Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates

Vincent Cattoir1, Laurent Poirel1, Vincent Rotimi3, Claude-James Soussy2 and Patrice Nordmann1*

1Service de Bactériologie-Virologie, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine Paris-Sud, Université Paris XI, 78, rue du Général Leclerc, 94275 K.-Bicêtre, France; 2Service de Bactériologie-Virologie-Hygiène, Hôpital Henri Mondor, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine de Créteil, Université Paris XII, Créteil, France; 3Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait

Received 21 February 2007; returned 11 April 2007; revised 17 April 2007; accepted 11 May 2007

Objectives: To develop a rapid and reliable single-tube-based PCR technique for detecting simultaneously the plasmid-mediated quinolone resistance qnrA, qnrB and qnrS genes. Methods: After multiple alignments, primers were designed to detect known qnr variants (six for qnrA-, six for qnrB- and two for qnrS-like genes). They were used for screening a collection of 64 expanded-spectrum β-lactamase (ESBL)-producing enterobacterial isolates from Kuwait, collected from 2002 to 2004, as ESBL genes have been often associated with qnr genes. Sequencing was performed to identify qnr and associated ESBL genes. Results: In optimized conditions, all positive controls (used separately or mixed) confirmed the specificity of the PCR primers. Out of 64 isolates, only 3 isolates were positive for a qnrB-like gene (4.7%), whereas no qnrA-like and qnrS-like gene was detected. A qnrB2 gene was detected in an Enterobacter cloacae K34 (SHV-12+) isolate, whereas qnrB1-like (termed qnrB7) and qnrB6-like (termed qnrB9) genes were identified from E. cloacae K37 (SHV-12+) and Citrobacter freundii K70 (VEB-1b+) isolates, respectively.

Conclusions: We report here a fast and reliable technique for rapid screening of qnr-positive strains to be used for epidemiological surveys. A low prevalence of qnr determinants among ESBL-producing Enterobacteriaceae was identified in the study with Kuwaiti isolates.

Keywords: qnrA, qnrB, qnrS, Kuwait, Middle East

Introduction

Plasmid-mediated quinolone resistance was originally reported in a Klebsiella pneumoniae clinical isolate from the USA in 1998.1 This qnrA gene responsible for this resistance (now termed qnrA1) codes for a 218 amino acid protein belonging to the pentapeptide family that protects DNA from quinolone binding to gyrase and topoisomerase IV.2 QnrA confers resistance to quinolones such as nalidixic acid and increases MIC values of fluoroquinolones up to 20-fold.2 The QnrA determinant has been reported worldwide from unrelated enterobacterial species and six variants of QnrA are known (QnrA1 to QnrA6).2 Recently, two other plasmid-mediated quinolone resistance genes, namely, qnrB and qnrS, have been identified that code for QnrB (six variants) and QnrS (two variants) belonging also to the pentapeptide repeat family and sharing 41% and 60% amino acid identity with QnrA, respectively.2 Several surveys, based on molecular approaches consisting of PCR and sequencing, indicate a high rate of the association between Qnr-positive and ESBL-positive isolates.2,3 However, in
view of the genetic heterogeneity of those genes encoding Qnr determinants (94% to 99%, 85% to 99% and 90% nucleotide identity for qnrA-, qnrB- and qnrS-like genes, respectively), it is likely that their prevalence may be underestimated, as a consequence of a lack of sensitivity of the molecular tools that are used. In addition, performing such studies by amplifying separately the qnrA-, qnrB- and qnrS-like genes is time-consuming and expensive. A first multiplex PCR-based method has been described by Robicsek et al. and was applied for screening a collection of ceftazidime-resistant Enterobacteriaceae clinical isolates from the USA. The primers used for the detection of qnrB-like genes did not fully match all six variants, particularly for the reverse primer that mismatched at the 3′ end with qnrB5 and qnrB6 genes.

The purpose of the present work was to design an updated, simple and rapid multiplex PCR technique for the detection of the qnrA-, qnrB- and qnrS-like genes. It was applied to screen a collection of ESBL-producing enterobacterial isolates from Kuwait, a region of the world with unknown distribution of Qnr-like determinants.

Materials and methods

Bacterial isolates

For optimization of the multiplex PCR technique, well-characterized Qnr-positive strains were used as positive controls: Escherichia coli Lo qnrA1,2,3 Shevanella algae KB-1 qnrA3+, S. algae KB-2 qnrA4+ and S. algae KB-3 qnrA5+; K. pneumoniae B1 qnrB1+, Enterobacter cloacae B2 qnrB2+, E. coli B4 qnrB4+, E. coli B5 qnrB5+ and E. coli B6 qnrB6+ (P. Nordmann, unpublished results); E. coli S7 qnrS1+3 and E. coli A37 qnrS2+ (P. Nordmann, unpublished results); and E. cloacae S1 carrying both qnrB4+ and qnrS1+.3

ESBL-producing enterobacterial isolates (n = 64), collected from the University Hospital of Kuwait City from 2002 to 2004, were identified by using the Vitek2 Analyzer (bioMérieux SA, Marcy-l’Étoile, France). They included 29 E. coli, 19 K. pneumoniae, 6 Proteus mirabilis, 4 E. cloacae, 3 Enterobacter aerogenes, 2 Citrobacter freundii and 1 Serratia marcescens clinical isolates. These isolates were non-repetitive and only a single isolate per patient was retained.

Susceptibility testing

ESBL production was suggested by results of the Vitek2 Analyzer and confirmed by disc diffusion and synergy tests performed on Mueller–Hinton agar-containing plates. MICs of quinolones, fluoroquinolones and β-lactams were determined using the Etest method, according to the manufacturer’s recommendations (AB Biodisk, Solna, Sweden). MIC breakpoints used for susceptibility and resistance to nalidixic acid and ciprofloxacin were ≤8 and ≥32 mg/L and ≤1 and ≥4 mg/L, respectively, as recommended by the CLSI (formerly NCCLS).5

Multiplex PCR technique

Rapid DNA preparation was performed by a boiling technique that includes a heating step at 100°C of a single colony in a total volume of 100 µL of distilled water followed by a centrifugation step of the cell suspension. On the basis of a sequence alignment of the qnrA-, qnrB- and qnrS-like genes, pairs of primers were designed to amplify internal fragments with sizes of 580, 264 and 428 bp, respectively (Table 1). A pair of degenerated primers was specifically designed to amplify the six variants of qnrB, despite the high polymorphism of this gene (Table 1). Total DNA (2 µL) was subjected to multiplex PCR in a 50 µL reaction mixture containing 1× PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl2, 200 µM each deoxynucleotide triphosphate, 20 pmol of each of the six primers (Table 1) and 2.5 U of Taq polymerase (Applied Biosystems, Courtabeuf, France). Amplification was carried out with the following thermal cycling profile: 10 min at 95°C and 35 cycles of amplification consisting of 1 min at 95°C, 1 min at 54°C and 1 min at 72°C and 10 min at 72°C for the final extension. DNA fragments were analysed by electrophoresis in a 2% agarose gel at 100 V for 1 h in 1× TAE [40 mM Tris–HCl (pH 8.3), 2 mM acetate and 1 mM EDTA] containing 0.05 mg/L ethidium bromide.

PCR amplification and sequencing of qnr and ESBL genes

To further characterize the qnrB-like genes, additional PCR experiments were performed by using primers described by Jacoby et al. followed by direct sequencing of both strands. Molecular identification of the ESBL genes was carried out for the isolates found to be Qnr-positive, as described previously.3

Results and discussion

Positive controls (used separately or mixed) yielded expected bands and confirmed the specificity of the PCR primers used (Figure 1). Both the qnrB4 and qnrS1 genes were

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence (5′→3′)</th>
<th>Gene</th>
<th>Position&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Size of PCR-amplified product (bp)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>QnrAm-F</td>
<td>AGAGGATTTTCCTACGCCAGG</td>
<td>qnrA1 to qnrA6</td>
<td>30–49</td>
<td>580</td>
<td>This study</td>
</tr>
<tr>
<td>QnrAm-R</td>
<td>TGCCAGGCCAGATCTTGAC</td>
<td>qnrA1 to qnrA6</td>
<td>589–608</td>
<td>264</td>
<td>This study</td>
</tr>
<tr>
<td>QnrBm-F</td>
<td>GGMMATHGAAATTCCGCCACCTG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>qnrB1 to qnrB6</td>
<td>283–302</td>
<td>428</td>
<td>This study</td>
</tr>
<tr>
<td>QnrBm-R</td>
<td>TTTGCYGGYCCCGAGTCGAA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>qnrB1 to qnrB6</td>
<td>526–545</td>
<td>428</td>
<td>This study</td>
</tr>
<tr>
<td>QnrSm-F</td>
<td>GCAAGTTCTAATGACGAGGTT</td>
<td>qnrS1 to qnrS2</td>
<td>137–156</td>
<td>534–563</td>
<td>This study</td>
</tr>
<tr>
<td>QnrSm-R</td>
<td>TCTAACCAGCTGAGTTCCGCG</td>
<td>qnrS1 to qnrS2</td>
<td>137–156</td>
<td>428</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>F, sense primer; R, antisense primer.

<sup>b</sup>Nucleotide numbering begins at the initiation codon of the qnrA1, qnrB1 and qnrS1 genes, respectively.

<sup>M</sup> = A or C; <sup>H</sup> = A or C or T; <sup>Y</sup> = C or T.
unambiguously detected in \textit{E. cloaca}e S1 clinical isolate (Figure 1, lane 8). Given the high genetic diversity between the different \textit{qnr} genes and the sequence variability within the \textit{qnrA} (94\% to 99\%), \textit{qnrB} (85\% to 99\%) and \textit{qnrS} genes (90\%), the use of degenerate primers (especially for the detection of \textit{qnrB}-like genes) might be preferred and therefore amplification of unknown variants might be obtained.

Only three isolates (two \textit{E. cloacae} and one \textit{C. freundii}) isolates were positive for a \textit{qnrB}-like gene (4\%, whereas no \textit{qnrA}-like or \textit{qnrS}-like gene was detected. The \textit{qnrB}-like gene from \textit{E. cloacae} K34 isolate was \textit{qnrB}2 (445/445 bp), which was mostly identified from the \textit{qnrB}-positive enterobacterial isolates from the USA. The \textit{qnrB}-like genes from \textit{E. cloacae} K37 and \textit{C. freundii} K70 isolates coded for proteins showing 97\% and 96\% amino acid identity with \textit{qnrB}1 and \textit{qnrB}6, respectively (corresponding to nucleotide identity of 434/445 and 397/447, respectively). These two novel \textit{QnrB} variants were designated \textit{QnrB7} and \textit{QnrB8}, respectively (GenBank accession no. EU026242 and EU026243, respectively). Only \textit{QnrB} determinants were identified from that collection from Kuwait. \textit{QnrA} has been reported worldwide.\textsuperscript{2}

Both \textit{E. cloacae} K34 and K37 were of intermediate susceptibility to nalidixic acid and remained susceptible to fluoroquinolones, whereas \textit{C. freundii} K70 was fully susceptible to nalidixic acid and fluoroquinolones (Table 2). Interestingly, no mutation was detected in the quinolone-resistance-determining regions of \textit{gyrA} and \textit{parC} genes for these isolates that may have led to additional chromosome-encoded resistance to fluoroquinolones (data not shown). Therefore, other resistance mechanisms may be associated in these strains, such as membrane impermeability and/or efflux pumps overexpression.

\textit{E. cloacae} K34 and \textit{E. cloacae} K37 isolates produced ESBL SHV-12, whereas \textit{C. freundii} K70 isolate produced ESBL VEB-1b. Although an association between the \textit{QnrA} and VEB-1 determinants has been identified, we found here a \textit{QnrB}-like gene associated with the \textit{bla}_{\text{VEB-1b}} gene.\textsuperscript{8} Association between \textit{QnrB}-like determinants (\textit{QnrB}1, \textit{QnrB}2 and \textit{QnrB}5) and ESBLs (SHV-12 and CTX-M-15) has been recently reported from enterobacterial clinical isolates as well as association between \textit{QnrB4}}


