries, including Italy (Cannatelli *et al.*, 2014; Giordano *et al.*, 2018). Owing to extended antibiotic resistance, infections caused by KPC-Kp are difficult to treat and are usually associated with high mortality rates. Old therapeutic molecules, such as colistin, regained popularity as a "last-line" drug to treat these infections (Biswas *et al.*, 2012; Loho and Dharmayanti, 2015; Tzouvelekis *et al.*, 2014). Colistin is an antimicrobial polypeptide endowed with bactericidal activity by interacting with the lipid A component of the lipopolysaccharide (LPS): this electrostatic interaction leads to competitive displacement of divalent cations Ca2+ and Mg2+ from LPS, resulting in disruption of the outer membrane and cell death (Ah *et al.*, 2014; Biswas *et al.*, 2012; Choi and Ko, 2015; Abiola Olumuyiwa Olaitan *et al.*, 2014). However, due to the increasing use of colistin therapy in clinical setting, a spread of colistin-resist-

Key words:
Colistin resistance, *Klebsiella pneumoniae*, Illumina, nanopore, mgrB, PhoQ.

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ant Kp (CoRKp) strains has recently been reported worldwide (Amann *et al.*, 2019; Carlet *et al.*, 2012; Codjoe and Donkor, 2017). To date it is described that Kp isolate may develop resistance to colistin mostly through modification of the LPS biosynthetic pathway by the addition of cationic charges, such as the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A, which decreases the affinity between colistin and its target (Cheng *et al.*, 2010; Jaidane *et al.*, 2018; Abiola O. Olaitan *et al.*, 2014; Wright *et al.*, 2015).

In fact, an increasing number of studies have demonstrated that modifications on PhoPQ, PmrAB and CrrAB two-component systems and on the small transmembrane protein MgrB lead to the addition of L-Ara4N on LPS and consequently to colistin resistance (Aghapour *et al.*, 2019; Campos *et al.*, 2004; Falagas *et al.*, 2010; Gunn *et al.*, 2000; Thompson *et al.*, 2013; Winfield and Groisman, 2004; Zaman *et al.*, 2018). In addition to these chromosomal mechanisms, plasmid-encoded mobile colistin resistance genes (*mcr*) have recently been reported as a transmissible resistance mechanism in Enterobacterales, including Kp, causing great medical concern worldwide (Petrosillo *et al.*, 2019).

Despite the progress made to understand the mechanisms behind colistin resistance, there are still many unanswered questions.

In this scenario, the implementation of Next-Generation Sequencing (NGS) technologies plays an important role for a deeper characterization of microbial agents in hospital or community settings in a short period of time and with high accuracy (Motro and Moran-Gilad, 2017; Wick *et al.*, 2019). In particular, the coupling of Illumina and Nanopore systems is gaining importance for the reconstruction of high-quality assembled genomes. In fact, the combination of short-read high-accuracy Illumina sequencing data with long-read Nanopore sequencing data improves the assembly and the analysis of the sequenced genome, possibly enabling a deeper characterization of resistance-associated mutations (Oxford Nanopore Technologies, 2018; Wick *et al.*, 2017).

In this study, four colistin-resistant Kp clinical isolates were collected and sequenced by second and third generation NGS approaches consisting of Illumina and Nanopore hybrid analysis. Following this approach, a novel mutation involved in colistin resistance was identified.

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility testing

From February 2017 to May 2018, four MDR Kp clinical isolates resistant to colistin and eight MDR Kp isolates susceptible to colistin.

The isolates were identified in San Raffaele Microbiology Laboratory by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI_TOF MS) performed with bioMérieux Vitek MALDI-TOF MS apparatus (bioMérieux Vitek 2, Marcy l'Etoile, France). Antimicrobial susceptibility was determined with an automated VITEK®2 system (bioMérieux, Marcy-l'Étoile, France) using antibiotic panels with the following antimicrobial agents: amikacin, amoxicillin/clavulanic acid, cefepime, cefotaxime, ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, piperacillin/tazobactam, tigecycline and trimethoprim/sulfamethoxazole. Gradient test (E- test, bioMérieux, Marcy-l'Étoile, France) was used to confirm Vitek 2 results when antimicrobial susceptibility testing revealed an MDR pattern or unusual results. Moreover, a microdilution susceptibility test, Sensititre® (Thermofisher Scientific, Waltham, MA, USA), was used for antimicrobial MIC determination.

The minimum inhibitory concentrations (MICs) of all tested molecules, but tigecycline, were interpreted according to EUCAST guidelines; for tigecycline, FDA breakpoints were used (https://www.fda.gov/drugs/development-resources/tigecycline-injection-products).

DNA extraction

The bacterial genome was isolated from overnight cultures on Mueller-Hinton agar plates using commercially available QIAamp DNA Mini Blood (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

Whole-Genome Sequencing

The extracted DNA was used for Illumina (Illumina, San Diego, CA, USA) and Nanopore sequencing (Oxford Science Park, Oxford, UK). Two separate genomic DNA libraries were prepared according to the requirements of the two platforms.

For Illumina sequencing, the purity of the extracted DNA was checked with NanoDropND-1000 (ThermoFisher Scientific, Waltham, MA, USA) and quantified using Qubit™ 4 Fluorometer (Invitrogen™, Carlsbad, CA, USA). Subsequently, the DNA sequencing library was generated using Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The library generated was sequenced with MiSeq Reagent Kit v3 (600-cycles) (Illumina, San Diego, CA, USA) on MiSeq platform.

For Nanopore sequencing, the library was prepared using ONT 1D ligation sequencing kit (SQK-LSK108, Oxford Nanopore Technologies, Oxford, UK) with the native barcoding expansion kit (EXP-NBD103, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions. To maximize DNA recovery, 1 µg of DNA from each isolate was treated with the end-repair/dA tailing module and eluted in 24 µl Agencourt AMPure XP (Beckman

Coulter, Villepinte, France). Following the barcode ligation reaction, the DNA was purified using AMPure XP beads (Beckman Coulter, Villepinte, France) and eluted in 10 µl of H_2O. For library pooling, the equimolar amount of each barcoded sample was calculated based on 5 µl of DNA sample with the lower concentration, and 50 µl of this pooled DNA sample was used for adapter ligation. The library was loaded onto an R9.4 flow cell and sequenced for 48 h.

Whole-Genome Sequencing analysis

A combination of Nanopore and Illumina reads was used to generate the complete genome sequence of the 12 clinical isolates.

As outlined in figure 1, the quality of Illumina raw data was assessed using FastQC (v0.11.8) (Babraham Bioinformatics). Reads were further corrected, quality trimmed, and *de novo* assembled using SPAdes software (v.3.12.0) (Center for Algorithmic Biotechnology, St. Petersburg State University) and, successively, the assembled sequences were visualized using Bandage (Bioinformatics Application for Navigating De novo Assembly Graphs Easily).

As showed in figure 1, for Nanopore analysis the raw data (fast5 files) were basecalled using Guppy (v2.2.3) (Computational Biology Research Center, Tsukuba, Japan), which generated FastQ files with a read accuracy of Q8.9 and consensus accuracy of Q22.8. Epi2Me (v.desktop 2019.7.0) (Oxford Nanopore Technologies, Oxford, UK) was run for demultiplexing the FastQ files and then the reads were corrected and trimmed using Canu (v1.8) (National Human Genome Research Institute, National Institutes of Health). Only the sequences that passed these two steps were assembled using Canu assembler (National Human Genome Research Institute, National Institutes of Health) and visualized using Bandage (Phillippy *et al.*, 2020).

Hybrid genome assembly and analysis

The hybrid genome assembly was performed by aligning the short-high accuracy Illumina reads with the Nanopore assembled reads using BWA-MEM algorithm (v. 0.7.17) (Sanger Institute) (Quainoo *et al.*, 2017). Multiple rounds of error corrections were performed using Pilon (v1.2.3) (Broad Institute) until no error remained. The quality of the assembled genomes was evaluated using Quast v4.0 (Quality Assessment Tool for Genome Assemblies, Algorithmic Biology Lab) based on standard assembly quality metrics (assembly size, total number of contigs, N50, maximum contig length, mean contig length).

Clonal sequence types (STs), resistance and virulence genes were determined using online databases including the MLST analysis (Center for Genomic Epidemiology database, https://cge.cbs.dtu.dk/services/MLST/), ResFinder (v.4.0) (Center for Genomic Epidemiology, https://cge.cbs.dtu.dk/services/ResFinder/) and Kleborate (https://github.com/katholt/Kleborate), respectively (Wick *et al.*, 2018).

Whole-genome-based Kp capsule polysaccharide-based typing (K-type) was performed using the

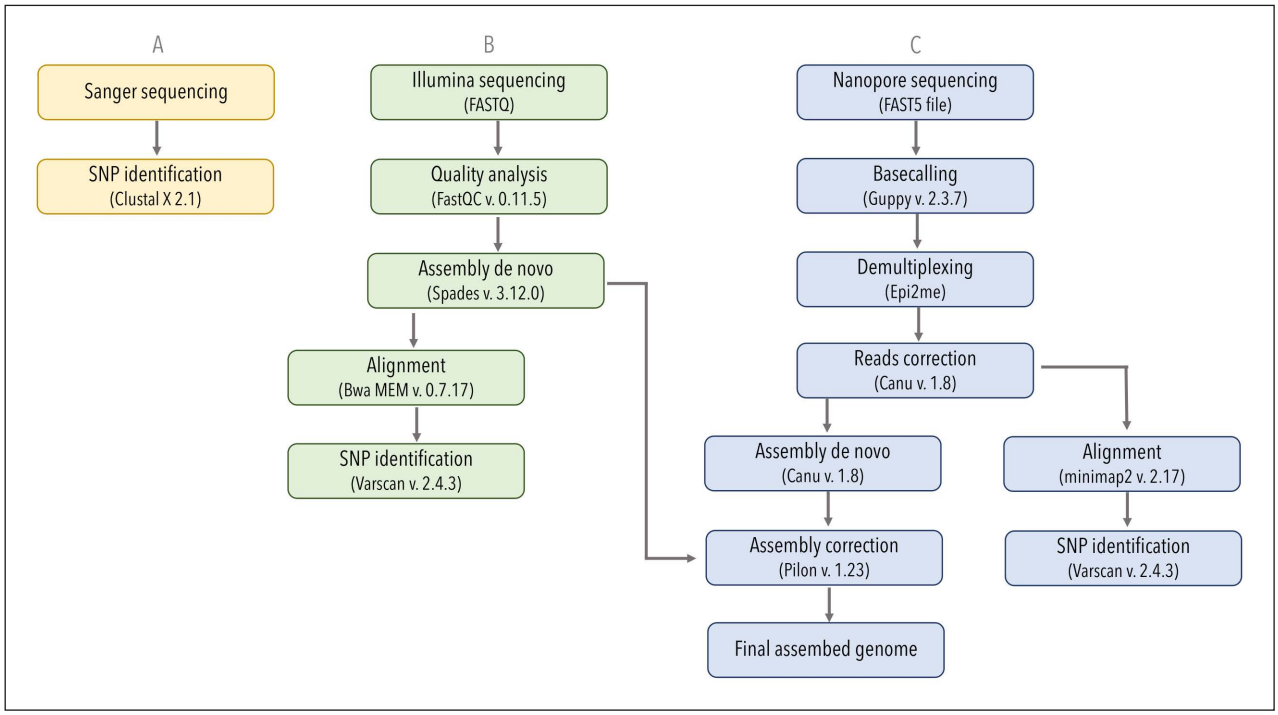

Figure 1 - *Bioinformatic flowchart of the three pipelines used for genome assembly and for the identification of mutations related to colistin resistance mechanisms.*

Kaptive Web database analysis based on a set of common genes that encode the core capsule biosynthesis machinery (e.g., galF, wzi, wza, wzb, wzc, gnd and ugd).

Variant calling and mutations identification

Two different variant calling analyses were performed using Illumina and Nanopore FastQ reads (*Figure 1*).

Using BWA-MEM (v. 0.7.17) (Sanger Institute) and minimap (Minimap2 aligner v. 2.17) (Broad Institute), Illumina and Nanopore reads were aligned with the reference sequence 18CPO060 (GenBank CP034778.1) colistin-susceptible Kp strain (Mataseje *et al.*, 2019). The aligned reads were visualized using Tablet (v. 1.19.05.28) (The James Hutton Institute) and SNPs were called using Varscan (v. 2.4.3) (University of Washington in St. Louis) with specific parameters: minimum coverage 10, minimum frequencies variation 0.20 and p-value 0.05. The Nanopore identified SNPs were compared to the SNPs detected by Illumina systems and the parameter of minimum frequencies variation was increased from 0.20 to 0.75 to increase Nanopore accuracy (*Figure 1c*) (Wick., 2017).

Sanger sequencing

phoQ and *mgrB* were amplified using KAPA HiFi HotStart ReadyMix (ROCHE, Basel, Switzerland) kit. PCR products were analyzed by electrophoresis on 1% agarose gel and size-specific expected bands were purified using QIAquick GEL Extraction Kit (QIAGEN, Hilden, Germany). The purified DNAs were sequenced by Sanger method on an automatic sequencer ABI 3100 genetic analyzer DNA Sequencer (Applied Biosystem, Foster City, CA, USA). The obtained nucleotide sequences were aligned to the reference sequence 18CPO060 (GenBank CP034778.1) using Clustal X (v. 2) (Conway Institute UCD, Dublin) (*Figure 1*).

Complementation assay

The complementation assay was performed using previously described protocols (Haeili *et al.*, 2017; Jayol *et al.*, 2014; Pitt *et al.*, 2018). Briefly, wild type *mgrB* and *phoQ* genes were amplified by PCR using KAPA HiFi HotStart ReadyMix (ROCHE, Basel, Switzerland) and a pair of specific primers for each gene. The amplified fragments were cloned into the high-copy-number plasmid pCR-BluntII-TOPO encoding resistance to kanamycin and zeocin using the Zero Blunt TOPO PCR cloning kit (Invitrogen™, Carlsbad, CA, USA). The obtained pTOPO-*mgrB* and pTOPO-*phoQ* were amplified and purified from *E. coli* TOP10 cells using the QIAprep spin miniprep column kit (QIAGEN, Hilden, Germany) and the two plasmids were used to transform the four colistin-resistant Kp clinical isolates that were previously made electrocompetent (Fournet-Fayard *et al.*, 1995). The recovery of these transformations was plated on LB agar disks supplemented with zeocin (100 µg/ml) and kanamycin (50 µg/ml) and incubated overnight at 37°C. From each plate, five colonies were screened to verify the presence of the cloned gene by PCR and sequencing, and only the positive clones were further tested for colistin MIC using broth microdilution (BMD) MIC Strip Colistin (Merlin®, Bornheim, Germany) following the manufacturer's instructions. Each MIC test was performed using colistin-resistant Kp clinical isolates transformed with the wild type gene that harbor the mutation, a positive control (clinical isolates CoRKp) and a negative control (Kp transformed with the non-mutated gene).

RESULTS

Antimicrobial susceptibility profiles

Twelve MDR Kp isolated at San Raffaele Hospital were included in this study and microbiological and clinical information were recorded. The isolates were cultured from different biological samples (6 rectal swabs, 1 urine, 2 broncho-alveolar lavages, 3 blood samples) collected from patients admitted to different wards (4 in Intensive Care Units, 4 in Hematology, 1 in General Medicine, 1 in Cardiology, 1 in Gastroenterology and 1 Outpatient).

Antimicrobial susceptibility test showed the heterogeneity of the 12 Kp isolates and their MDR profile. Only isolate Kp #2 was fully resistant to all tested antibiotics, except for tigecycline (MIC 2 µg/ml). The other 11 isolates expressed a susceptible profile to at least two antibiotics. In detail, isolates Kp #1, #4, #11 featured lower MIC values to tigecycline and were susceptible to gentamicin, amikacin and colistin, respectively; isolates Kp #6, #7, #9 were susceptible to colistin, gentamicin and tigecycline with Kp #9 featuring susceptibility also to imipenem. As Kp #6, #7, #9, also Kp #5 was susceptible to colistin, gentamicin, but showed a resistant phenotype to tigecycline; isolates Kp #3, #10, #12 were susceptible to colistin, tigecycline and amikacin with the isolate Kp #12 showing lower MIC values to carbapenems. Isolate Kp #8 was susceptible to amikacin, gentamicin and featured a lower MIC to carbapenems (*Table 1*).

Whole-genome sequencing and genome assembly

Illumina sequencing generated 46,536,998 raw reads with an average length of 35-130 bp and an average Phred quality score (Q score) ~Q36, while 1,958,810 barcoding reads were obtained from Nanopore sequencing run with an average sequence length of 5,204 bp and an average Quality Score value of 10.18. The obtained quality scores are a sign of the high-qual-

Table 1 - *Clinical and microbiological features for the 12 Kp isolates. AMK, Amikacin; AMC, amoxicillin-clavulanic acid; FEP cefepime; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; CST, Colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TZP, piperacillin-tazobactam; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole. BAL1: Bronchoalveolar lavage.*
The antibiogram, excluding tigecycline, were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. For tigecycline the breakpoints issued by the FDA were used (https://www.fda.gov/drugs/development-resources/tigecycline-injection-products). Moreover, intermediate phenotype was considered as "susceptible increased exposure" following EUCAST guidelines.

Sample	*Ward*	*Source*	*AMK*	*AMC*	*FEP*	*CTX*	*CAZ*	*CIP*	*CST*	*GEN*	*IPM*	*MEM*	*TZP*	*TGC*	*SXT*
1	Medical ICU	Rectal swab	≥64	≥32	8	8	≥64	≥4	8	2	≥32	≥32	≥128	1	≥320
2	Medical ICU	Rectal swab	16	≥32	≥64	≥64	≥64	≥4	16	≥16	>32	>32	≥128	2	80
3	Haematology	Blood	≤2	≥32	≥64	≥64	≥64	≥4	<0.5	≥16	≥32	≥32	≥128	0.25	≥320
4	Haematology	Rectal swab	4	≥32	≥64	≥64	16	≥4	16	≥16	≥16	≥16	≥128	1	≥320
5	Medical ICU	Bronchial aspirate	16	>8	>32	>4	>128	>2	<0.25	2	>16	>64	>128	>4	>128
6	Medical ICU	Blood	16	>8	>32	>4	128	>2	<0.25	1	>16	64	>128	1	>128
7	Cardiology	Blood	16	>8	>32	>4	>128	>2	<0.25	1	>16	32	>128	1	>128
8	Haematology	Rectal swab	8	>8	>32	>4	>128	>2	8	≤1	2	8	>128	>4	>128
9	Haematology	Rectal swab	16	>8	>32	>4	64	>2	<0.25	≤1	2	64	>128	1	>128
10	Outpatients Units	Urine	≤4	>8	>32	>4	64	>2	<0.25	≤1	16	>64	>128	0.25	<10
11	General medicine	Rectal swab	>16	>8	>32	>128	>128	>2	<0.25	>4	>16	64	>128	0.5	<10
12	Gastroenterology	BAL 1	8	>8	>32	>4	>128	>2	<0.25	≤1	8	8	>128	0.5	>80

resistent; intermediate; susceptible

ity level of the sequencing run (Supplemental *Table S1*) (Illumina, 2011; Tyler *et al*., 2018).
Coupling the results acquired from Nanopore and Illumina sequencing, we reconstructed a single high accuracy contig of ~5.3 Mb associable with Kp chromosomal sequence for 11 out of 12 isolates, and as expected, we identified different shorter contigs probably related to different plasmid sequences (*Ta-*

Table 2 - *Features of assembled genomes obtained after hybrid analysis and Pilon correction. The parameters reported define the quality of the assembled sequences in term of contigs length, % of GC and contiguity (L50 parameter). For all isolates, except for MDR KP #9, contigs of ~5.3 Mb in size with a % GC content of 57% corresponding to that commonly observed for* K. pneumoniae *were obtained.*

Kp	*Contigs ≥25000 bp*	*Contigs ≥50000 bp*	*Largest contigs bp*	*Other contigs*	*GC (%)*	*L50*
#1	6	4	**5,401,407**	276,149; 136,430; 115,273; 39,100; 28,042	56.97	**1**
#2	3	3	**5,374,636**	284,384; 141,021	57.09	**1**
#3	3	3	**5,500,813**	146,496; 81,838	57.23	**1**
#4	5	3	**5,553,323**	150,997; 101,278; 43,133; 36,460	57.13	**1**
#5	3	3	**5,353,843**	238,468; 136,097	57.14	**1**
#6	5	3	**5,354,880**	245,044; 146,638; 45,869; 45,083	57.07	**1**
#7	4	3	**5,387,073**	230,239; 161,956; 27,007	57.10	**1**
#8	4	2	**5,297,620**	242,572; 38,459; 24,268	57.28	**1**
#9	9	6	**2,860,201**	1,937,936; 591,880; 268,527; 138,282; 50,872; 44,044; 43,452; 31,505	57.11	**2**
#10	5	4	**5,330,581**	233,059; 100,417; 53,440; 31,031; 9,743	56.79	**1**
#11	3	2	**5,461,239**	185,679; 34,615; 18,048; 14,201; 10,712; 10,529; 10,430	56.93	**1**
#12	5	3	**5,359,710**	199,376; 174,352; 36,401; 30,482; 22,003	56.80	**1**

ble 2). For sample Kp #9, only a contig of 2.8 Mb was reconstructed due to technical problems during the assembly phase. For this reason, we excluded this isolate from subsequent analysis.

Multi-locus sequence type (MLST), antimicrobial resistance and virulence analysis

To analyze the genotypic differences among isolates of interest, we first performed *in silico* multi-locus

Table 3 - *Antimicrobial resistance genes identified by in silico analysis.*

	ST	*aminoglycoside*	*Beta-lactam*	*carbapenem*	*quinolone*	*fosfomycin*	*phenycol*	*sulphamide*	*tetracycline*	*trimethoprim*
1	258	aac(6')-Ib-cr, aadA2 aadA2b aph(3')-Ia	blaSHV-182, blaTEM-1A, blaOXA-9	blaKPC-3	aac(6')-Ib-cr, oqxA oqxB	fosA	catA1	sul1		
2	307	aac(3)-IIa aac(6')-Ib-cr aac(6')-Ib-cr	blaCTX-M-15, blaOXA-1, blaSHV-106, blaOXA-9 blaSHV-106 blaSHV-28 blaTEM-1A blaSHV-28	blaKPC-3	qnrB1 aac(6')-Ib-cr oqxA oqxB	fosA	catB3			dfrA14
3	11	aac(3)-Iia, aph(3")-Ib, aac(6')-Ib-cr aph(6)-Id	blaCTX-M-15, blaSHV-182, blaOXA-1 blaTEM-1B	blaKPC-2	aac(6')-Ib-cr, qnrB1 oqxA oqxB	fosA	catB3	sul1 sul2		dfrA14
4	11	aac(3)-IIa aac(6')-Ib-cr aadA2b aph(3")-Ib aph(6)-Id	blaCTX-M-15, blaOXA-1 blaSHV-182 blaTEM-1B	blaKPC-11 blaKPC-12 blaKPC-17 blaKPC-18 blaKPC-2 blaKPC-21 blaKPC-24 blaKPC-26 blaKPC-3 blaKPC-30 blaKPC-33 blaKPC-35 blaKPC-5 blaKPC-6	aac(6')-Ib-cr, qnrB1 oqxA oqxB	fosA	catB3	sul1 sul2		dfrA14
5	512	aph(3')-Ia aadA2	blaSHV-182, blaOXA-9 blaSHV-182 blaTEM-1A	blaKPC-3	oqxA oqxB	fosA	catA1	sul1		
6	512	aac(6')-Ib-cr, aac(6')-Ib aadA2 aph(3')-Ia	blaOXA-9 blaOXA-9 blaSHV-182 blaTEM-156 blaTEM-1A	blaKPC-3	aac(6')-Ib-cr oqxA oqxB	fosA	catA1	sul1		
7	512	aac(6')-Ib aac(6')-Ib aadA2 aph(3')-Ia aac(6')-Ib-cr	blaSHV-182, blaTEM-1A, blaOXA-9	blaKPC-3	aac(6')-Ib-cr oqxA oqxB	fosA	catA1	sul1		
8	15	aac(6')-Ib-cr aadA2 aph(3')-Ia	blaCTX-M-15, blaOXA-1 blaSHV-106 blaSHV-28 blaTEM-1B		aac(6')-Ib-cr oqxA	fosA	catB3	sul1	tet(A)	
10	307		blaSHV-106, blaSHV-28	blaKPC-2	oqxA oqxB	fosA				
11	101		blaSHV-106, blaSHV-28	blaKPC-3	oqxA oqxB	fosA				
12	307		blaCTX-M-15, blaSHV-106 blaSHV-28	blaKPC-2	oqxA qnrB1 oqxB	fosA				dfrA14

sequence type (MLST), antimicrobial resistance and virulence gene analysis.

The MLST analysis is based on the seven standard housekeeping loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) and it allowed the identification of the different sequence types (STs). In detail, the 11 Kp clinical isolates showed six different STs: ST307 (Kp #2, #10 and #12), ST11 (Kp #3 and #4), ST258 (Kp #1), ST15 (Kp #8), ST512 (Kp #5, #6 and #7) and ST101 (Kp #11). The four colistin-resistant isolates (Kp #1, #2, #4 and #8) belonged to four different STs.

Regarding antimicrobial resistance investigation, the *in silico* analysis is fully coherent with the antimicrobial phenotypic assay for beta-lactam and carbapenems. Indeed, all isolates excluding Kp #8 harbored at least one beta-lactam (*blaCTX-M-15*, *blaTEM-1B*, *blaSHV* and *blaOXA-1*) and carbapenem resistance gene (*blaKPC-2*, *blaKPC-3* most frequently). Moreover, they all carried the fosfomycin (*fosA*) and quinolones (*aac(6')-lb-cr*, *oqxA*, *oqxB*, *qnrB1*, *qnrS1*) resistance genes, while the genes involved in resistance to aminoglycosides (*aac(3)-lla*, *aac(6')-lb-cr*, *aadA16*, *aph(3')-lb*, *aph(6)-ld*, *rmtC*), sulphonamides (*sul1*, *sul2*), trimethoprim (*dfrA14/27*) and phenicol (*catB3/catA2*) were identified in all isolates excluding Kp #10, #11 and #12; only the Kp #8 isolate harbored the *tetA* gene, which confers resistance to tetracycline (*Table 3*).

This analysis did not detect the presence of colistin resistance genes (*mcr 1-9*) in any of the 11 Kp isolates, highlighting that chromosomally-mediated mechanisms might play a prominent role in resistance of the four CoRKp isolates.

We also investigated the possible involvement of capsular types in the alteration of colistin interaction by analyzing the hypervirulent phenotype of the resistant isolates. *In silico* analysis revealed five different KL types (polysaccharide capsule and lipopolysaccharide O antigen) belonging to classical strains of Kp and the absence of the hypervirulent genes *rmpA/A2* (Elaf S and Flayyih, 2018).

Variant calling and mutations identification

We used Illumina high-throughput sequencing to identify the alterations of the main genes described as involved in colistin-resistant phenotype in Kp (*pmrA*, *pmrB*, *crrA*, *crrB*, *phoP*, *phoQ* and *mgrB* genes). Variant calling analysis of these genes revealed several nucleotide polymorphisms compared with reference sequence of Kp 18CPO060 (GenBank CP034778.1), a recently well described colistin-susceptible Kp strain (Mataseje *et al.*, 2019).

We identified four mutations exclusively in resistant isolates, while the others were shared by both resistant and susceptible isolates among the same ST.

In detail, isolates Kp #2, #10 and #12 (ST307) shared A41T and L231M neutral mutations on *pmrA* gene, while only Kp #2 colistin-resistant isolate harbored a 29nt truncated form of *mgrB*. Isolates Kp #5, #6, #7 (ST512) and Kp #3, #4 (ST11) showed only the neutral point mutation R256G on *pmrB,* while Kp #4, colistin-resistant isolate, harbored a complete deletion of *mgrB* (Giordano *et al.*, 2018; Haeili *et al.*, 2017; Jaidane *et al.*, 2018). The resistant isolate Kp #8 (ST15) carried a partial deletion of *mgrB* gene, the neutral mutation A246T on *pmrB* and the mutation Q295L on *crrB* gene observed across all ST512 isolates.

Table 4 - *List of different variations on genes involved in colistin resistance mechanisms identified in Kp isolates using NGS and Sanger sequencing. The mutations shown in bold are associated with colistin resistance mechanisms.*

Kp	*ST*	*mgrB*	*pmrA*	*pmrB*	*phoP*	*phoQ*	*crrA*	*crrB*	*mcr1-9*	*Col MIC (mg/L)*
#1	ST258	#	#	R256G	#	**V24G**	#	#	#	8
#2	ST307	**29nt-trunc**	A41T	L213M	#	#	#	#	#	16
#3	ST11	#	#	R256G	#	#	#	#	#	<0.5
#4	ST11	**Δ(12.702nt)**	#	R256G	#	#	#	#	#	16
#5	ST512	#	#	R256G	#	#	#	Q295L	#	<0.25
#6	ST512	#	#	R256G	#	#	#	Q295L	#	<0.25
#7	ST512	#	#	R256G	#	#	#	Q295L	#	<0.25
#8	ST15	**Δ – 24nt (2.107nt)**	#	A246T	#	#	#	Q295L	#	8
#10	ST307	#	A41T	L213M	#	#	#	#	#	<0.25
#11	ST101	#	#	#	#	E121K	#	#	#	<0.25
#12	ST307	#	A41T	L213M	#	#	#	#	#	<0.25

The colistin-resistant Kp #1 (ST258) isolate harbored only the point mutation V24G on *phoQ*, which had never been identified in previous studies on colistin resistance, and which was confirmed by the gold standard Sanger sequencing.
Lastly, the susceptible Kp #11 (ST101) isolate carried the variants E121K on *phoQ,* not previously identified, and the role of which was not further investigated in this study considering the susceptible phenotype of the isolate (*Table 4*).
In addition to these data, as expected, Nanopore sequencing detected numerous SNPs that were not observed in Sanger and/or Illumina analysis even after increasing the minimum variant allele frequency parameter from 0.20 to 0.75. Nanopore did not identify V24G on *phoQ* gene due to the low base accuracy for SNPs identification of long-reads sequencing. Nevertheless, the advantage of long reads to span entire regions of repetitive DNA (57% of Kp genome is covered by GC repeats) allowed us to confirm the deletions on *mgrB*.

Complementation assay

To determine if resistance to colistin could be associated to the *mgrB* deletions and to the mutation V24G on *phoQ*, we performed a complementation assay by restoring each resistant isolate with the wild type genes (Haeili *et al.*, 2017; Jayol *et al.*, 2014). The complementation with *mgrB* restored the colistin susceptibility profile in the resistant isolates harboring truncated or deleted *mgrB*, while the complementation with *phoQ* used as negative control did not. Regarding the strain that harbored the single point mutation V24G, the susceptibility phenotype to colistin was restored after transformation with the wild type gene (*Figure 2*). These results highlight the role of these mutations in colistin resistance mechanisms.

DISCUSSION

Colistin has recently regained importance, playing a major role in the therapeutic management of severe infections caused by carbapenemase-resistant Kp isolates. Although resistance to colistin has been widely reported worldwide, there is still a lack of understanding of the mechanisms responsible for this resistance. We focused this study on the identification of novel mutations involved in colistin resistance by characterizing the complete genome sequence of four CoRKp isolates and using eight CoSKp as controls, combining Illumina and Nanopore WGS.
To provide a relative genetic profile among different isolates, *in silico* multi-locus sequence typing (MLST) was performed and six different sequence types (STs) were identified, among which the most representative clones responsible for the global spread of KPC (ST258 and ST512) and also other STs less common in our area (e.g., ST11, ST15, ST101 and ST307) (Di Tella *et al.*, 2019; Loconsole *et al.*, 2020).
Before investigating the involvement of chromosomal genes associated with colistin resistance mechanisms, antimicrobial resistance analysis and virulome profile were performed to exclude the presence of plasmid-encoded mobile colistin resistance genes (*mcr 1-9*) and of hypervirulent capsular polysaccha-

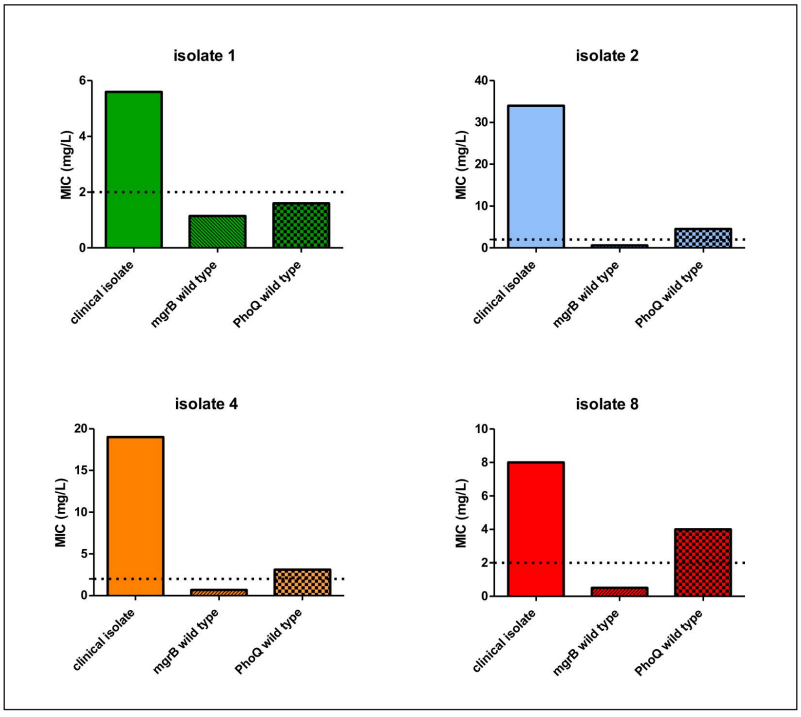

Figure 2 - *Colistin MIC results obtained by broth microdilution test before and after complementation assay performed with plasmid pTOPO-*phoQ *and pTOPO-*mgrB *for the four colistin-resistant Kp isolates.*

ride types (Keynan and Rubinstein, 2007; Liu *et al.*, 2016; Pan *et al.*, 2013).

In silico antimicrobial resistance analysis revealed a wide variety of antibiotic resistance genes in all isolates; plasmid encoded *mcr* genes were not detected in the four CoRKp genomes. This analysis confirmed the phenotypical MDR of all clinical isolates and demonstrated the colistin-resistance profile mediated by chromosomal genes (Aris *et al.*, 2020; Gunn, 2008; Kempf *et al.*, 2013) We also characterized the virulome to define the capsular type and the presence or the absence of mucoid phenotype regulator *rmpA/A2* genes, which could affect colistin effectiveness (Elaf S and Flayyih, 2018; Fasciana *et al.*, 2019). In this analysis, we did not detect the presence of hypervirulent capsular polysaccharide types and of *rmpA/A2* genes, confirming that the capsule of the resistant isolates did not interfere with colistin interaction (Elaf S and Flayyih, 2018; Fasciana *et al.*, 2019).

Having ruled out the involvement of the capsular types and plasmidic encoding genes for colistin resistance mechanisms, we investigated the role of chromosomally encoded genes already described in the literature as related to colistin resistance mechanisms (Cannatelli *et al.*, 2014; Cheng *et al.*, 2010; Kidd *et al.*, 2017; Abiola O. Olaitan *et al.*, 2014; Winfield and Groisman, 2004). We focused our attention on the chromosomal genes *pmrA, pmrB, phoP, phoQ, crrA, crrB*, and *mgrB*. Illumina and Nanopore variant calling analysis identified various alterations that could explain the colistin resistance phenotype. High accuracy Illumina sequencing allowed us to detect the novel point mutation V24G on *phoQ* for the Kp #1 CoRKp (ST258), while Nanopore long-reads system showed two truncated forms of the *mgrB* gene for Kp #2 and Kp #8 and complete deletion of *mgrB* for Kp #4.

The importance of these mutations was verified by complementation assay, confirming their involvement in colistin resistance: the susceptibility to colistin was restored for isolates Kp #2, #4, #8 after complementation with wild type *mgrB* gene and for isolate Kp #1 after complementation with wild type *phoQ*. However, for the Kp #1 isolate susceptibility was also restored after complementation with *mgrB* wild type form. This could be explained by the hypothesis that the mutation V24G could decrease interaction between PhoQ-MgrB, and an overexpression of MgrB could restore the pathway and the susceptible profile. A decrease of the MIC, but not a restoration of susceptibility phenotype, was also observed for Kp #2, #4 and #8 after the transformation with *phoQ* gene. These data showed the importance of *phoQ* in the colistin susceptible phenotype for these isolates, but the resistance profile was not associated to this gene.

In conclusion, an accurate detection of mutations involved in colistin resistance remains a long-standing problem. Although the sample size of this study was limited, since we opted for deeper hybrid analysis on a restricted number of clinical isolates, the present study allowed the identification of a novel mutation causing colistin resistance which could be further monitored in the future.

Data availability

Whole genome Illumina and nanopore sequencing reads for the *Klebsiella pneumoniae* colistin resistant isolate #1 (bam format) have been deposited in the NCBI database (BioProject accession number PRJNA750627).

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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