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Vertical and horizontal dissemination of an IncC plasmid harbouring rmtB 16S rRNA methylase gene, conferring resistance to plazomicin, among invasive ST258 and ST16 KPC-producing Klebsiella pneumoniae

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A B S T R A C T

Objectives: Carbapenem resistance in Klebsiella pneumoniae is a major clinical challenge. Aminoglycosides remain an important asset in the current therapeutic arsenal to treat these infections. We examined aminoglycoside resistance phenotypes and genomics in a collection of 100 invasive KPC-producing K. pneumoniae isolates sequentially collected in a Brazilian tertiary hospital between 2014 and 2016.

Methods: Aminoglycoside susceptibility testing was performed. We used a combined long-read (MinION) and short-read (Illumina) whole-genome sequencing strategy to provide a genomic picture of aminoglycoside resistance genes, with particular emphasis on 16S rRNA methyltransferases and related plasmids.

Results: 68% of the strains were resistant to gentamicin and 42% to amikacin, with 35% resistant to both of these commonly used aminoglycosides. We identified the 16S rRNA methyltransferase gene rmtB in 30% of these isolates: 97% (29/30) belonged to sequence type 258 (ST258) and a single isolate to the emergent ST16 clone. In ST258 and ST16 the rmtB gene was located on large IncC plasmids of 177 kb and 174 kb, respectively, highly similar to a plasmid previously identified in Proteus mirabilis in the same hospital. Moreover, 99% of the isolates remained susceptible to the veterinary-approved drug apramycin, currently under clinical development for human medicine.

Conclusion: Such findings in geographically and temporally related isolates suggest a combination of vertical clonal spread as well as horizontal interspecies and intraspecies plasmid transfer. This broad rmtB dissemination in an endemic setting for KPC-producing clones is worrisome since it provides resistance to most clinically available aminoglycosides, including the novel aminoglycoside-modifying enzyme-resistant plazomicin.

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1. Introduction

Antimicrobial resistance in Enterobacteriales has become a global challenge. Carbapenem-resistant Enterobacteriales were designated as a critical priority pathogen by the World Health Organization (WHO) in 2017 [1] because of their increasing incidence, high mortality and the relative lack of therapeutic options. In this context, last-resort antimicrobial agents such as polymyxins and aminoglycosides have regained popularity [2], especially in low- and middle-income countries where novel β-lactam/β-lactamase inhibitor combinations are not widely available. Acquisition of aminoglycoside resistance by carbapenemase-producing Enterobacteriales, and more particularly by Klebsiella pneumoniae, is thus of major concern.

Aminoglycosides are protein synthesis inhibitors with good bactericidal activity against a broad spectrum of bacterial species.
They bind to the aminoacyl-tRNA recognition site of the 16S rRNA constituting the 30S subunit of the ribosome and thus block protein synthesis. Aminoglycoside resistance may be caused by various mechanisms that can co-exist within single bacteria. Impaired permeability and increased efflux pumps decrease aminoglycoside susceptibility [3,4]. In addition, antibiotic inactivation by aminoglycoside-modifying enzymes (AMES) and enzymatic target site modification by 16S rRNA methyltransferases (RMFs) lead to higher drug resistance levels [3,4]. It is important to note that genes encoding enzymatic mechanisms of resistance are carried by mobile genetic elements, including plasmids, that easily disseminate between bacterial isolates including markedly different Enterobacteriales species.

Since aminoglycosides are commonly co-prescribed in the treatment of KPC infections, we investigated the susceptibility to aminoglycosides, including the novel agents plazomicin and apramycin, among invasive KPC-2-producing K. pneumoniae isolates. In addition, we studied the mechanisms of aminoglycoside resistance and molecularly characterised 16S RMT-harbouring plasmids.

2. Methods

2.1. Isolates

The 100 isolates tested in this study were obtained from a 3-year (2014–2016) cohort of KPC-2-producing K. pneumoniae bloodstream infection isolates from a Brazilian public teaching hospital located in the city of São Paulo. Clinical features associated with these cases have been previously published by our collaborative group [5]. Each isolate was cultured from a different patient. They had been tested by PCR for blaKPC positivity in the routine microbiology laboratory.

2.2. Determination of minimum inhibitory concentrations (MICs)

MICs were determined for amikacin, gentamicin and apramycin using the agar dilution method following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. In contrast, plazomicin MICs were determined using MIC Test Strip (Liofilchem®). EUCAST breakpoints were used for MIC interpretation of gentamicin and amikacin MICs. Since no EUCAST breakpoints are available for plazomicin and apramycin, the US Food and Drug Administration (FDA) breakpoint and epidemiological breakpoints from the European Food Safety Authority (EFSA) were applied for plazomicin (susceptible, ≤2 mg/L) and apramycin (susceptible, ≤8 mg/L), respectively. Escherichia coli strain ATCC 25922 was used as a quality control for all MIC determinations.

2.3. Whole genome sequencing (WGS)

In addition to the genomes already available from Andrey et al. [5] and Martins et al. [6], additional isolates were sequenced in order to obtain the complete genomic data set for the 100 isolates included in this study. DNA libraries were prepared using a Nextera XT DNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) and were sequenced using an Illumina MiSeq instrument (Illumina Inc.). Quality control of raw sequence reads included fastqc v.0.11.2, and quality and adaptor trimming were performed using Trimm Galore (0.4.3). Genome assemblies were performed using Newbler v.3.0 (454 Life Sciences, Branford, CT, USA) and SPAdes v.3.8.0 using default parameters [7]. For representative isolates P05 [belonging to sequence type 16 (ST16)] and P43 (belonging to ST258) (both carrying 16S RMT rmtB-bearing plasmids), total genomic DNA was sequenced using long-read (MiniION; Oxford Nanopore Technologies, Oxford, UK), allowing, in combination with Illumina raw short reads, hybrid de novo assembly using Unicycler v.0.4.0 [8–10]. This strategy allowed the generation of complete circularised sequences of the chromosome and corresponding plasmids for these two isolates. WGS data used in this study are available under BioProject accession nos. PRJNA510003, PRJNA628956, PRJNA629307, PRJNA628957, PRJNA629309, PRJNA628954, PRJNA628953 and PRJEB41225.

2.4. Antimicrobial resistance genes

Acquired aminoglycoside resistance genes were identified from the WGS data using the ResFinder online tool from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) [11]. Cut-off values of 80% coverage and 95% identity were used. For the AMES aac(6’)-Ib and aac(6’)-Ib-cr, when the ResFinder pipeline revealed <100% nucleotide identity, a manual analysis was conducted to determine whether the sequences carried mutations conferring cross-resistance to fluoroquinolones. Sequences were extracted on UGENE [12], translated and aligned using MUSCLE, with 45 sequences described by Ramirez and Tolmasky [13] used as references.

2.5. Sequence type and core genome multilocus sequence typing (cgMLST) analysis

The sequence type (ST) of isolates was determined from the WGS data by MLST analysis built in the Kleborate pipeline (https://github.com/katholt/Kleborate) using the 7-locus scheme described at the K. pneumoniae BIGSdb hosted at the Institut Pasteur (https://bigd.bpasteur.fr/klebsiella/klebsiella.html). The clonality and relatedness of the isolates was determined by cgMLST analysis implemented in the chewBBACA pipeline [14]. Briefly, the gene prediction algorithm Prodigal was trained using K. pneumoniae subsp. pneumoniae HS11286. A reference cgMLST data set for the species complex (K. pneumoniae, K. variicola and K. quasipneumoniae) was downloaded from https://www.cgmmlst.org/ and used to determine the cgMLST in our strains. The resulting cgMLST data were analysed using the goeBURST algorithm [15] to obtain a minimum spanning tree using PHYLOViZ [16].

2.6. Plasmid alignment

Plasmids carrying the 16S RMT rmtB gene in strains P05 and P43 were used to perform an NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and similar plasmids harbouring rmtB were further compared in genomic alignments using BLAST Ring Image Generator (BRIG) [17]. The rmtB-harbouring plasmid from Proteus mirabilis accession no. MF150118, previously isolated in the same hospital [18], was used as a reference and aligned with rmtB plasmids accession nos. MF150084, CP027695, CP028896 and CP031122. Additionally, CGView Comparison Tool [19] was used to view the percentage identity of the plasmids by performing a blast search using MF150118 as reference.

2.7. Conjugation experiments

To determine the ability of the rmtB plasmid to be transferred by conjugation, solid mating assays were conducted. Briefly, the donor strain harbouring the rmtB plasmid was mixed at a ratio of 1:3 with the recipient sodium azide-resistant E. coli strain J53. After centrifugation and re-suspension in a minimal volume, cells were spotted on LB agar and incubated at 37°C for 3 h. Cells were scraped, re-suspended in LB, serially diluted and plated on Chromogenic UTI Agar containing 150 μg/mL sodium azide and 50 μg/mL amikacin. The presence of rmtB was confirmed by PCR using the primers rmtB-F 5’-GTCTTTCGCGGCGATGTA-3’ and rmtB-R 5’-ATGCAAATGCGCGCTGTAAT-3’ [20].

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3. Results

3.1. Resistance to gentamicin and amikacin of a KPC-2-producing Klebsiella pneumoniae collection

The 100 KPC-2-producing *K. pneumoniae* isolates analysed in this study comprised a large array of STs, including several internationally disseminated clones and accounting for a large genetic variability [ST258 (n = 31), ST11 (n = 28), ST16 (n = 16), ST437 (n = 11), ST15 (n = 6), ST101 (n = 3), ST307 (n = 2), ST29 (n = 1), ST524_2LV (n = 1) and ST265_3LV (n = 1)]. Antimicrobial susceptibility results showed that 68% of the strains were resistant to gentamicin (MIC_{50/90} 128/>128 mg/L) and 42% to amikacin (MIC_{50/90} 8/>128 mg/L) (Fig. 1A). Moreover, 35% of the strains were resistant to both commonly prescribed aminoglycosides.

3.2. Variety of aminoglycoside-modifying enzymes (AMEs)

Analysis of the 100 genomes revealed the presence of various AME-encoding genes with frequencies varying according to the ST, as shown in Table 1. Detailed data for each isolate are presented in Supplementary Table S1. Only nine strains did not carry any AME and were fully susceptible to aminoglycosides. AME- and RMT-encoding genes not mentioned in the table were not detected in this collection. The most common AME in our collection was the aminoglycoside O-nucleotidyltransferase (ant(3')]-Ib) and aminoglycoside acetyltransferase-encoding (aac(3)-IId) that confers streptomycin resistance. The gene encoding this enzyme was detected in most of the ST258, ST11, ST16 and some of the ST15 isolates.

Four different aminoglycoside O-phosphotransferases were identified: (i) *aph(3')*-Ib, also known as *strA*, and (ii) *aph(6')*-IId, also known as *strB*, both conferring streptomycin resistance; (iii) *aph(3')*-Ia; and (iv) *aph(3')*-Vla present only in a few ST11 and ST437 strains in our collection conferring low-level resistance to amikacin (MICs, 16–32 mg/L).

Various aminoglycoside *N*-acetyltransferase-encoding genes, belonging to the subclass *aac(3)-II* and *aac(6')*-Ib catalysing acetylation at position 3' and 6', respectively, were identified. Different sequences were identified for *aac(3)-II*: some *aac(3)-IIa* and some *aac(3)-IId*. In strain P43, we identified a variant sequence highly similar to *aac(3)-Ild* but carrying the previously unreported mutations V259F and R280L compared with the reference sequence EU022314. Regarding *aac(6')*-Ib, at least 45 distinct sequences have been reported [13] and the nomenclature remains unclear. Herein, strains carrying both W104R and D181Y mutations (positions determined from sequence accession no. AF477974) conferring cross-resistance to fluoroquinolones [21] were classified as *aac(6')*-Ib-cr, while strains carrying wild-type sequences of the highly conserved central region of 181 amino acids [13] were classified as *aac(6')*-Ib, regardless of their variable N-terminus, which were previously shown to have little impact on the aminoglycoside resistance phenotype [13,22,23]. All strains analysed above except for one carried either the wild-type or both mutations described. P16 carried one of the two mutations (W104R) required to extend the enzyme inactivation spectrum to fluoroquinolones.

3.3. Vertical and horizontal dissemination of the 16S rRNA methyltransferase *rmtB* in KPC-2-producing Klebsiella pneumoniae ST258

Although aminoglycoside resistance was present in nearly all STs identified in this study, the ST258 lineage showed the highest resistance rate (94%) and the highest resistance levels (MIC_{90} >128 mg/L) both to gentamicin and amikacin. Genomic analysis of ST258 isolates revealed the presence of the gene encoding 16S RMT RmtB that confers resistance to all 4,6-disubstituted 2-deoxystreptamine (DOS) aminoglycosides. The *rmtB* gene was also present in one ST16 isolate (P05), suggesting possible horizontal transfer within different STs of the same species. The *rmtB* gene was the only

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**Fig. 1.** (A) Distribution of amikacin and gentamicin minimum inhibitory concentrations (MICs) for each sequence type (ST) in *Klebsiella pneumoniae* strains carrying *bla*<sub>KPC-2</sub>. (B) Distribution of apramycin and plazomicin MICs in *K. pneumoniae* strains carrying (red) or not carrying (grey) the 16S rRNA methyltransferase *rmtB* gene.
identified 16S RMT within this collection and was not detected in other lineages. The genetic relatedness of these 100 isolates inferred from cgMLST data is shown in Fig. 2.

The complete circular genomic assembly of isolate P43 (ST258) showed that rmtB is carried on a large multiresistance plasmid (size 177 kb), named pP43-rmtB, belonging to the IncC incompatibility group and distinct from the blaKPC-2-harbouring plasmid. The plasmid carrying rmtB in isolate P05 (ST16) was found to be highly similar to pP43-rmtB, except for two 2-kb deletions (98% coverage; 99.94% nucleotide identity). Both plasmids were aligned with the rmtB-carrying plasmids found in public databases (Fig. 3).

Interestingly, this plasmid was different from the rmtB plasmid belonging to the IncFIIk group previously identified in a K. pneumoniae isolate in the same hospital in 2014 (accession no. MF150084) [18]. However, it showed >99% nucleotide identity with a plasmid previously identified in P. mirabilis [18] (accession no. MF150118), also circulating in the same hospital. Detailed plasmid comparison and nucleotide identity percentages are shown in Supplementary Fig. S1. In addition, a highly similar plasmid was found in two strains from a sequenced collection (USA) as well as a similar plasmid from China, suggesting a wider dissemination of this multidrug resistance plasmid. Plasmid alignment is shown in Fig. 3 using the 176-kb plasmid MF150118 found in P. mirabilis as reference. These findings suggest multiple genetic events, including both interspecies horizontal dissemination of this plasmid among Enterobacteriales and at least one intraspecies transfer event within a carbapenemase-producing K. pneumoniae strain. Importantly, the plasmid was stable in the KPC-2-ST258 epidemic clone (96% of the rmtB-positive tested isolates), which disseminated clonally in this hospital.

Mating experiments showed conjugal transfer of the IncC-rmtB plasmids from K. pneumoniae P05 and P. mirabilis A64421

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**Table 1**

Gentamicin and amikacin minimum inhibitory concentrations (MICs) and percentage of rmtB- and aminoglycoside-modifying enzyme (AME)-encoding genes according to the Klebsiella pneumoniae sequence type (ST).

<table>
<thead>
<tr>
<th>ST</th>
<th>n</th>
<th>Gentamicin MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Amikacin MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>RMT gene</th>
<th>AME-encoding genes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td>rmtB</td>
<td>aph(3')-Ia</td>
</tr>
<tr>
<td>ST11</td>
<td>28</td>
<td>128</td>
<td>128</td>
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<tr>
<td>ST16</td>
<td>6</td>
<td>0.5</td>
<td>128</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
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<td>16</td>
<td>0.5</td>
<td>64</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ST101</td>
<td>3</td>
<td>128</td>
<td>128</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ST258</td>
<td>31</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
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<td>2</td>
<td>16</td>
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<td>8</td>
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<tr>
<td>ST437</td>
<td>11</td>
<td>128</td>
<td>&gt;128</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>ST265_3LV</td>
<td>1</td>
<td>0.25</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>ST254_2LV</td>
<td>1</td>
<td>&gt;128</td>
<td>-</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>128</td>
<td>&gt;128</td>
<td>8</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

n, number of isolates; MIC<sub>50</sub> and MIC<sub>90</sub>, MIC that inhibits 50% and 90% of tested isolates according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints; RMT, 16S rRNA methyltransferase.

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Fig. 2. Minimum spanning tree showing the genetic relatedness of 100 Klebsiella pneumoniae isolates in this study determined from core genome multilocus sequence typing (cgMLST) data. Strains are colour coded by sequence type (ST), and outlined strains with red dots indicate rmtB-plasmid bearing strains.
donors into *E. coli* J53 at a frequency of $7.5 \times 10^{-5}$ and $5 \times 10^{-5}$, respectively. In contrast, conjugative transfer could not be obtained for the *K. pneumoniae* P43 IncC-rmtB plasmid under our conditions.

Additional antimicrobial resistance genes identified on the plasmids are represented by red arrows in Fig. 3. Besides rmtB, this multidrug resistance plasmid carries various AMEs [aac(3)-II, aadA2, aph(3’)-Ib and aph(6)-Id], the macrolide resistance gene erm(42), the β-lactamase *bla*<sub>TMP-1</sub> and the extended-β-lactamase (ESBL) *bla*<sub>CTX-M-14</sub>, the dihydropteroate synthase variants *sul1* and *sul2* conferring sulfonamide resistance, the tetracycline efflux pump *tet(C)*, the dihydrofolate reductase *dfrA12* conferring trimethoprim resistance, the chloramphenicol efflux pump * floR*, and the tunicamycin resistance gene.

### 3.4. Exploration of alternative aminoglycosides

Two alternatives aminoglycosides (apramycin and plazomicin) were tested against the 100 isolates in this collection. The MICs obtained are shown in Fig. 1B and detailed results are given in Supplementary Table S1. While the results showed that the 16S RMT rmtB is associated with high levels of plazomicin resistance with MICs > 256 mg/L, all isolates but one remained susceptible to apramycin with MICs between 4 mg/L and 8 mg/L, independently of the presence of rmtB. One ST11 isolate (rmtB-negative) showed high MICs both to apramycin (MIC, 16 mg/L) and plazomicin (MIC, 3 mg/L). Increased efflux pump activity, impaired membrane permeability or a combination thereof could have led to increased apramycin and plazomicin MICs since no AME- or 16S RMT-encoding genes were identified in this isolate.

### 4. Discussion

Antibiotic resistance is a major challenge to modern medicine. Despite their age and toxicity profile, aminoglycosides remain an important and necessary pillar to treat infections caused by carbapenem-resistant Enterobacteriales. However, the increase in resistance observed worldwide requires close epidemiological follow-up. We assessed aminoglycoside susceptibility, since a high...
level of resistance would seriously compromise available therapeutic options for KPC-2-producing *K. pneumoniae* invasive infections. Therefore, here we examined the aminoglycoside resistance genomic and phenotypic characteristics of 100 carbapenem-resistant invasive KPC-producing *K. pneumoniae* isolates previously collected in a tertiary hospital in São Paulo, Brazil, during a 3-year period. All strains produced the KPC-2 carbapenemase. When clonal complex 258 (CC258), with ST258, ST11 and ST437, was the most prevalent, we described the emergence of a ST16 clone associated with high mortality rates [5]. This collection encompasses a large genetic variability, since international *K. pneumoniae* epidemic clones such as ST15, ST101, ST307 and ST29, besides CC258, were also identified as KPC-2-producers in this hospital.

Worryingly, we observed a high prevalence of resistance to gentamicin and amikacin. As previously reported in many other epidemiological studies [13], we identified a high variety of AMEs. More surprisingly, 30% of the strains also carried the 16S RMT *rmtB*, which confers resistance to most systemic aminoglycosides available in clinical settings. This enzyme has been frequently associated with the *bla* _shv-1_ Carbapenemase gene as they can be carried on the same plasmid [2,24]. In contrast, reports of *rmtB* in KPC-2-producing *K. pneumoniae* are less frequent but were reported mainly in the ST11 lineage in Asia, primarily in China but also in South Korea and Japan [25–28]. In 2019, a nationwide study conducted in Greece found that 15 of 200 KPC-positive isolates carried the *rmtB* gene [29]. However, little is known about their genetic contexts and horizontal dissemination dynamics.

Here we report the likely intraspecies (within KPC-producing *K. pneumoniae* clones) and interspecies (with *P. mirabilis*) transfer of an *rmtB*-carrying multiresistance IncC plasmid. At least one ST16 KPC-producing *K. pneumoniae* isolate had acquired this plasmid. We could show conjugative properties for this IncC-*rmtB* plasmid, at least from *P. mirabilis* and *K. pneumoniae* to *E. coli*. The exact chronology of these conjugative events remains uncertain.

The efficient clonal interhospital and intrahospital dissemination of a KPC–RmtB-producing ST258 clone suggests that this plasmid is well adapted to this genetic background. As ST258 is one of the main KPC-producing nosocomial clones present worldwide [30,31], the presence of the 16S RMT *rmtB* gene in this clone is particularly worrisome in the context of global antimicrobial resistance. This KPC–RmtB-producing ST258 clone has been identified in several other Brazilian teaching hospitals including in São Paulo and Porto Alegre, although the plasmid structure is not known [32,33]. Such plasmid dissemination represents a major challenge in this setting owing to the few antimicrobial alternatives available for treatment of carbapenem–resistant invasive infections, especially in a country where polymyxin B resistance levels are high and novel β-lactamase inhibitors are not always available [5].

Looking for alternative active aminoglycosides, we tested the newly approved semisynthetic plazomicin and the veterinary-approved apramycin. Plazomicin is the latest clinically approved aminoglycoside specifically designed to overcome inactivation by the main AMEs [34] but not 16S RMTs. As expected, the strains carrying only AMEs were susceptible to plazomicin. The only AME able to inactivate this new molecule, *aac(2’)-I*, which had been described in *Providencia stuartii*, was not detected in these isolates by WGS. However, the isolates carrying *rmtB* were fully resistant to plazomicin [34]. Plazomicin remains an interesting therapeutic option in the absence of 16S RMTs.

Apramycin is a monosubstituted DOS aminoglycoside that is known to be resistant to most of the AMEs except *aac(3’)-IV*, which was not identified in any strain of our collection but was previously described in a few human infection cases [35,36]. Because of its unique structure, apramycin is not affected by methylation of the 16S rRNA in position G1405 (*E. coli* numbering) [37]. Two recent studies showed that this molecule has a good activity against carbapenem–resistant *Acinetobacter baumannii* and Enterobacteriales including *K. pneumoniae* [37,38]. Apramycin is currently in phase 1 clinical trials for its potential use in human medicine (ClinicalTrial.gov NCT04105205). Our results confirm the interest of further developing apramycin or structural analogues [39] in order to extend our therapeutic arsenal against carbapenemase-producing Enterobacteriales.

### 5. Conclusion

Antibiotic resistance is one of the main public–health challenges we currently face, and plasmids are the key players driving the horizontal dissemination of resistance genes among Gram-negative bacteria. Identification of the same multiresistance plasmids in different bacterial species and in different *K. pneumoniae* clones confirms their high potential for dissemination. Thus, detecting horizontal gene transfer events is an important step to understand the spread of resistance genes as well as to design further strategies to minimise its occurrence. More studies are needed to infer the frequency and direction of the horizontal transfer events in clinical settings.

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### Conflict of interest

ACG recently received research funding and/or consultation fees from Bayer, Eurofarma, Cristalia, Entasis Therapeutics, InfectoPharm, Merck Sharp & Dohme, Pfizer and Zambon. JS has received restricted research grants from and participated on advisory boards for bioMérieux and Debiopharm. All other authors declare no competing interests.

### Ethical approval

Not required.

### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jigar.2020.12.006.

### References


In this paper, the authors discuss the development and characterization of a new method for identifying and resolving antibacterial resistance patterns. They employ a combination of Next Generation Sequencing (NGS) and computational algorithms to analyze the genetic diversity of bacteria, specifically targeting the emergence of antibiotic-resistant clones. The study highlights the importance of using comprehensive genomic approaches to understand the evolution and spread of resistant strains, particularly in the context of Klebsiella pneumoniae and Enterobacteriaceae.

The authors detail the use of tools such as CLC Genomics Workbench, MCLUST, and R software to identify signatures of resistance genes and to infer evolutionary relationships among different bacterial strains. They also compare their findings with previous studies to provide a broader context for the current data.

The implications of their research are significant for both clinicians and public health officials, as it offers a more nuanced understanding of how resistance emerges and spreads, thereby informing strategies for containment and control.