Occurrence of \textit{qnrB1} and \textit{qnrB12} genes, mutation in \textit{gyrA} and \textit{ramR}, and expression of efflux pumps in isolates of \textit{Klebsiella pneumoniae} carriers of \textit{bla}_{KPC-2}

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Abstract

**Purpose.** The occurrence of quinolone-resistance genes (\textit{qnrA}, \textit{qnrB} and \textit{qnrS}), the presence of mutations in \textit{gyrA}, \textit{gyrB} and \textit{parC}, as well as the expression of efflux pumps (\textit{acrB} and \textit{acrF}) and mutations in the gene \textit{ramR}.

**Methodology.** Were investigated in 30 \textit{bla}_{KPC-2}-positive isolates of \textit{Klebsiella pneumoniae} taken from infection and colonization in hospital patients from Recife-PE, Brazil. The detection of the \textit{qnr}, \textit{acrB} and \textit{acrF} genes and analysis of the mutations in \textit{ramR} and the quinolone-resistance-determining regions of \textit{gyrA}, \textit{gyrB} and \textit{parC} were performed by PCR followed by DNA sequencing.

**Results.** Among the isolates analysed, 73.3% (\textit{n}=22) presented the \textit{qnrB} gene. For the DNA sequencing, six isolates (K3-A2, K12-A2, K25-A2, K27-A2, K19-A2 and K3-C2) were selected and the \textit{qnrB1} and \textit{qnrB12} variants were detected. This is the first ever report, to the best of our knowledge, of the presence of \textit{qnrB12} in \textit{K. pneumoniae}. This is also the first report, to the best of our knowledge, of the presence of \textit{qnrB1} or \textit{qnrB12} with \textit{bla}_{KPC-2} in \textit{K. pneumoniae} in Brazil. Mutations were observed in \textit{gyrA} S83 and in \textit{ramR}. All isolates presented genes for the \textit{acrB} and \textit{acrF} efflux pumps and the reverse transcription PCR performed showed that the pumps were being expressed.

**Conclusion.** KPC-2-positive isolates colonizing patients, which also showed \textit{qnrB}, mutation in \textit{gyrA} and efflux pumps, may be important reservoirs for disseminating these resistance mechanisms in the hospital environment.

INTRODUCTION

\textit{Klebsiella pneumoniae} is an important pathogen associated with a variety of infections related to health care. It mainly causes septicemia, pneumonia and urinary tract infections [1–3]. It is the main bacterial species producing the enzyme \textit{Klebsiella pneumoniae} carbapenemase (KPC), which confers resistance to all \beta-lactam antibiotics, including carbapenems [4–6]. Some bacteria that produce carbapenemase usually also present a reduced susceptibility to fluoroquinolones, which can be justified by the presence of the \textit{bla}_{KPC} accompanied by the \textit{qnr} gene in the same resistance plasmid [7].

The mechanisms of resistance to quinolones emerged from mutations of chromosomal genes that encode the DNA gyrase and topoisomerase IV [3] \textit{gyrA} and \textit{gyrB}, \textit{parC} and \textit{parE}, respectively, in regions called quinolone-resistance-determining regions (QRDRs) [3, 8]. Two other main mechanisms have also been reported, consisting of an active efflux mediated by the AcrAB-ToIC efflux system, chromosomally encoded, and the protection of the \textit{qnr} target proteins, most of which are plasmid-mediated quinolone resistance (PMQR) genes acquired by horizontal transfer [8, 9].

To date, different variants of the PMQR genes have been described: \textit{qnrA} (seven variants), \textit{qnrB} (74 variants), \textit{qnrC} (one variant), \textit{qnrD} (two variants), \textit{qnrS} (nine variants) and \textit{qnrVc} (seven variants) (www.lahey.org/qnrStudies/). In Brazil, PMQR genes have been detected in

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**Keywords:** \textit{Klebsiella pneumoniae}; \textit{qnrB1}; \textit{qnrB12}; efflux pump; \textit{gyrA} and \textit{ramR}.

**Abbreviations:** PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone-resistance-determining region; RT-PCR, reverse transcription PCR. The GenBank/EMBL/DDBJ accession numbers for the new sequences derived in this work are KP144334, KP144336, KP144337, KP144338, KP271121, KP184842, KP184843, KP184844, KP271120 and KP730602.
Enterobacteriaceae (K. pneumoniae, Escherichia coli and Enterobacter cloacae) bearing qnrB1, qnrB2, qnrB19 and qnrS1 with the co-occurrence of extended-spectrum β-lactamase [9–11].

The AcrAB efflux system, present in most species of Enterobacteriaceae, uses the outer membrane protein TolC, thus reducing susceptibility to macrolides, fluoroquinolones, chloramphenicol, trimethoprim and tetracyclines [12]. The AcrEF efflux pump is highly homologous to AcrAB in Salmonella enterica with an amino acid identity of 81.4% and similar substrate specificities, since the overexpression of AcrEF confers a similar effect to AcrAB.

The overexpression of ramA seems to be the cause of the overexpression of AcrAB in K. pneumoniae and Enterobacter cloacae [13], and the expression of ramA is negatively controlled by the regulator RamR leading to overexpression of the efflux pump AcrAB [12]. RamR is a protein of the TetR family of regulators (TFRs) and the repressor of the romA-ramA locus. Reports on Salmonella, Klebsiella and Enterobacter have shown that mutations located throughout the protein RamR lead to the non-suppression of the transcription of romA-ramA and subsequently to the overexpression of the AcrAB operon, resulting in a multiresistance phenotype [12, 14, 15].

Therefore, the objective of this study was to investigate the occurrence of qnr genes, the presence of mutations in gyrA, gyrB, parC and ramR genes, as well as to assess the presence and expression of efflux pumps in multi-resistant blaKPC positive isolates of K. pneumoniae, coming from colonization and infection in patients in public hospitals in Recife, Brazil.

METHODS

Bacterial isolates

An analysis was made of 30 hospital isolates of K. pneumoniae previously selected due to them being resistant to quinolones and positive for the blaKPC gene, coming from colonization (rectal swab), urinary tract infections or respiratory infections from three public hospitals in the city of Recife-PE, Brazil, between 2011 and 2012 (Table 1). The K. pneumoniae isolates selected are stored in the Bacteriology and Molecular Biology Laboratory of the Department of Tropical Medicine, Federal University of Pernambuco, Brazil, and were identified biochemically by the automated system Bactec 9120/BD Phoenix and typed as to their resistance profile, entrobacterial repetitive intergenic consensus PCR (ERIC-PCR), and the presence of the blaKPC gene [6]. For DNA sequencing of the genes found, six isolates (K3-A2, K12-A2, K25-A2, K27-A2, K19-A2 and K3-C2) were selected. These isolates were not clonally related and were resistant to at least 15 antimicrobials, including the four quinolones tested. The cultures were preserved in 20% (v/v) glycerol at −70 °C and, to conduct the analysis, they were grown in a brain heart infusion at 37 °C for 18 h.

Test of susceptibility to antimicrobials

The 30 K. pneumoniae isolates were cultured with Mueller–Hinton agar (Biolife) and the susceptibility of the isolates was detected by the automated BD Phoenix system, and the disc diffusion method for quinolones [16]. The following antimicrobials were used: amoxicillin/clavulanic acid (AMC); amoxicillin (AMO); amikacin (AMI); aztreonam (ATM); ceftazidine (CAZ); cefoxitin (CFO); cefotaxime (CTX); cefepime (CPM); piperacillin/tazobactam (PIT); polymyxin B (POL); trimethoprim/sulfamethoxazole (SUT); gentamicin (GEN); ciprofloxacin (CIP); norfloxacin (NOR); nalidixic acid (NAL); levofloxacin (LEV); imipenem (IPM); meropenem (MPM); and ertapenem (ERT) [16].

PCR and sequencing of the gyrA, gyrB, parC and ramR genes

Genomic DNA of 30 isolates was extracted directly from colonies suspended in 200 µl of distilled water. The suspension was heated at 100 °C for 10 min, centrifuged (5 min, 10 000 g) and 100 µl of the supernatant containing the DNA was kept at −20 °C until the time of use. After extraction, the DNA was quantified in a NanoDrop 2000c UV–visible spectrophotometer.

The gyrA, gyrB, parC and ramR genes were amplified in four K. pneumoniae isolates (K3-A2, K12-A2, K25-A2 and K27-A2), the primers of which are described in Table 2. In each PCR, a negative control and a positive control were included. The amplified products were subjected to electrophoresis on 1% agarose gel under constant voltage of 100 V, in Tris/boric acid/EDTA (TBE) buffer. The amplicons of the gyrA, gyrB, parC and ramR genes were purified (Wizard SV Gel and PCR Clean-Up System; Promega). After purification, they were quantified by NanoDrop and sequenced (Sequencer 3500 Genetic Analyzer; Applied Biosystems).

Analysis of mutations in the gyrA, gyrB, parC and ramR genes

For analysis of mutations in the gyrA, gyrB, parC and ramR genes, four isolates (K3-A2, K12-A2, K25-A2 and K27-A2) were selected. The sequences were compared with the sequences deposited in GenBank. The amino acid sequences of gyrA, gyrB and parC were analysed to identify precise mutations with the respective changes in their protein sequence (Sequence Manipulation Suite, www.bioinformatics.org/sms2/trans_map.html).

For analysis of the sequences of the ramR regulator, the sequences were compared with the sequence of the K. pneumoniae isolate MGH 78578 deposited in GenBank (accession number: NC_009648) [17]. The ramR amino-acid sequences were analysed to identify precise mutations that foster superexpression of the AcrAB-TolC efflux pumps. In the analysis of the sequence of the RamR regulator, mutations were investigated at positions 58 G→C and 104 G→C in accordance with Bialek-Davenet et al. [15] and at positions 40 A→T, 122 C→T, and 136 C→T.
## Table 1. Origin, presence of the qnrB, acrB and acrF genes, ERIC-PCR and resistance profile of KPC-2-positive K. pneumoniae isolates from Recife-PE, Brazil

<table>
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<tr>
<th>Isolate identification*</th>
<th>Origin</th>
<th>qnrB†</th>
<th>acrB†</th>
<th>acrF†</th>
<th>ERIC‡</th>
<th>Resistance profile§</th>
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<td>K3-A2</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>9E</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Rectal swab</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>19E</td>
<td>AMC, AMO, ATM, CAZ, CFO, CPM, CTX, CIP, LEV, NAL, NOR, ERT, GEN, PIT, SUT</td>
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<td>+</td>
<td>+</td>
<td>19E</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>23E</td>
<td>AMC, AMI(I), AMO, ATM, CAZ, CFO, CPM, CTX, CIP, LEV, NAL, NOR, ERT, GEN, IMP, MPM, PIT, SUT</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>25E</td>
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<td>+</td>
<td>+</td>
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*Identification of isolates: K, K. pneumoniae; A2, C2 and OC, public hospital.
†+, Presence of the gene; –, absence of the gene.
in accordance with Hentschke et al. [13]. The sequences for these analyses have been deposited in GenBank under the following accession numbers: KP144334, KP144337, KP144338 and KP271121.

Amplification of the qnr, acrB and acrF genes

The qnrA, qnrB, qnrS, acrB and acrF resistance genes were investigated using PCR on 30 K. pneumoniae isolates, the primers for which are described in Table 2.

Four positive isolates (K3-A2, K12-A2, K19-A2 and K3-C2) for the qnrB gene and four positive isolates (K3-A2, K12-A2, K25-A2 and K27-A2) for the amplicons of the acrB and acrF genes were purified (Wizard SV Gel and PCR Clean-Up System; Promega) and sequenced (Sequencer 3500 Genetic Analyzer; Applied Biosystems). All of the sequences were deposited in GenBank under the following accession numbers: KP144336, KP184842, KP184843 and KP184844.

RT-PCR for the acrB and acrF genes (efflux pumps)

The RNA of four K. pneumoniae isolates (K3-A2, K12-A2, K25-A2 and K27-A2), extracted using the SV Total RNA Isolation System (Promega) in accordance with the manufacturer’s instructions, was quantified by Nanodrop and stored in a deep-freezer at −70 °C. To evaluate the expression of the efflux pumps by means of the presence or absence of the acrB and acrF transcripts, qualitative reverse transcription PCR (RT-PCR) was undertaken using the Access RT-PCR System (Promega) in accordance with the manufacturer’s instructions. The amplified products were subjected to electrophoresis on 1% agarose gel under constant voltage of 100 V. In all reactions, a positive control and a negative control were used.

RESULTS

Susceptibility to antimicrobials profile

All 30 isolates of K. pneumoniae analysed showed resistance to amoxicillin, amoxicillin/clavulanic acid, cefepime, cefotaxime and piperacillin/tazobactam, amongst which 96.7% (n=29) were resistant to aztreonam and ceftazidime, 93.4% (n=28) to trimethoprim/sulfamethoxazole, 73.3% (n=22) to cefoxitin and 63.3% (n=19) to gentamicin. As to the quinolones tested, the isolates demonstrated 100% resistance to ciprofloxacin, 96.7% (n=29) resistance to nalidixic acid, 93.4% (n=28) resistance to norfloxacin and 60% (n=18) resistance to levofloxacin, as well as high levels of resistance to the carbapenems tested, there being 100% (n=30) resistance to ertapenem, 80% (n=24) resistance to meropenem and 73.3% (n=22) resistance to imipenem. The K. pneumoniae isolates analysed were more susceptible to polymyxin B and amikacin when compared to the other antimicrobials: only 6.7% (n=2) were resistant to amikacin and 10% (n=3) were resistant to polymyxin B (Table 1).

Analysis of mutation in the QRDRs of the gyrA, gyrB and parC genes

Analysis of gene mutations was performed on the representatives (K3-A2, K12-A2, K25-A2 and K27-A2) with

Table 2. Primers used in PCR and DNA sequencing for detection of gyrA, gyrB, parC, ramr, qnrA, qnrB, qnrS, AcrB and AcrF genes

<table>
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<th>Sequence (5′–3′)</th>
<th>Temperature (°C)</th>
<th>Reference</th>
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<td>[42]</td>
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*annealing temperature.
different clonal profiles by ERIC-PCR. The isolates were analysed for the presence of mutations at the Ser83 and Asp87 positions for the gyrA gene, Ser80 and Glu84 for the parC gene, and Ser359, Ser367 and Glu466 for the gyrB gene. In this study, of the four isolates tested, two (K25-A2 and K27-A2) presented mutation in gyrA in the Ser83 position where the exchange of one amino acid resulted in the change of a serine to a leucine. The mutated sequence has been deposited in GenBank under accession number KP271120.

The isolate K25-A2, which is fluoroquinolone-resistant, presented a mutation in the QRDR of gyrA and did not present any of the qnr genes investigated (Table 3). On the other hand, the isolate K27-A2 presents the qnrB gene associated with a mutation in the QRDR of gyrA (Table 3). No mutation in gyrB nor in parC was found at the positions analysed (Table 3).

qnr genes

In this study, the PCR analysis showed that 73.3 % (n=22) of the isolates showed the qnrB gene (Table 1). Sequencing of the selected isolates revealed the presence of the qnrB1 variant in two other isolates and the presence of the qnrB12 gene in another two isolates (Table 3). The isolates K3-A2 and K12-A2 showed both the qnrB1 and blaKPC-2 genes and the K19-A2 and K3-C2 isolates showed the qnrB12 and blaKPC-2 genes. No qnrA nor qnrS genes were found in the K. pneumoniae isolates analysed.

Efflux pumps and the ramR regulator

The investigation of efflux pumps AcrB revealed the presence of the acrB gene, which encodes the TolC pump, and the presence of the acrF gene, which encodes the AcrEF-TolC pump in all of the 30 K. pneumoniae isolates analysed. Using RT-PCR, the transcription of the acrB and acrF genes in the four isolates selected was confirmed, thus revealing the expression of both pumps investigated.

The K3-A2 and K12-A2 isolates, which express the two efflux pumps, showed no mutation in gyrA, but had the qnrB1 gene. The isolate K25-A2, also with two pumps, showed mutation in gyrA but did not carry any of the qnr genes tested, and the isolate K27-A2, as well as the two pumps, presented mutation in gyrA and the presence of qnrB (Table 3).

The isolate K3-A2 presented mutations at positions 40 and 122. The three other isolates analysed did not present mutation in ramR at the positions evaluated (Table 3). The mutated sequence has been deposited in GenBank with the following accession number: KP730602.

DISCUSSION

Since the introduction of fluoroquinolones, resistance to these agents in the family Enterobacteriaceae has become common and widespread. Nosocomial infections by K. pneumoniae are often treated with extended-spectrum cephalosporins, carbapenems and fluoroquinolones. However, the mechanisms of resistance such as qnr genes and carbapenemases were found to be promoting serious therapeutic problems [18].

The resistance profile of K. pneumoniae isolates of this study suggests that the high levels of resistance to quinolones and carbapenems are related to the conjunction of more than one resistance mechanism, which may be justified in this study because of the presence of blaKPC-2.
qnrB genes and efflux pumps and may be well-represented by the K3-A2, K12-A2, K19-A2 and K3-C2 isolates.

The data from this study reveal the presence of blaKPC-2 and qnrB1, as well as blakPC-2 and qnrB12 in K. pneumoniae isolates. This is the first report, to our knowledge, published about the presence of blakPC-2 with qnrB1 or with qnrB12 in Brazil. Other studies have demonstrated the presence of qnr genes together with other β-lactamases. Ferjani et al. [19], in Tunisia, detected qnrB1 associated with blactx-M-15 or blasIV-12. In France, Filippa et al. [20] found qnrB1 with blactx-M-15. In Brazil, Viana et al. [11] reported the association of qnrB1 with blactx-M-1, blatem-1, blasIV-11 or blaoxa-18/45-like.

In other countries there are also already some reports which associate qnr and blakPC. Chmelinský et al. [21] described, for the first time in Israel, the presence of blakPC and qnrB2 in the same plasmid of Enterobacter cloacae. In China, Jiang et al. [22] and Zhang et al. [23] report, respectively, the presence of blakPC-2 and qnrB4, and blakPC-2 and qnrB2, in K. pneumoniae isolates.

The isolates K19-A2 and K3-C2 presented the qnrB12 gene, which is the first report of this gene in K. pneumoniae. The qnrB12 gene have previously been described only in Citrobacter werkmanii in Germany [24] and in Citrobacter youngae in Finland [25]. This study demonstrates the emergence of this gene in isolates of K. pneumoniaeblaKPC-2 producers. It should be noted that in Citrobacter spp., qnrB12 seems to be a chromosomal gene. While most qnr genes are present in plasmids in enterobacteria, there are a few reports that suggest a chromosomal location [25]. Kehrenberg et al. [25] suggest that a plasmid harbouring the qnrB12 gene, similar to the pTN60012 plasmid of K. pneumoniae, may have been integrated into the chromosomal DNA of C. werkmanii.

Studies showed a high prevalence of qnr genes found in enterobacteria from fauces of healthy children in South America (Peru and Bolivia), 54% of which contained qnrB [25, 26]. In Brazil, Veras et al. [27] detected the blasIV gene in K. pneumoniae isolates from enteric microbiota of healthy children in Recife-PE. These studies show the importance of commensal bacteria as carriers of genes that confer resistance to antimicrobials.

In this study, 80% of the isolates resistant to quinolones and carriers of blakPC were from colonization that may serve as a source of clinical infection or as an important reserve for disseminating blakPC-2 and qnrB in the hospital environment. Colonization isolates can disseminate blakPC-2 and qnrB among other organisms of the same species or different species of bacteria in the same patient, since the blakPC-2 gene may be transmitted by plasmids from one strain to another [28]. Consequently, active surveillance of the spread of KPC-positive K. pneumoniae, and other carbapenem-resistant Enterobacteriaceae (CRE), has become an integral part of programmes designed to control the spread of CRE in various hospitals [29-33].

In this study, no mutations in the QRDRs of gyrB and parC were detected. These findings corroborate those of Paiwa et al. [9] who also did not detect any mutation in gyrB. According to Kim et al. [34] and Nam et al. [3], mutations in gyrB and parE are substantially less frequently detected and confer lower levels of resistance when compared with gyrA and parC. In this study, a mutation was detected in gyrA (S83L) in two isolates of K. pneumoniae. Machuca et al. [35] have shown that a single substitution in gyrA increases the values of the minimum inhibitory concentrations (MICs) for fluoroquinolones by up to 16 times, a fact that may explain the resistance to fluoroquinolone, even without the presence of the qnr gene in the K25-A2 isolate. Interactions between mutations in QRDRs and qnr may result in a higher level of resistance to fluoroquinolone. The qnr gene alone confers low-level resistance to quinolones. Its main contribution is to facilitate the development of mutations in QRDRs [36, 37]. Machuca et al. [35] observed that the presence of any qnr gene increases the levels of MICs in all genotypes of E. coli and that combinations of only one chromosomal mechanism, with mutations in QRDRs, with qnr genes increased the values of the MICs from four to 32 times and from 32 to 128 times greater than the genetic equivalent without the qnr gene.

Overexpression of efflux pumps may also play a big role in the resistance level. It is believed that the efflux systems by themselves produce low levels of resistance to different classes of antimicrobial agents, including quinolones, chloramphenicol and β-lactam [15], and become clinically relevant when combined with mutations in target enzymes or membrane changes [38]. In this study, we found that 73.3% of isolates, besides efflux pumps, presented qnrB genes. It was also detected that the isolate K3-A2 showed mutation in ramR which can lead to an overexpression of the AcrAB efflux pump. The location of the mutations in the ramR sequence, detected here, corroborate both Hentschke et al. [13] and Bialek-Davenet et al. [15], since mutations in the locations described in both studies are presented. Points mutations occur in different positions and these genetic changes are distributed throughout the ramR gene, which can lead to further modification of the amino acid, a premature 'stop codon' or truncated synthesis of the protein RamR [15].

Hentschke et al. [13] found precise mutations in the ramR gene in eight of 17 mutants and these mutations proved to be responsible for the overexpression of both ramA and acrB genes. Bialek-Davenet et al. [15] report that the ramR gene controls the expression of the efflux system in K. pneumoniae. However, the locations of the mutations in the sequence found in their study were different from those described by Hentschke et al. [13].

The AcrEF efflux system, also detected in all the isolates of K. pneumoniae analysed in this study, may confer an advantage on the organisms because AcrEF may be necessary for the resistance to medications when AcrAB does not work.
Further investigations of the regulation of multidrug efflux systems are needed to elucidate the biological significance of their regulatory networks [39].

The colonization isolate K3-A2, besides being the carrier of the blaKPC-2 gene, also showed the qnrB1 gene, an expression of both efflux pumps analysed, and mutation in ramR, which may result in overexpression of the AcrAB efflux pump. Additionally, all the other isolates presented at least one resistance mechanism to quinolone associated with blaKPC-2, which can already cause failure in therapy. In Brazil, there are no reports in the literature surveyed of the AcrAB-ToIC, AcrEF-ToIC efflux systems and their regulators in *K. pneumoniae*.

This study shows patients colonized with *K. pneumoniae*-carriers of the blaKPC-2 gene, qnrB, mutation in gyrA, expression of efflux pumps and mutation in ramR. Colonized patients usually require long-term medical care and can play an important role in the spread of these extremely resistant pathogens. The high prevalence of these isolates in intensive care unit patients and significant mortality rates associated with infection by *K. pneumoniae* blaKPC carriers demonstrate the importance of identifying, isolating and treating these patients early [40, 41].

In this study, the emergence of different resistant mechanisms to quinolones in *K. pneumoniae* KPC-2-positive isolates from patients hospitalized in Recife, Brazil was observed. These findings underscore the need for systematic studies aimed at a better understanding of these mechanisms in the resistance and virulence of these bacteria, to prevent any spread of these associations and so enable infection control measures to be adopted early, thus interrupting the spread of these resistance mechanisms and increasing patient survival.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**Ethical statement**
It was not necessary to submit the work to the ethics committee since we do not have the patients’ data or records.

**References**


