Short Communication

Expansion of KPC-producing *Klebsiella pneumoniae* with various *mgrB* mutations giving rise to colistin resistance: the role of ISL3 on plasmids

Cesira Giordano a, Simona Barnini a, Constantinos Tsioutis b,c, Monika A. Chlebowicz d, Effie V. Scoulica c, Achilles Gikas c, John W. Rossen d, Alexander W. Friedrich d, Erik Bathoorn a

a Bacteriology Unit, Azienda Ospedaliero–Universitaria Pisana, via Paradisa 2, 56124, Pisa, Italy
b Department of Internal Medicine, Infectious Diseases Unit, University Hospital of Heraklion, Voutes, 71110, Heraklion, Crete, Greece
c School of Medicine, European University Cyprus, 6 Diogenis Street, Engomi, Nicosia 1516, Cyprus
d University of Groningen, University Medical Center Groningen, Department of Medical Microbiology, Groningen, The Netherlands

**ABSTRACT**

*mcr*-1 has been reported as the first plasmid-encoded gene conferring colistin resistance. In KPC-producing *Klebsiella pneumoniae* (KPC-KP), however, colistin resistance is rapidly emerging through other mechanisms. Resistance is frequently due to disruption of the *mgrB* gene by insertion sequences, e.g. ISL3. The aim of this study was to investigate the expansion of *mgrB*-mutated KPC-KP isolates. In addition, the localisation and targets of ISL3 sequences within the core and accessory genome of common KPC-KP lineages were identified. A total of 29 clinical *K. pneumoniae* isolates collected from Italian patients were randomly selected. Whole genome sequences were analysed for resistance genes, plasmids and insertion sequences. In addition, 27 colistin-resistant KPC-KP isolates from a previous study from Crete (Greece) were assessed. Clonal expansion of KPC-KP isolates with various mutations in *mgrB* among all lineages was observed. In two Italian MLST ST512 isolates and eight Greek ST258 isolates, an identical copy of ISL3 was inserted in *mgrB* nucleotide position 133. ISL3, a transposable restriction–modification system of 8154 nucleotides, was located on pKpQIL-like plasmids and may transpose into the chromosome. In four isolates, chromosomal integration of ISL3 in diverse inner membrane proteins other than *mgrB* was identified. Colistin resistance is most often explained by clonal expansion of isolates with mutated *mgrB*. pKpQIL-like plasmids, which are omnipresent in KPC-KP, carry insertion sequences such as ISL3 that have *mgrB* as a target hotspot for transposition. Transposition of insertion sequences from plasmids and subsequent clonal expansion may contribute to the emerging colistin resistance in KPC-KP.

© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

*Klebsiella pneumoniae* has emerged as an important multidrug-resistant nosocomial pathogen worldwide. In some countries, e.g. Italy, Greece and some parts of the USA, carbapenemase-producing *K. pneumoniae* (CPKP) are epidemic and represent a significant proportion (ca. 40%) of *K. pneumoniae* isolates [1–3]. CPKP have become a major clinical concern owing to their rapidly increasing resistance to nearly all currently available antibiotics [4]. Owing to extended antibiotic resistance of these strains, infections caused by CPKP are difficult to treat and are usually associated with high mortality rates [5]. Colistin represents a last-line antibiotic choice in the treatment of bacterial infections by carbapenemase-producing Gram-negative isolates [6], representing a valid alternative treatment when carbapenems are not effective. However, increasing colistin use has led to resistance, especially during therapy [7]. In Italy, the rate of colistin-resistant *K. pneumoniae* rose from 36% in 2011 to 50% in 2015, leading to a worrisome recurrence of clinical outbreaks [8,9].

The majority of known resistance mechanisms in *K. pneumoniae* involve lipopolysaccharide (LPS) modification mainly occurring at the level of the outer membrane. An increasing number of studies have demonstrated that modifications of the PmrA/B and PhoQ/P two-component systems as well as inactivation of MgrB are able to decrease the affinity of colistin for LPS. PmrA/B and PhoQ/P regulate the *pmrHFIJKLM* operon, which controls modification of the...
outer membrane. The 144-bp \textit{mgrB} gene encodes a small transmembrane protein that exerts negative feedback on the abovementioned pathway by interaction with the sensor kinase PhoQ at the periplasmic level [10]. Different mutations occurring in the \textit{mgrB} gene have already been described as a major cause of colistin resistance in \textit{K. pneumoniae}. In resistant strains, complete and partial \textit{mgrB} gene deletions [8,11], non-synonymous single nucleotide polymorphisms (SNPs) [12] and inactivation by various insertion sequence (IS) elements have been found [8,12,13]. The latter event has been repeatedly detected in colistin-heteroresistant nosocomial isolates and involves \textit{mgrB} gene disruption by a variety of insertion sequences. However, we have little insight into the prevalence of these IS elements in the plasmids of clinical isolates and their transposition patterns.

The present study aimed to investigate vertical and horizontal expansion of mutations occurring in \textit{mgrB}, in particular those occurring through insertion of ISL3. Furthermore, the study aimed to identify the positions in the genome that constitute key targets of ISL3 sequences in colistin-resistant CPKP. For this purpose, whole genome sequences of various common nosocomial CPKP lineages isolated in two different regions, namely Pisa (Italy) and Crete (Greece), were analysed and compared.

\section{Materials and methods}

A total of 29 clinical \textit{K. pneumoniae} isolates resistant to colistin were randomly selected from unique patients at Azienda Ospedaliero–Universitaria Pisana (Pisa, Italy) during 2015–2016. Various biological samples were cultured on common isolation media in routine diagnostics and were incubated at 37 °C. Suspected colonies were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonics GmbH, Bremen, Germany). Antimicrobial susceptibility testing for amikacin, amoxicillin/clavulanic acid, ampicillin/sulbactam, cefepime, cefotaxime, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, levofloxacin, meropenem, nitrofurantoin, piperacillin/tazobactam, tigecycline and trimethoprim/sulfamethoxazole (SXT) were performed by broth microdilution assay (SensititreTM; Thermo Fisher, Waltham, MA). Minimum inhibitory concentrations (MICs) were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Colistin-resistant isolates were stored in glycerol at –80 °C for DNA extraction. Total genomic DNA was extracted from fresh cultures using an UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer’s instructions. The concentration and purity of the extracted DNA were determined with a Qubit® 2.0 fluorometer using the dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA). A DNA library was prepared using Nextera XT v.01 (Illumina Inc., San Diego, CA) according to the manufacturer’s instructions and was then run on a MiSeq system (Illumina Inc.) to generate 250-bp paired-end reads. De novo assembly was performed using CLC Genomics Workbench v.9.5.2 (Qiagen, Hilden, Germany) after quality trimming (Qs ≥ 20) [14]. Multilocus sequence typing (MLST) as well as whole-genome MLST (wgMLST) comparison based on an ad hoc scheme including 4891 alleles were performed using SeqSphere v.3.4.0 ( Ridom GmbH, Münster, Germany). Assembled genomes were uploaded to the web tools ResFinder 2.1 [15] to identify acquired resistance genes as well as to PlasmidFinder 1.3 [16] to detect plasmids. The sequences are available from the European Nucleotide Archive (study PRJEB198808). The DNA sequences of \textit{mgrB} (GI: 695277517), \textit{phoP} (GI: 378976159), \textit{phoP} (GI: 378976159), \textit{pmrA} (HG7942341.1:3372–4043) and \textit{pmrB} (HG7942341.1:2271–3368) were used as references for detecting gene mutations associated with colistin resistance. Nucleotide positions refer to the coding sequence of the genes, starting with the first base of the start codon. PROVEAN software tool was used to predict whether sequence variants at the nucleotide level resulted in amino acid substitutions with an impact on the biological function of proteins [17]. IS elements were identified using ISfinder [18]. The ISL3 reference sequence (GI: \texttt{NC_0099650}) was BLASTed against the whole genome sequence of all isolates. The contigs of plasmid sequences of interest, detected by PlasmidFinder, were mapped against complete reference sequences of plasmids pKpQIL-10 (\texttt{KJ146687.1}) and pKPN-IT (\texttt{JN233704.1}). BLAST Ring Image Generator (BRIG) was used to display plasmid sequence comparisons [19].

In addition, 19 colistin-resistant isolates were selected from a previously characterised collection of 34 randomly assessed clinical KPC-producing \textit{K. pneumoniae} (KPC–KP) isolates collected between 2010–2014 in Crete (Greece) and deposited at the European Nucleotide Archive (study PRJEB10561) for molecular comparison [12].

\section{Results}

\subsection{Isolate characteristics}

The 29 isolates from Italy belonged to three different sequence types (2 to ST307, 2 to ST37 and 25 to ST512). All isolates were resistant to colistin with MICs ranging from 4 mg/L to >8 mg/L (Table 1). Of the 29 isolates, 27 were resistant to carbapenems with meropenem MICs of >8 mg/L. Isolates from five patients (17%) were resistant to all tested antibiotics, and in 10 other patients (34%) only one antibiotic tested susceptible (tigecycline, fosfomycin or SXT) (Supplementary Table S1). The \textit{bla}\textsubscript{KPC}-3 carbapenemase gene was identified in 28 isolates. In two isolates, both the \textit{bla}\textsubscript{KPC}-3 and \textit{bla}\textsubscript{IMP}-1 carbapenemase genes were detected (Supplementary Table S2). In addition, different combination of \textit{B}–lactamase genes encoding CTX-M-15, OXA-1, OXA-9, SHV-11, SHV-28 and TEM-1A were detected. Several aminoglycoside resistance genes, including the \textit{aac(6')-Ib-cr} gene responsible for low-level resistance to fluoroquinolones and aminoglycosides, were detected in all isolates except two. The \textit{dfrA12}, \textit{dfrA14} and \textit{dfrA17} genes associated with resistance to trimethoprim were detected in 22 isolates. The plasmid-encoded colistin resistance gene \textit{mcr-1.2} associated with an IncX4 plasmid was detected in one isolate. Mutations of selected genes (\textit{mgrB}, \textit{phoP}, \textit{phoQ}, \textit{pmrA} and \textit{pmrB}) associated with resistance to colistin are presented in Table 1. The \textit{mgrB} gene was mutated in 22 colistin-resistant isolates. In 16 isolates, all belonging to ST512, there was a deletion of 11 nucleotides (\texttt{Δ109–119}) in \textit{mgrB} predicted to lead to a truncated \textit{MgrB} protein of 37 amino acids with a G37Y mutation. In two ST37 isolates there was a point mutation (\texttt{A7T}) creating a stop codon. Disruption of \textit{mgrB} by insertion sequences was detected in four isolates: in two ST512 isolates a copy of the complete insertion sequence \textit{ISKpn26} (\textit{ISS} family) was detected at nucleotide position 75; and in another two ST512 the intact insertion sequence \textit{ISKpn25} (\textit{ISL3} family) was inserted at nucleotide position 133. Point mutations in the \textit{phoP} gene were detected in two isolates. In one of these isolates, the nucleotide mutation T602C leading to amino acid substitution I201T was observed, predicted to have a deleterious impact on the protein function. No mutations were detected within the \textit{phoQ} gene. In two ST307 isolates, a point mutation \texttt{G121A} was detected in the \textit{pmrA} gene, resulting in amino acid change A41T that is potentially deleterious. Furthermore, in one of the latter isolates, an additional \textit{G385C} (amino acid A129P) mutation in the \textit{pmrB} gene was detected, predicted to be deleterious. In one ST512 isolate a three-nucleotide in-frame insertion in \textit{pmrB} at position 403 was detected. Finally, no mutations in the abovementioned genes were found in four colistin-resistant isolates with MICs ranging from 4–8 mg/L.
Non-synonymous nucleotide mutations and their positions in the reference coding sequence are presented.

MLST, multilocus sequence typing; COL, colistin; MIC, minimum inhibitory concentration; R, resistant.

publication characteristics of these isolates have been reported in a previous paper from Italy. Also, two Greek ST258 isolates and two Italian ST512 isolates from Greece, and a cluster of two isolates among the ST512 isolates from Italy were included. Among the Greek isolates, there was a cluster of seven isolates from Greece with the G110C substitution, and two ST37 isolates were observed, all belonging to ST512, collected in different hospitals. Among the Greek isolates, there was a cluster of seven ST258 isolates with disruption of the mgrB by ISL3.

3.2. Investigation of clonal expansion by whole-genome multilocus sequencing (wgMLST)

The 22 Italian isolates with mutations in the mgrB, together with 19 isolates from the previous Greek study, were selected for wgMLST comparison. The selected isolates from the Greek study with mutations in mgrB or phoQ are shown in Supplementary Table S3. The characteristics of these isolates have been reported in a previous publication [12]. Fig. 1 shows a minimum spanning tree based on allelic mismatch between these isolates. The different MLST types are separated by an intermittent line. ST512 and ST258 are closely related to each other (minimum spanning of 127 alleles difference), whereas ST37 and ST307 are distinct from all presented MLST types (minimum spanning of >3000 alleles difference).

Five clonally related clusters of isolates with identical mutations in mgrB were observed. The red arrows point out two clusters of isolates with identical insertions of ISL3 in nucleotide position 133 of mgrB: a cluster of eight isolates among the ST258 isolates from Greece, and a cluster of two isolates among the ST512 isolates from Italy. Also, two Greek ST258 isolates and two Italian ST512 isolates showed identical disruption of mgrB at nucleotide position 75 by ISL3.

Other clusters of isolates with identical mutations in mgrB included a clonal cluster of 16 ST512 isolates from Italy harbouring the 11-nucleotide deletion in mgrB, a cluster of three ST258 isolates from Greece with the G110C substitution, and two ST37 isolates from Italy with the A77 substitution. Among Italian isolates, a clonal cluster of isolates harbouring the 11-nucleotide deletion in mgrB was observed, all belonging to ST512, collected in different hospitals. Among the Greek isolates, there was a cluster of seven ST258 isolates with disruption of the mgrB by ISL3.

3.3. Position of ISL3 in the genome: plasmid position and chromosomal targets

ISL3, a transposable insertion sequence of 8154 nucleotides, is referred to as a type I restriction–modification system harbouring a transposase (100% identity with ISL3 family transposase WP_004152342.1), a DNA sequence specificity subunit (100% identity with WP_004152344.1), a restriction endonuclease subunit (100% identity with WP_004152343.1) and a DNA methyltransferase subunit (100% identity with WP_004152345.1). As shown in Fig. 2, the complete ISL3 sequence was localised on a pKpQIL-like plasmid, which was detected in all five MLST types. The ISL3 sequence was not detected in the two isolates (ST512 and ST37) in which the pKpQIL-like plasmid was absent. Intact ISL3 was not present on all other detected plasmids, including IncFIB(K) (26 isolates), CoRnAI (22 isolates), IncFlI(K) (28 isolates), IncX3 (17 isolates), IncN (2 isolates), IncQ1 (1 isolate), IncX4 (1 isolate), IncFAI (1 isolate) and IncFIB (AP00019182) (2 isolates). Sequence comparison between the ISL3 on the pKpQIL-like plasmids and ISL3 inserted in mgrB shows a 100% match between the 8154 nucleotides.

Chromosomal integrations of ISL3 in positions other than mgrB were identified in five isolates (Table 2). In two Italian ST512/ KPC-3 isolates, ISL3 was inserted in inner membrane protein genes murC and sbmA, respectively. One Greek ST258 isolate showed two ISL3 insertions: one in the yidL gene, an AraC family putative transcriptional regulator, and one in the inner membrane protein gene yidC. In a second Greek isolate, ISL3 was inserted in the inner membrane protein gene gaa, and in a third isolate it was inserted in the intergenic region between the two-component system genes ycgF and ycgZ.
4. Discussion

Clonal expansion of KPC-KP isolates with various mutations in \textit{mgrB} in all lineages was shown. De novo acquisition of colistin resistance can occur through transposition of insertion sequences with \textit{mgrB} as a target. Genetic comparison of KPC-KP lineages showed that disruption of the chromosomal \textit{mgrB} gene may be caused by transposition of the IS\textsubscript{L3} insertion sequence carried by pKpQIL-like plasmids. This IS\textsubscript{L3}-carrying plasmid is omnipresent in KPC-KP, both in KPC-2-KP lineages isolated in Greece and in KPC-3-KP isolates originating from Italy. Horizontal dissemination of plasmids harbouring insertion sequences, e.g. IS\textsubscript{L3}, could facilitate the
acquisition of colistin resistance de novo. The observed subsequent clonal expansion contributes to the emergence of colistin resistance in KPC-KP in Southern Europe.

Colistin resistance in KPC-KP was sporadically reported in the early days of the KPC-KP outbreak from 2007–2010 [9,12]. In recent years, resistance to colistin with MICs varying from 4 mg/L to 256 mg/L has been reported in studies from Greece and Italy [12,20]. Insertional inactivation of \textit{mgrB} by insertion sequences was most often associated with colistin resistance. Vertical clonal expansion of colistin-resistant KPC-2-KP by \textit{mgrB} has been reported in a recent study [12], and part of these data are also shown in the present study. The involvement of plasmids in colistin resistance was shown in two recent reports [21,22]. We now add to this the horizontal and vertical dissemination of colistin resistance by plasmids carrying ISL3 causing disruption of \textit{mgrB}. A plasmid origin of insertion sequences targeting \textit{mgrB} has been suggested by Poirot et al [13]. They

Fig. 2. Genome comparison of ISL3-carrying pKpQIL-like plasmids in Italian and Greek KPC-producing \textit{Klebsiella pneumoniae} isolates. Plasmids are grouped by colour according to different multilocus sequence typing (MLST) types and countries where the isolates have been detected. The subunits of the complete ISL3 sequence are shown in red in the outer ring plasmid.
observed that mgrB truncations with identical IS5 insertion elements occurred among clonally unrelated isolates. The present study confirms this observation and identified the plasmid carrying these sequences. We focused on ISL3, however IS5 was also frequently detected on the same pKpQIL-like plasmids. In five isolates from Italy, disruption by IS5 of the traA gene coding for the pilus assembly protein carried by pKpQIL was detected. Chromosomal targets of IS5 were also detected: in two other Italian isolates, besides the two isolates with disrupted mgrB, IS5 was found to disrupt a chromosomal hypothetical protein.

In this study, it was shown that ISL3 not only targets mgrB but also other inner membrane protein-coding sequences such as marC, yfdC, igaA and sbmA. This frequent insertion of ISL3 in mgrB and other inner membrane protein sequences suggest that these targets are not random but are hotspots for integration of ISL3. In this study, mgrB was disrupted by ISL3 at the identical nucleotide position 133 in two different lineages (Italian ST512 and Greek ST258), which shows that this insertion sequence recombines at specific target sequence sites. This has also been described for IS5 [13]. Thus, insertion sequences may functionally disrupt inner membrane proteins and thereby confer antibiotic resistance.

Transposable insertion sequences may have a general role in antibiotic resistance modification in Enterobacteriaceae. For instance, mgrB was found truncated by IS5 family in another Klebsiella species, namely Klebsiella oxytoca, resulting in colistin resistance, which is similar to reports in K. pneumoniae [13,23]. In this study, a BLASTn search was performed to identify the presence of ISL3 in other Enterobacteriaceae. ISL3 family transposases are present in other Gram-negative isolates, including Escherichia coli (KCN999035), Citrobacter amalonaticus (CP011133.1) Raoultella ornithinolytica (CP013340.1) and Pantoea ananatis (CP014207.1) with identities of 100%, 96%, 96% and 93%, respectively. These ISL3 sequences appear to have evolved within the same genera. Future studies may give answer to the question whether horizontal transmission of insertion sequences may occur among different genera.

In conclusion, an international collaborative study on the molecular epidemiology of KPC-KP resulted in identification of clonal clusters with various mutations in mgrB leading to resistance to colistin. Expansion of a pKpQIL-like plasmid was demonstrated. Insertion sequences originate from this plasmid and target mgrB in KPC-KP lineages. Dissemination of ISL3 on pKpQIL plasmids that transpose into the same position in mgrB may explain colistin resistance in clonally unrelated isolates with identical mutations in mgrB. This is cause for serious concern for public health as colistin is among the few remaining treatment options for infections by multidrug-resistant Gram-negative pathogens.

Funding: This study was partly supported by the Innovation Fund of the Department of Medical Microbiology of the University Medical Center Groningen (Groningen, The Netherlands).

Competing interests: None declared.

Ethical approval: Not required.

---

**Table 2**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Country</th>
<th>Insertion</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1084</td>
<td>Italy</td>
<td>marC</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>1235</td>
<td>Italy</td>
<td>sbmA</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>554</td>
<td>Greece</td>
<td>yidL</td>
<td>Arac family putative transcriptional regulator</td>
</tr>
<tr>
<td>546</td>
<td>Greece</td>
<td>yfdC</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>517</td>
<td>Greece</td>
<td>igaA</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>2 isolates</td>
<td>Italy</td>
<td>mgrB</td>
<td>Inner membrane protein</td>
</tr>
<tr>
<td>10 isolates</td>
<td>Greece</td>
<td>mgrB</td>
<td>Inner membrane protein</td>
</tr>
</tbody>
</table>

**Appendix. Supplementary data**

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ijantimicag.2017.10.011.

**References**


[10] Lippe AM, Goulian M. Feedback inhibition in the PhoQ/PhoP signaling system which is similar to reports in other Gram-negative isolates, including other Enterobacteriaceae. IS 5 disrupts mgrB and other chromosomal hypothetical proteins.


