

Molecular Epidemiological Insights into Colistin-Resistant and Carbapenemases-Producing Clinical *Klebsiella pneumoniae* Isolates

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Purpose: Carbapenemases-producing *Klebsiella pneumoniae* are challenging antimicrobial therapy of hospitalised patients, which is further complicated by colistin resistance. This study describes molecular epidemiological insights into colistin-resistant and carbapenemases-producing clinical *K. pneumoniae*.

Patients and methods: Cultures collected from 26 hospitalised patients during 2014–2017 in the main hospital in Molise Region, central Italy, were characterized. The minimum inhibitory concentration for 19 antibiotics was determined, including carbapenems and colistin. Prevalence of resistance-associated genes was investigated through PCR, detecting *bla*_{KPC}, *bla*_{GES}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{OXA-48}, *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, and *mcr*-1,2,3,4,5,6,7,8. The *mgrB* gene was also analysed in colistin-resistant strains by PCR and sequencing assays. *K. pneumoniae* were typed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Results: Twenty out of 26 *K. pneumoniae* were phenotypically resistant to carbapenems and 19 were resistant to colistin. All isolates harbored *bla*_{KPC}, and *bla*_{SHV}, *bla*_{TEM} and *bla*_{VIM} were further the most common resistance-associated genes. In colistin-resistant strains, *mcr*-1,2,3,4,5,6,7,8 variants were not detected, while mutations and insertion elements in *mgrB* were observed in 68.4% (n=13) in 31.6% (n=6) isolates, respectively. PFGE revealed 12 clusters and 18 pulsotypes at 85% and 95% cut-off, while the Sequence Types ST512 (n=13, 50%), ST101 (n=10, 38.5%), ST307 (n=2, 7.7%) plus a novel ST were detected using MLST.

Conclusion: All *K. pneumoniae* showed a multidrug-resistant phenotype, particularly to carbapenems and colistin. According to national data, *bla*_{KPC} was the prevailing carbapenemase, followed by *bla*_{VIM}, while *bla*_{TEM} and *bla*_{SHV} were among the most frequent beta-lactamases. Consistent with previous reports in Italy, ST512 was the most common clone, particularly during 2014–15, whilst ST101 became dominant in 2016–17. Colistin resistance was mainly associated with deleterious mutations and transposon in the *mgrB* gene. Improvements of surveillance, compliance with infection prevention procedures and antimicrobial stewardship are essential to limit the spread of resistant *K. pneumoniae*.

Keywords: antimicrobial resistance, carbapenems, central Italy, genetic relatedness, hospital infections, *mgrB* gene

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Introduction

Klebsiella pneumoniae is the most clinically relevant *Klebsiella* species.¹ The 2011–2012 Point Prevalence study of the European Centre for Disease Prevention and Control identified *K. pneumoniae* causing 6.8% of hospital-acquired infections

(HAIs), which represented the second most frequent *Enterobacteriaceae* after *Escherichia coli*.² In 2005, almost all European regions were carbapenem-resistant *K. pneumoniae* (CR-*Kp*) free. From 2005 to 2015, CR-*Kp* has emerged in several countries, reaching rates of 40–60%.³ In the TOTEM study, CR-*Kp* was classified as the most critical antimicrobially resistant pathogen and a leading cause of nosocomial infections, mainly in intensive care units (ICUs).⁴ Furthermore, among 5331 bacteremia cases due to carbapenemase-producing *Enterobacteriaceae*, 96.8% was attributed to *K. pneumoniae*.⁵ At EU/EEA level, in 2017, 34% of *K. pneumoniae* notified to the European Antimicrobial Resistance Surveillance Network were resistant to at least one of the antimicrobial groups under surveillance, including carbapenems.⁶

Carbapenemases production, particularly KPC, represents the most prevalent mechanism for carbapenems resistance in *K. pneumoniae*,^{5,7} but the carbapenemases GES, NDM, IMP, VIM, and OXA-48 can also be involved.⁸ *K. pneumoniae* carrying the gene *bla*_{KPC} are endemic in Italy since nearly 90% of CR-*Kp* are KPC producers, followed by *bla*_{VIM} (9.2%) and *bla*_{OXA-48} (1.3%).^{3,9,10} In *K. pneumoniae*, extended spectrum beta-lactamases (ESBLs) have been also detected, being involved in oximinocephalosporin resistance and able to hydrolyze beta-lactams.¹¹ During 1990–2000, *K. pneumoniae* has become the major ESBL-carrying pathogen in hospital outbreaks, mostly carrying *bla*_{TEM} and *bla*_{SHV}.¹²

The increasing prevalence of multidrug-resistant (MDR) Gram-negative bacteria has led to re-introduction of colistin, especially for infections sustained by *K. pneumoniae*.⁴ Nevertheless, in the last years, colistin resistance has also emerged in CR-*Kp* with rates as high as 36%.^{3,13,14} In this case, resistance is due to structural modifications of lipopolysaccharide (LPS) that is the target for colistin. Resistance could be attributed to *mcrB* inactivation by down-regulation of the Pmr system involved in LPS modification, neutralizing the negative charge and decreasing colistin binding,^{15,16} or through plasmid-mediated *mcr* (*mcr*-1,2,3,4,5,6,7,8 variants).^{17–23}

Management of CR-*Kp* infections is associated with long hospitalizations and poor outcomes^{1,14} and complicated by MDR emergence, which severely limits antimicrobial treatment options.¹⁴ Since *K. pneumoniae* is among the most frequent agents in nosocomial settings,⁸ identification of outbreaks due to MDR strains is crucial. In this scenario, molecular typing enabling strains comparison is required for detecting epidemics and tracking infection sources and

factors involved in the transmission.²⁴ For *K. pneumoniae* molecular characterization, the foremost approaches rely on pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) systems.²⁵

In this study, clinical *K. pneumoniae* isolated in the main hospital in Molise Region, central Italy, were characterized to evaluate MDR patterns, genetic differences and relationships, and prevalence of carbapenem resistance determinants, as well as to elucidate the mechanisms involved in colistin resistance.

Materials and Methods

K. pneumoniae Cultures and DNA Extraction

Twenty-six *K. pneumoniae* cultures isolated within the “Alert Organism” surveillance system during 2014–2017 were collected from the main hospital for acute care in Molise Region, central Italy. The hospital at the time of the study had a total of 336 beds, 320 for acute care and 16 for ICU.²⁶ Additionally, there were 19 total wards: ten of medicine and surgery specialties, four of pediatrics and two of ICU specialties, followed by single wards of gynecology/obstetrics, geriatrics, psychiatry, rehabilitation, and mixed specialties.

The selection criteria for the tested strains were non-replicates cultures, and a KPC phenotype evaluated with the Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF) assay, as reported by the hospital laboratory. *K. pneumoniae* cultures were mostly recovered from aspirated bronchial (n=9, 35.0%) samples, urine (n=6, 23%), rectal swab (n=4, 15.4%), and blood cultures (n=3, 11.5%) (Table 1). Fifteen (57.7%) isolates were from patients admitted to ICU: 60% male, overall mean age 73±12.6 years (median 78.5, range 44–89 years). Clinical specimens were cultured and purified on McConkey agar plates (Biolife, Milan, Italy) incubated at 37°C overnight. DNA was extracted using Maxwell[®] 16 Cell DNA Purification Kit (Promega, Milan, Italy).

Antimicrobial Susceptibility Testing

The susceptibility to nineteen antimicrobials was evaluated by the hospital Microbiology laboratory using BD Phoenix[™] Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, United States). The minimum inhibitory concentrations (MICs) were calculated for imipenem, ertapenem and meropenem

Table 1 Patient's Clinical Data and Characteristics of Analyzed Strains

Strain	Isolation date	Age	Gender	Sample	Ward	Clinical Data and Risk Factors
KP3	16/08/2014	85	M	Blood culture	Medicine	Infection/sepsis (fever)
KP4	18/08/2014	79	M	Bronchial aspirate	ICU	Infection Invasive procedures
KP5	30/10/2014	63	M	Urine	ICU	Urinary tract infection
KP6	20/10/2014	40	F	Urine	Urology	Urinary tract infection
KP7	19/11/2014	75	M	Prosthetic liquid	Orthopaedic	Infection/sepsis
KP8	17/11/2014	84	M	Bronchial aspirate	ICU	Pulmonary infection Invasive procedures
KP9	09/12/2014	76	M	Bronchial aspirate	ICU	Pulmonary infection Invasive procedures
KP10	14/12/2014	70	M	Blood culture	Infectious disease	Infection/sepsis
KP11	15/12/2014	63	M	Blood culture	Urology	Infection/sepsis
KP12	05/01/2015	85	F	Urine culture	ICU	Urinary tract infection
KP14	05/01/2015	74	M	Bronchial aspirate	ICU	Infection/sepsis Invasive procedures
KP18	12/01/2015	75	F	Bronchial aspirate	ICU	Pulmonary infection Invasive procedures
KP19	12/01/2015	81	M	Bronchial aspirate	ICU	Pulmonary infection
KP25	25/01/2016	56	M	Ulcer swab	Diabetology	Diabetic foot with amputation
KP27	01/02/2016	60	M	Bronchial aspirate	ICU	Urinary tract infection
KP28	29/02/2016	59	M	Urine culture	ICU	Urinary tract infection Invasive procedures
KP29	02/03/2016	71	F	Rectal swab	ICU	Colonization
KP31	11/09/2016	50	M	Rectal swab	ICU	Colonization
KP32	20/02/2017	82	F	Urine culture	Infectious disease	Urinary tract infection
KP34	06/03/2017	56	M	Bronchial aspirate	ICU	Pulmonary infection
KP36	03/04/2017	78	F	Bronchial aspirate	ICU	Urinary tract infection Invasive procedures
KP39	09/11/2017	58	F	Stent	Nephrology	Not available
KP40	06/11/2017	59	F	Urine	Nephrology	Urinary tract infection Invasive procedures
KP41	07/11/2017	78	M	Cyst	Orthopaedic	Not available
KP42	07/11/2017	87	M	Rectal swab	ICU	Colonization
KP43	19/12/2017	57	M	Rectal swab	ICU	Colonization

(carbapenems); ampicillin, amoxicillin-clavulanate, piperacillin and piperacillin-tazobactam; ceftazidime, cefuroxime and cefotaxime; amikacin and gentamicin; ciprofloxacin and levofloxacin; fosfomycin, trimethoprim-sulfamethoxazole, tigecycline, and tobramycin. The microdilution method was used for colistin (polymyxin) MIC determination. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoints.²⁷

Detection of Resistance-Associated Genes

Genes involved in carbapenems resistance were detected through single PCR assays, targeting *bla*_{KPC}, *bla*_{GES}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{OXA-48} and *bla*_{NDM-1} genes.^{28,29} Amplifications were performed in 25 µL volume with 2 µL DNA template, 1X PCR Master Mix (Promega Corporation) and 1 µM of each primer. Target genes were amplified at specific conditions: 94°C 2 mins; 35 cycles: 94°C 1 min, 45°C (*bla*_{IMP})/52°C (*bla*_{KPC})/54°C

(*bla*_{GES})/56°C (*bla*_{VIM/OXA-48})/60°C (*bla*_{NDM-1}) 40 sec, 72°C 1 min; 72°C 5 mins. PCR amplicons were electrophoretically separated (1.0–1.5% m/v concentration, 1X TAE buffer at 100 V for 1 hr), including 100 bp DNA ladder (Promega). Positive and negative control were used in each batch of reactions.

K. pneumoniae isolates were also screened for *bla*_{SHV}, *bla*_{TEM}, and *bla*_{CTX-M} genes by Multiplex PCR assays, using previously described oligonucleotides and specific cycling conditions.¹¹ The amplified products were resolved by agarose gel electrophoresis (1.5% m/v concentration, 1X TAE buffer at 100 V for 1 hr) including a 100 bp DNA ladder (Promega) and controls in each batch of reactions.

Molecular Analysis of Colistin Resistance

The colistin-resistant (col-R) isolates were screened by singleplex PCRs for the presence of *mcr*-1,¹⁷ *mcr*-2,¹⁸ *mcr*-3,¹⁹ *mcr*-4,²⁰ *mcr*-5,²¹ *mcr*-6,²² *mcr*-7,²³ *mcr*-7.1²³ and *mcr*-8.²² Amplifications were performed in 25 µL volume using 2 µL DNA, 1X PCR master mix (Promega Corporation) and

primers at 1 μ M. Amplicons were characterized after agarose gel electrophoresis (1.5% m/v concentration, 1X TAE buffer at 100 V for 1 hr) including a 100 bp DNA ladder (Promega).

PCR analysis of *mgrB* was performed using *mgrB_Ext_F* and *mgrB_Ext_R* primers targeting *mgrB* coding sequence and some flanking regions, as previously reported.¹³ Amplifications were carried out in 25 μ L volume using 5 μ L DNA template, 1X PCR master mix (Promega) and oligonucleotides at 2 μ M.

Colistin-sensitive (col-S) strains were used as a negative control carrying wild-type *mgrB*. PCR products were characterised using agarose gel electrophoresis (1.5% m/v concentration, 1X TAE buffer at 100 V for 1 hr) with a 100 bp DNA ladder (Promega). Amplicons longer than the expected molecular weight (253 bp) suggested the presence of an Insertion Sequence (*IS*), and were analyzed by Sanger sequencing (Eurofins Genomics, Germany GmbH, Ebersberg, Germany), including col-S strains as control. Sequences were analyzed with Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov/Blast.cgi) and processed with BioEdit v7.0.5.

The nucleotide sequences of wild-type *mgrB* in KP25 and KP42 isolates (GenBank Accession numbers MN389772 and MN389773, respectively), as well as those of col-R strains without *IS*s (KP5, KP6, KP7, KP9, KP10, KP28, KP31, KP34, KP36, KP39, KP40, KP41 and KP43) were deposited at BankIt/GenBank (Accession numbers: MN389775, MN389774, MN389776, MN389777, MN389778, MN389779, MN389780, MN389781, MN389782, MN389783, MN389784, MN389785, and MN389786, respectively).

To translate DNA sequences, EMBOSS Transeq (<https://www.ebi.ac.uk/Tools/st/emboss>) tool was used. The amino acid sequences were analyzed with Protein Variation Effect Analyzer (PROVEAN, <http://provean.jcvi.org/index.php>) allowing prediction by algorithm of the functional impact for all classes of sequence variations.³⁰ The change in the alignment score was considered as a measure of change in similarity caused by variation and thus to protein functionality.

Molecular Typing by PFGE and MLST

For PFGE, bacterial DNA was digested with *Xba*I (Fermentas, Milan, Italy) according to PulseNet protocol using conditions of pulse times from 5 to 40 sec over 24 hrs at 6.0V/cm and at 14°C.³¹ Pulsotypes were

analyzed through BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), and dendrograms were generated using Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA).⁹ The similarity band patterns interpretation was performed according to Tenover criteria,^{24,32} setting 85% and 95% similarity *cut-off* for identifying similar restriction patterns and clusters, respectively. A validated MLST scheme was used,³³ and PCR products were sequenced by Sanger method (Eurofins Genomics) to identify allelic profiles and assign the Sequence Type (ST). The allelic combination was analysed on Pasteur platform (http://bigsdbs.pasteur.fr/perl/bigsdbs/bigsdbs.pl?db=pubmlst_klebsiella_seq_def_public).

Results

Antimicrobial Resistance Profiles in *K. pneumoniae* Cultures

K. pneumoniae cultures showed a multi-carbapenem-resistant phenotype, with all resistant to ertapenem, 22 (84%) to meropenem, and 20 (77%) to imipenem. Twenty isolates were resistant to all carbapenems tested. In addition, nineteen (73%) strains were col-R. No isolates were susceptible to ampicillin, amoxicillin-clavulanate, ceftazidime, ciprofloxacin, cefotaxime, cefuroxime, levofloxacin, piperacillin and piperacillin-tazobactam. Moreover, 81% (n=21) isolates showed resistance to tobramycin, while 16 (62%) were resistant to trimethoprim, 16 (61.5%) to amikacin, 8 (31%) to tigecycline, 5 to gentamicin, and 2 to fosfomicin.

Prevalence of Resistance-Associated Genes

All *K. pneumoniae* harbored *bla*_{KPC}, and 69.2% (n=18) were *bla*_{VIM} positive. A high proportion of isolates also carried ESBLs. The *bla*_{SHV} and *bla*_{TEM} were found in 96.2% (n=25) and 88.4% (n=23), respectively, and 84.6% (n=22) harbored both genes. The *bla*_{CTX-M} was only found in two strains. None of the strains carried *bla*_{GES}, *bla*_{NDM-1} or *bla*_{OXA-48}.

Prevalence of *mcr* Variants and *mgrB* Analysis

None of the 19 col-R isolates showed plasmid *mcr*-1,2,3,4,5,6,7,8 variants. Colistin resistance was also investigated through *mgrB* analysis, and the initial evaluation using agarose gel analysis, considering that the expected amplicon for wild-type *mgrB* has a 253 bp size.¹³ PCR products were sequenced to identify *IS* or mutations involved in colistin resistance.

Amplicons longer than the expected size were observed in six (31.6%) out of the 19 col-R isolates, and sequencing confirmed the presence of transposon. The most common insertion element detected in five cultures belonged to *IS5*-like family (1056 bp), while *ISKpn14* element was found in KP11 (Figure 2). Furthermore, KP8, KP12, KP14, KP18 and KP19 isolates with *IS5*-like elements were all grouped in the PFGE cluster VIII.

In col-R strains with 253 bp amplicon, an identical deletion Δ g19 causing frameshift mutation and premature MgrB termination was found in KP9 and KP10 (n=2, 10.5%) isolates; missense mutations t95→g translated into V32G were found in 42.1% (n=8; KP5, KP31, KP34, KP36, KP40, KP41 and KP43) isolates; missense mutations c62→a translated as T21N occurred in KP28, as well as missense mutation g60→a translated into W20Stop was found in KP31 (Figures 3 and 4). After open reading frame identification, sequence analysis with PROVEAN was reported in Table 2.

K. pneumoniae Molecular Epidemiology

PFGE revealed 12 clusters at 85% cut-off similarity (Figure 1): cluster VIII was the most common, grouping 9 (34.6%) isolates, followed by cluster V with three isolates, and clusters III, IV, and VI, each including two cultures. Dendrogram analysis at 95% similarity revealed 18 pulsotypes (PTs), with PT12 as the prevalent (n=4 isolates,

15.3%), followed by PT1, 3, 8 and PT10, each associated with two strains. PFGE discriminatory power was of 96%, as calculated by Simpson's Index of Diversity.³⁴

Three STs were identified (discriminatory power=0.61): the ST512 as the most common (n=13, 50%), followed by ST101 (n=10), and ST307 (n=2). It was not possible to define ST for KP10. ST512 was the most frequently detected during 2014–2015 (84.6%), while ST101 was the predominant during 2016–2017 (61.5%).

Discussion

The rapid spread of antibiotics resistance is nowadays a major concern causing untreatable infections in humans. A rising of MDR rate would lead to 10 million people dying every year by 2050, which exceeds the 8.2 million estimated deaths due to cancer.³⁵

This study describes the AMR profiles and molecular epidemiological insights concerning colistin-resistant CR-*Kp* isolated during 2014–2017. *K. pneumoniae* were most commonly isolated from patients aged ≥ 60 years who were treated in the ICU, where invasive procedures with devices at risk of generating biofilms formation play a crucial role in the occurrence of CR-*Kp*.^{9,36,37}

Twenty-one antimicrobial susceptibility patterns were found, underlining high inter-strain diversity. All cultures had an MDR pattern, with high percentages of carbapenem (76.9%) and colistin resistance (73%). Conversely, 92% of cultures were susceptible to fosfomycin, which has been recently evaluated for treating extensively drug-resistant (XDR) pathogens, although resistance associated to *fosA* gene is emerging and can be transferred between *Enterobacteriaceae*.³⁸

The increased application of colistin therapy for infections due to MDR Gram-negative bacteria has contributed to the spread of transmissible resistance, and may speed up the progression from XDR to Pan-drug Resistant (PDR) *Enterobacteriaceae*.^{4,14,39} In our study, prevalence of col-R *K. pneumoniae* was higher than that reported in other Italian studies, ranging between 36.1% and 50%.^{9,40–42}

Results regarding ESBLs presence are in line with other studies, where plasmid-acquired *bla*_{TEM} and *bla*_{SHV} were frequently associated with *Klebsiella* spp. infections.^{43,44} Conversely, *bla*_{CTX-M} enzymes have become the most prevalent in *E. coli*, with potential to spread beyond the hospital environment in other species.⁴⁵ The identification of ESBL-producing *Klebsiella* in hospital settings should be followed by infection control interventions, with

Table 2 PROVEAN Analysis of the Single Amminoacid Change of MgrB Protein

Strain	Variant	Description	PROVEAN score	Prediction (cut off= -2.5)
KP5 KP7 KP31 KP34 KP36 KP40 KP41 KP43	V32G	Valine in position 32 is mutated in Glycine	-7	Deleterious
KP28	T21N	Threonine in position 21 is mutated in Asparagine	-3.23	Deleterious

Notes: Variants and their description are reported in the second and third columns, respectively. PROVEAN score is a measure of the change in protein structure: if the score is equal or below the predefined threshold (cut off = -2.5), the variant is predicted to have a "deleterious" effect; if above, the variant is predicted to have a "neutral" effect.²⁷ In the latter column, there is a prediction of the mutation effect on the protein functionality.

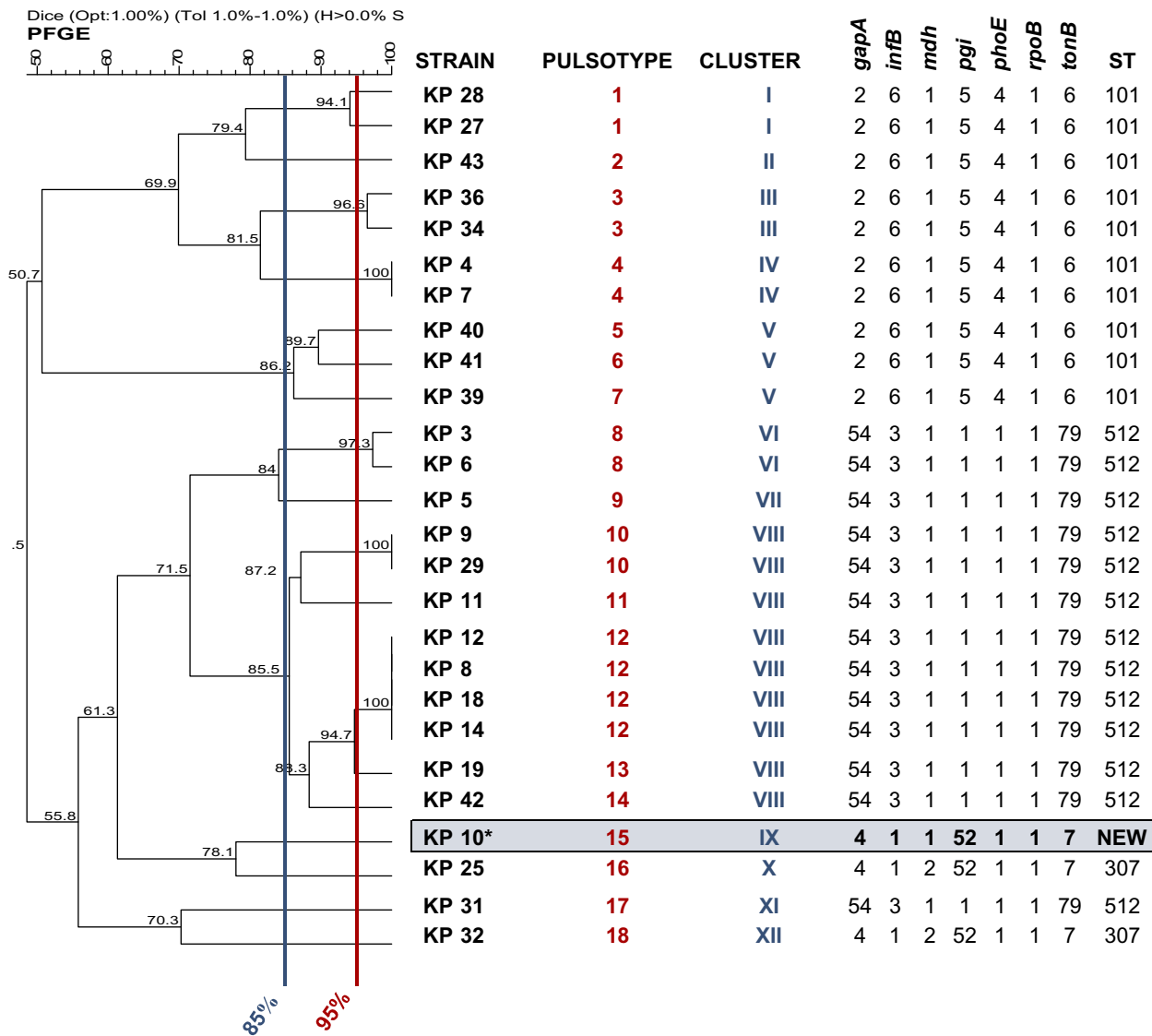


Figure 1 PFGE dendrogram (Dice coefficient) and MLST results for 26 clinical *K. pneumoniae* isolates.
Note: The new ST is highlighted in blue.
Abbreviation: ST, Sequence Type.

reinforcement of hand hygiene of primary importance, followed by compliance with guidelines on antibiotic stewardship, and removal of contaminated devices.⁴⁶

Concerning carbapenemases encoding genes, *bla*_{VIM} and *bla*_{KPC} genes were detected in 70% and 100% isolates, respectively, which is consistent with the endemic KPC circulation reported in Italy.⁵ Globally, the most worrying scenario is the increasing spread and dissemination of KPC-producing *K. pneumoniae* of clonal complex CC258 and CC512 being responsible for several outbreaks, unlike VIM carbapenemase, which is currently not widely diffused in Italy.^{8,47} Furthermore, *K. pneumoniae* producing NDM-1 or OXA-48 were not

detected, similarly to IMP and GES, according to national data reporting sporadic cases.⁴⁵

The increasing occurrence of col-R strains is considered a global concern. In Italy, a retrospective study (from January 2010 to June 2014) reported a threefold increase of colistin-resistance rate in KPC-producing *K. pneumoniae* in blood isolates, and 51% mortality at 30 days due to bloodstream infections.⁴⁸

In our study, *mcr*-1,2,3,4,5,6,7,8 were not detected in col-R strains, which is consistent with previous reports,⁴² being more frequently detected in *E. coli* than in *K. pneumoniae*.⁴⁹ Approximately 95% of col-R isolates carried alterations in *mgrB*, which is likely to be

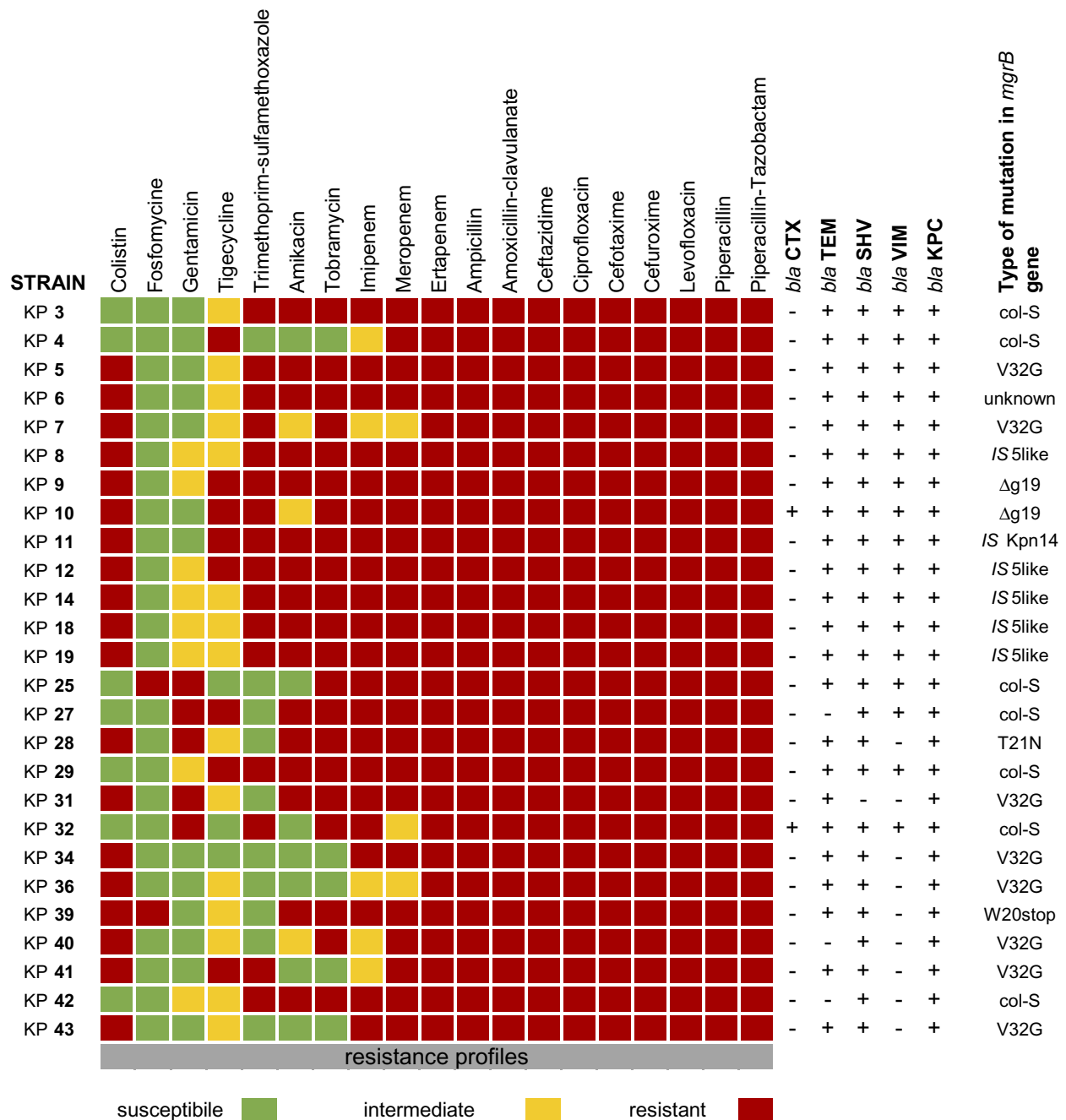


Figure 2 Antimicrobials resistance phenotypes and antimicrobial resistance genes profiles for 26 *K. pneumoniae* isolates. **Abbreviations:** col-S, colistin-sensitive; Δg19, deletion of guanine in position 19; V32G, Valine in position 32 is mutated in Glycine; T21N, Threonine in position 21 is mutated in Asparagine; W20stop, Tryptophan is mutated in stop codon; unknown, no mutation in *mgrB* gene.

responsible for the colistin-resistant phenotypes. Inactivation of *mgrB* throughout *IS*s especially by *IS5*-like and *ISKpn14* elements was detected in six out of 19 col-R strains. These mechanisms were reported elsewhere,^{13,16} and *IS*s transfer within genomes and plasmids has been considered a common driver of diversity and acquisition of antibiotic resistance.⁵⁰ Furthermore, it

has been reported that plasmids transfer between strains within the gut is a potential mechanism of indirect acquisition of colistin resistance.⁵¹ As assessed in vitro, *IS* interrupting *mgrB* and conferring colistin resistance was initially located on a plasmid.⁵²

In three isolates, a truncated MgrB due to one single nucleotide deletion causing frameshift mutation and

	10 20 30 40 50
WT (Cannatelli 2014)	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP25 (WT-this work)	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP42 (WT-this work)	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP5	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP6	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP7	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP9	GTGAAAAAAT TACGGTGG-T TTTACTGATA GTCATCATAG CAGGCTGCCT
KP10	GTGAAAAAAT TACGGTGG-T TTTACTGATA GTCATCATAG CAGGCTGCCT
KP28	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP31	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP34	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP36	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP39	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP40	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP41	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT
KP43	GTGAAAAAAT TACGGTGGGT TTTACTGATA GTCATCATAG CAGGCTGCCT

	60 70 80 90 100
WT (Cannatelli 2014)	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP25 (WT-this work)	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP42 (WT-this work)	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP5	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP6	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP7	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP9	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP10	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP28	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP31	GTTGCTGTGA ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP34	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP36	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP39	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP40	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP41	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT
KP43	GTTGCTGTGG ACTCAGATGC TTAACGTAAT GTGCGACCAG GATGTTTCAGT

	110 120 130 140 144
WT (Cannatelli 2014)	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP25 (WT-this work)	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP42 (WT-this work)	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP5	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP6	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP7	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP9	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP10	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP28	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP31	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP34	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP36	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP39	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP40	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP41	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA
KP43	TTTTTCAGCGG CATTTCGACT ATTAATAAAT TTATTCCGTG GTAA

Figure 3 Alignment of FASTA *mgrB* sequence in col-R *K. pneumoniae* without ISs compared with wild-type (WT) sequences in col-S isolates.

Notes: WT strains in blue; mutation highlighted in red.

premature termination was found, as reported elsewhere.⁵³ In addition, nine isolates had a non-functional MgrB due to one amino acid change (V32G,

T21N and W20Stop), as observed in other studies.^{1,9,53}

Hence, colistin resistance was linked to alterations in *mgrB* because complementation studies with a wild-type

	WT (Cannatelli 2014)	KP25 (WT)	KP42 (WT)	KP6	KP5	KP7	KP34	KP36	KP39	KP40	KP41	KP43	KP28	KP31	KP9	KP10
1	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
2	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
3	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
4	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
7	W	W	W	W	W	W	W	W	W	W	W	W	W	W	F	F
8	L	L	L	L	L	L	L	L	L	L	L	L	L	L	Y	Y
9	L	L	L	L	L	L	L	L	L	L	L	L	L	L	stop	stop
10	I	I	I	I	I	I	I	I	I	I	I	I	I	I		
11	V	V	V	V	V	V	V	V	V	V	V	V	V	V		
12	I	I	I	I	I	I	I	I	I	I	I	I	I	I		
13	I	I	I	I	I	I	I	I	I	I	I	I	I	I		
14	A	A	A	A	A	A	A	A	A	A	A	A	A	A		
15	G	G	G	G	G	G	G	G	G	G	G	G	G	G		
16	C	C	C	C	C	C	C	C	C	C	C	C	C	C		
17	L	L	L	L	L	L	L	L	L	L	L	L	L	L		
18	L	L	L	L	L	L	L	L	L	L	L	L	L	L		
19	L	L	L	L	L	L	L	L	L	L	L	L	L	L		
20	W	W	W	W	W	W	W	W	W	W	W	W	W	W	stop	
21	T	T	T	T	T	T	T	T	T	T	T	T	T	N		
22	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q		
23	M	M	M	M	M	M	M	M	M	M	M	M	M	M		
24	L	L	L	L	L	L	L	L	L	L	L	L	L	L		
25	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
26	V	V	V	V	V	V	V	V	V	V	V	V	V	V		
27	M	M	M	M	M	M	M	M	M	M	M	M	M	M		
28	C	C	C	C	C	C	C	C	C	C	C	C	C	C		
29	D	D	D	D	D	D	D	D	D	D	D	D	D	D		
30	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q		
31	D	D	D	D	D	D	D	D	D	D	D	D	D	D		
32	V	V	V	V	G	G	G	G	G	G	G	G	G	V		
33	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q		
34	F	F	F	F	F	F	F	F	F	F	F	F	F	F		
35	F	F	F	F	F	F	F	F	F	F	F	F	F	F		
36	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
37	G	G	G	G	G	G	G	G	G	G	G	G	G	G		
38	I	I	I	I	I	I	I	I	I	I	I	I	I	I		
39	C	C	C	C	C	C	C	C	C	C	C	C	C	C		
40	T	T	T	T	T	T	T	T	T	T	T	T	T	T		
41	I	I	I	I	I	I	I	I	I	I	I	I	I	I		
42	N	N	N	N	N	N	N	N	N	N	N	N	N	N		
43	K	K	K	K	K	K	K	K	K	K	K	K	K	K		
44	F	F	F	F	F	F	F	F	F	F	F	F	F	F		
45	I	I	I	I	I	I	I	I	I	I	I	I	I	I		
46	P	P	P	P	P	P	P	P	P	P	P	P	P	P		
47	W	W	W	W	W	W	W	W	W	W	W	W	W	W		
48	stop	stop	stop	stop	stop	stop	stop	stop	stop	stop	stop	stop	stop	stop		

Figure 4 FASTA alignment of MgrB amino acid sequence in col-R *K. pneumoniae* without ISs compared with wild-type (WT) sequence of col-S strains. **Notes:** WT MgrB in col-S strains in light blue; non-functional MgrB without ISs in col-R strains in green; truncated MgrB in col-R strains in yellow.

mgrB demonstrated that susceptibility to colistin can be successfully restored.⁵³ In our study, col-R KP6 showed a wild-type *mgrB*, suggesting mutations in other colistin-resistance-related genes within Pmr signaling system or by alternative mechanism(s).

The presence of identical *mgrB* alterations in isolates from the same ward and assigned to the same ST and PFGE profile supports the clonal expansion and cross-transmission in hospital setting.⁵³

PFGE revealed high level of strains diversity, and results from MLST indicated the circulation of ST512, ST101 and ST307. In the tested isolates, ST512, a single-locus variant of ST258, the most frequently detected clone responsible for KPC global spread,⁵⁴ was the most common that is consistent with studies elsewhere in Italy.^{9,37,39} For KP10 strain, the ST was not assigned, being found a monoallelic variant of ST307 (4-1-1-52-1-1-7 instead of 4-1-2-52-1-1-7); thus, further analyses are needed to confirm the novel ST. Interestingly, MLST revealed that ST512 was the most frequently detected in 2014–2015, while ST101 prevailed during 2016–2017, suggesting a changed circulation in the latest years in our hospital. Remarkably, PFGE cluster VIII grouped 77% of col-R cultures, four of which isolated from patients within the ICU as indistinguishable pulsotypes, all carried transposons in *mgrB* and were isolated during Christmas season holidays (December 2014–January 2015). In particular, the cluster VIII included the cultures KP8 (isolation data 17/11/14), KP12 and KP14 (isolation data 12/1/14), and KP18 (isolation data 5/1/15) (Table 1), belonging to a group of strains isolated during an outbreak in the ICU ward, which was likely related to a low level of compliance to standard hygiene procedures because of reduced personnel availability, and underlined the likelihood of bacterial persistence in the hospital environment.¹⁰

The discriminatory abilities of PFGE and MLST were compared by the number of unique STs and number of clusters identified. PFGE showed good discriminatory power, and it is still considered a reference method for the epidemiological investigations of infectious diseases, including nosocomial outbreaks.⁵⁵ PFGE, generating genome-wide DNA fingerprints with rare-cutter restriction enzymes, is also a cost-effective method; nevertheless, it is labor-intensive and may lack comparability between laboratories due to operator errors in identifying bands particularly when shifted or weak on PFGE gel image analysis. In our study, MLST was less discriminating than PFGE, as found elsewhere.²⁴ Anyway, MLST is

considered the most suitable genotyping method for strains comparison, further providing data within laboratories, and appropriate for global and long term or evolutionary studies rather than local epidemiology.⁵⁵

Conclusions

To our knowledge, this is the first study concerning colistin and carbapenems resistance characteristics in clinical *K. pneumoniae* isolates from the Molise Region, central Italy. Although focusing on topic investigated elsewhere, our findings can be useful to better understand the most significant concerns on hospital infections by *K. pneumoniae* at a local level, and can support the molecular epidemiology data related to CR-Kp both nationally and internationally, hence contributing to complete the framework of the epidemiology of this microorganism.

This study confirms that CR-Kp infections are most commonly detected in ICU patients due to their critical conditions and invasive procedures like catheterization or tracheostomy. In our setting, the KPC enzyme remains the predominant carbapenemase in *K. pneumoniae*, followed by VIM. The highest prevalence of KPC was linked with ST512 prevalence, although a switching towards ST101 circulation was detected. A high level of colistin resistance was found, more than the rate reported in other studies, likely due to an overuse of colistin in our hospital setting, and it is associated with acquisition of insertion elements or accumulation of deleterious mutations in the *mgrB* gene. Further investigations are warranted to clarify the entire role of Pmr signaling system in col-R strains.

In conclusion, infections with carbapenem-resistant organisms, particularly when KPC-producing, are widely distributed, and antimicrobial treatments selected should be critically evaluated, since an optimal therapy is not yet defined.⁵⁶ In light of the lack of novel antimicrobial agents for the treatment of difficult healthcare infections, the implementation of proper prevention strategies and adequate staffing is essential to control the spread of MDR *K. pneumoniae*.²⁶ Moreover, the routine application of molecular analyses for rapid and accurate detection of determinants and mutations conferring resistance is crucial to reduce and control the burden of MDR bacterial infections⁴⁰ and to guide best-choice therapy for better patient outcomes, as well as to elucidate epidemiology and dynamics of dissemination in the hospital environment.

Certainly, while, on one hand, not all the infections are associated with modifiable factors, evidences suggest that

the spectrum of situations in which currently it is possible to intervene is broader than in the past. Furthermore, triggering and modifiable factors are mostly due to inadequate healthcare practices, particularly to the failure in applying standard and specific precautions for infectious diseases to avoid unnecessary procedures, the inappropriate use of antibiotics, and the lack of human and technological resources to be committed in the care and prevention.

Ethics

A formal institutional review board process for the ethical approval of this research was not required; thus, it is not available since no experimental, clinical or diagnostic procedures other than ones required for clinical management of the patients were performed. Furthermore, patients' anonymous information were provided from the microbiology hospital laboratory, which isolated the strains. The study completely followed the principles outlined in the Declaration of Helsinki.

Data Availability

All data generated or analysed during this study are included in the manuscript .

Acknowledgment

The authors are grateful to Dr. Jim McLauchlin, Public Health England, London, UK, for the useful comments and revisions provided to this work.

Author Contributions

All authors substantially contributed to conception and design, acquisition, analysis and interpretation of data, drafted and critically revised the article for important intellectual content, approved the final version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Disclosure

The authors report no conflicts of interest in this work.

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