Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil

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Objectives: The aim of this study was to determine the spread of plasmid-mediated quinolone resistance determinants [qnr-like, aac(6)-Ib-cr and qepA genes] among nalidixic acid-resistant enterobacterial strains isolated from outpatients from Southeast Brazil, their transferability and the genetic structures associated with the qnr genes.

Methods: The qnrA, qnrB and qnrS genes were screened by a multiplex PCR-based technique from 257 non-repetitive nalidixic acid-resistant enterobacterial isolates collected from January 2000 to May 2005. Conjugation experiments were performed to determine whether the qnr-carrying plasmids were self-transferable. Genetic structures surrounding the qnr genes were analysed by PCR and cloning. The aac(6)-Ib-cr and qepA genes were screened among qnr-positive strains.

Results: Six qnrB-like-positive isolates (2.3%) were detected, whereas no qnrA- or qnrS-positive isolates were detected. Three Escherichia coli and two Klebsiella pneumoniae isolates harboured a qnrB2 gene and a single Citrobacter freundii isolate had the qnrB8 gene. One qnrB2-positive isolate also had the extended-spectrum β-lactamase blaCTX-M-2 gene. All these isolates also possessed chromosomal substitutions in gyrase- and topoisomerase-encoding genes, explaining their high-level resistance to quinolones.

Conclusions: This study constitutes the first epidemiological survey of the three known Qnr determinants among Brazilian isolates and shows their low prevalence in that country, with the qnrB2 gene being mostly identified.

Keywords: Qnr, Latin America, prevalence

Introduction

The qnr-like genes involved in plasmid-mediated quinolone resistance (PMQR) encode proteins of the pentapeptide repeat family that interfere with the action of quinolones on bacterial DNA gyrase and topoisomerase IV, resulting in low levels of quinolone resistance.1

Four main types of the qnr genes, qnrA, qnrB, qnrC and qnrS, have been identified. PMQR associated with the qnr gene (later termed qnrA1) was detected first in the USA in 1994 from a Klebsiella pneumoniae isolate2 and has been reported among enterobacterial species in Asia, in several countries in Europe and, recently, in South America.1,3–5 The qnrB gene was identified first in an isolate from India, then in the USA, Korea, Kuwait, France and Taiwan.1,6,7 The qnrS1 gene was detected for the first time in a Shigella flexneri isolate from Japan and has been found in Enterobacteriaceae from Germany, the USA, Taiwan, Vietnam and France.1 Very recently, the novel qnrC gene was identified from a Proteus mirabilis clinical isolate from China encoding QnrC1, sharing <68% amino acid identity with the other Qnr determinants.8

Six variants of QnrA have been identified worldwide and 19 variants of the qnrB gene are known, whereas only 3 variants of the qnrS gene are known so far.9 The qnrA gene has been

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associated with sul1-type integrons.\textsuperscript{3,4,7} The qnrB genes are associated with ESBL-encoding genes and are often located near a specific orf1005.\textsuperscript{3} Garnier et al.\textsuperscript{10} described a qnrB2 determinant in a new complex sul1-type integron from Salmonella enterica serovar Keurmassar. In the few qnrS1-positive plasmids, the qnrS1 gene was not identified as part of an integron, but in a plasmid bracketed by inverted repeats with insertion sequence-like structures that may be responsible for its mobilization.\textsuperscript{11} Interestingly, the qnrS2 gene was identified as part of a singular Mic structure in Aeromonas spp. isolates.\textsuperscript{12} Notably, QnrS1 and QnrB4 determinants have been reported together in a single Enterobacter cloacae isolate.\textsuperscript{7}

In Brazil, the qnrA gene has been identified on a plasmid encoding an SHV-5 from an E. cloacae isolate,\textsuperscript{2} but the qnrB and qnrS genes have never been identified. Here, the prevalence of the qnr genes was determined among a collection of oxyimino-cephalosporin-susceptible and -resistant enterobacterial isolates, all being resistant to nalidixic acid and isolated from outpatients in Southeastern Brazil.

Materials and methods

Bacterial isolates and susceptibility testing

Two hundred and fifty-seven non-duplicate nalidixic acid-resistant enterobacterial isolates were collected from outpatients from January 2000 to May 2005 in a private laboratory located in the Southeast region of Brazil (Juiz de Fora, Minas Gerais State). All isolates were identified both by conventional techniques and by MiniAPI, a semi-automated assay (bioMérieux, Marcy l’Étoile, France). Species distribution was as follows: Escherichia coli (n = 194), K. pneumoniae (n = 32), Klebsiella oxytoca (n = 11), P. mirabilis (n = 6), E. cloacae (n = 5), Serratia marcescens (n = 2), Enterobacter aerogenes (n = 2), Providencia stuartii (n = 2), Citrobacter freundii (n = 1), Morganella morgani (n = 1) and Proteus vulgaris (n = 1). The antibiotic susceptibility of the enterobacterial isolates was determined first by the disc diffusion method on Mueller–Hinton agar plates with β-lactam and non-β-lactam antibiotic-containing discs. The MICs of β-lactams, quinolones and fluoroquinolones were determined by an agar dilution method\textsuperscript{13} (CLSI) and the Etest technique according to the manufacturer’s recommendations (AB Biodisk, Solna, Sweden). E. coli ATCC 25922, azide-resistant E. coli J53 and the electrocompetent E. coli TOP10 (Invitrogen) were used as the control for susceptibility testing and as hosts in conjugation and transformation experiments, respectively. E. coli Lo, K. pneumoniae B1 and E. coli S7 were used as qnrA-, qnrB- and qnrS-positive controls, respectively.\textsuperscript{6}

PCR amplification and sequencing

The presence of the qnrA, qnrB and qnrS genes was detected by multiplex PCR technique as described previously.\textsuperscript{6} The presence of the aac(6’)-lb gene was found by using primers AAC6-A (5’TTCGATGCTCTTGATGGCTA-3’) and AAC6-B (5CTC GAATTGCTGGCTTTT-3’) to produce a 482 bp product that was subsequently sequenced to determine whether it corresponded to the aac(6’)-lb-cr variant. The search for qepA used primers QepA-F (5CGTGGTGCTGGAGTTCTC-3’) and QepA-R (5CTGACAGTACTCCGTATG-3’), amplifying a 403 bp fragment. Detection of ESBLs and amplification of quinolone resistance-determining region (QRDR) of the gvrA and parC genes in qnrB isolates was performed using primers and amplification conditions as described previously.\textsuperscript{6} After PCR amplification, all DNA fragments were purified with the Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France). Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide and deduced protein sequences were analysed with software available over the Internet at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). The nucleotide sequences of the QRDR in the gvrA and parC genes obtained for qnrB-positive isolates were compared with those determined from reference sequences deposited in GenBank databases.

Conjugation, electroporation, plasmid analysis and hybridization

Conjugation experiments using an azide-resistant E. coli J53 (AzIR\textsuperscript{6}) as a recipient strain were performed in liquid culture media.\textsuperscript{6,14} Transconjugants were selected on Trypticase Soy (TS) agar plates containing sodium azide (100 mg/L) plus nalidixic acid (6 mg/L). Colonies were tested by PCR using specific primers for the qnrB gene. Plasmid DNAs of donor isolates and E. coli transconjugants were extracted using the Kieser technique.\textsuperscript{15} Transformation experiments were performed using an E. coli TOP10 as a recipient strain for qnrB-positive isolates that presented non-conjugative plasmids. Recombinant strains were selected onto nalidixic acid-containing (3 mg/L) TS agar plates and their plasmids extracted by the Kieser technique. DNA–DNA hybridization experiments were performed as reported\textsuperscript{16} after Southern transfer with a probe consisting of a 264 bp PCR fragment generated from all qnrB-positive isolates. Labelling of the probe and signal detection was carried out using a non-radioactive labelling and detection kit according to the manufacturer’s instructions (Gene Images Random Prime, GE Healthcare).

Cloning experiments and PCR mapping

Whole-cell DNA of qnrB isolates was extracted as described previously.\textsuperscript{14} Genetic structures surrounding the qnrB gene in enterobacterial isolates were cloned by restricting total DNA with EcoRI or KpnI, ligated into restricted plasmid pBK-CMV (Strategene, France) and transforming recombinant plasmids into E. coli TOP10. Recombinant plasmids were selected on TS agar plates containing nalidixic acid (3 mg/L) and kanamycin (30 mg/L). Recombinant plasmid DNA was extracted with a Plasmid Midi Kit (Qiagen) and sequenced on both strands. PCR mapping was performed to identify the genetic structures surrounding the qnrB gene.

Results and discussion

Prevalence of Qnr determinants

Out of 257 non-repetitive nalidixic acid-resistant enterobacterial isolates, six qnrB-positive isolates (2.3%) were identified, including three E. coli, two K. pneumoniae and one C. freundii (Table 1). All isolates were negative for the qnrA and qnrS genes. The qnrB-positive isolates were recovered from urinary tract infections. Five isolates had an identical qnrB2 sequence. C. freundii 79 possessed a qnrBS gene identical to that found in a C. freundii isolate from Ireland (accession number EF576718). The search for qepA and aac(6’)-lb-cr genes among those qnrB-positive isolates remained negative.
The prevalence of Qnr determinants from those Brazilian strains was low and did not significantly increase during the studied period; a single qnrB2-positive isolate (C. freundii 79) was detected in 2003, three qnrB2-positive isolates (E. coli 113, K. pneumoniae 230 and K. pneumoniae 234) in 2004 and two qnrB2-positive isolates (E. coli 342 and E. coli 371) in 2005. This low prevalence was consistent with a recent study conducted from 2002 to 2004. Only QnrA determinants were previously found in enterobacterial isolates from Brazil.

Antibiotic susceptibility
Seventy-two percent of the 257 nalidixic acid-resistant isolates showed a decrease in susceptibility to fluoroquinolones (ciprofloxacin, ofloxacin and levofloxacin). The qnrB-positive isolates were resistant to amoxicillin, ticarcillin, gentamicin and nalidixic acid and were susceptible to amoxicillin/clavulanate and expanded-spectrum cephalosporins, except for a single isolate (K. pneumoniae 230) that was resistant to cefotaxime and cefepime. Isolates C. freundii 79 and E. coli 113 were susceptible to fluoroquinolones according to the CLSI criteria, but all qnrB2 isolates had ciprofloxacin MIC ≥ 0.5 mg/L and/or nalidixic acid MIC ≥ 48 mg/L. Synergy between oxynino-cephalosporins and clavulanate was detected in K. pneumoniae 230 consistent with the production of an ESBL, and a corresponding gene was identified by PCR and sequencing as blaCTX-M-2. Therefore, only one of the six qnrB-positive isolates expressed an ESBL phenotype.

Transfer of quinolone resistance and plasmid characterization
Plasmids ranging from 7 to 154 kb and three to seven plasmids per isolate were identified. Transfer of the quinolone resistance marker by conjugation was obtained for three out of the six qnrB-positive isolates (Table 1). The transferable plasmids of E. coli 113, K. pneumoniae 234 and E. coli 342 were 55, 55 and 154 kb, respectively. The other three qnrB-bearing plasmids were not transferable, and high-molecular weight plasmids (124 and 64 kb) were visualized in donor strains and confirmed to carry the qnrB genes by hybridization assays (data not shown). The plasmid carrying the blaCTX-M-2 gene in K. pneumoniae 230 was not associated with the qnrB2 gene.

QRDR sequencing
Substitution at codon 83 was detected in the QRDR of the gyrA gene in C. freundii 79 (Thr-83→Ile) and E. coli 113 (Ser-83→Phe). Substitutions at codons 83 and 87 in gyrA and codon 80 in parC genes were found in K. pneumoniae 230, K. pneumoniae 234 (Ser-83→Phe, Asp-87→Ala and Ser-80→Ile), E. coli 371 and E. coli 342 (Ser-83→Leu, Asp-87→Asn and Ser-80→Ile). These substitutions explained the difference in MIC values for quinolones and fluoroquinolones for qnrB-positive isolates.

Genetic environment of the qnrB genes
A psp operon coding for putative phage shock proteins and a partial copy of the 3’-end segment (3’-CS) of class I integrons were identified in the vicinity of the qnrB2 gene for the five positive isolates (Figure 1). The 3’-extremity was determined and a sap operon coding for a putative peptide transport system permease followed by a putative carbon starvation gene of S. flexneri was identified. The dfrA25 gene cassette was the single gene cassette identified inside the class 1 integron structure in E. coli 113, E. coli 342, K. pneumoniae 230 and K. pneumoniae 234. The dfr17 and aada5 gene cassettes encoding sulphonamide and aminoglycoside resistance, respectively, were identified in E. coli 371 (Figure 1). In all qnrB-positive isolates,

### Table 1. Characterization of qnrB-positive isolates and MICs of quinolones, fluoroquinolones and ceftazidime for clinical strains, their transconjugants (Tc) or recombinant plasmids (Tf), and the E. coli J53 recipient strain

<table>
<thead>
<tr>
<th>Isolatea</th>
<th>Qnr determinant</th>
<th>Date of isolation</th>
<th>Plasmid size (kb)</th>
<th>MIC (mg/L)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NAL</td>
</tr>
<tr>
<td>C. freundii 79</td>
<td>QnrB2</td>
<td>05/12/2003</td>
<td>no plasmid</td>
<td>&gt;256</td>
</tr>
<tr>
<td>E. coli 113</td>
<td>QnrB2</td>
<td>19/02/2004</td>
<td>55c</td>
<td>48</td>
</tr>
<tr>
<td>Tc 113</td>
<td></td>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>K. pneumoniae 230d</td>
<td>QnrB2</td>
<td>04/10/2004</td>
<td>124</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tf 230</td>
<td></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>K. pneumoniae 234</td>
<td>QnrB2</td>
<td>13/10/2004</td>
<td>55c</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tc 234</td>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>E. coli 342</td>
<td>QnrB2</td>
<td>29/09/2005</td>
<td>154c</td>
<td>&gt;256</td>
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<td>Tc 342</td>
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<td>16</td>
</tr>
<tr>
<td>E. coli 371</td>
<td>QnrB2</td>
<td>08/03/2005</td>
<td>65</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tf 371</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>4</td>
<td>0.12</td>
</tr>
</tbody>
</table>

aTc, transconjugant; Tf, transformant.
bNAL, nalidixic acid; OFL, ofloxacin; NOR, norfloxacin; CIP, ciprofloxacin; GEN, gentamicin; CAZ, ceftazidime.
cConjugative plasmid.
dblaCTX-M-2 producer.
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![Diagram](image_url)

Figure 1. Genetic organization of the qnrB2-containing complex sul1-type integrons. (a) *E. coli* 113, *K. pneumoniae* 230, *K. pneumoniae* 234, and *E. Coli* 342; (b) *E. coli* 371; and (c) *S. enterica* serovar Keurmassar, previously described. The genes and their transcription orientations are represented by arrows and the black rectangles correspond to 59be recombination sites. *cs* is the gene encoding a putative protein involved in carbon starvation, and *sap* is a gene encoding a putative peptide transport system permease.

except *C. freundii* 79, a putative recombinase gene was present at the left-hand boundary, as found by Garnier et al. The *qnrB2* gene was inserted in the opposite direction with respect to the *orf513* transposase gene (Figure 1). It was not possible to determine the structures surrounding the *qnrB8* gene in *C. freundii* 79, and the lack of plasmid identification in that isolate suggested a chromosomal location of that gene.

This study constitutes the first report of the QnrB determinant among Latin American isolates. Interestingly, the *qnrB2* gene was identified in non-ESBL-producing isolates arising from a community setting. This report further suggests that the most prevalent Qnr-like determinants may not be QnrA but rather QnrB.

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Transparency declarations

None to declare.

References

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