Real-Time PCR Assays for Quantification of qnr Genes in Environmental Water Samples and Chicken Feces

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Real-time PCR assays were developed for the enumeration of plasmid-mediated quinolone resistance (PMQR) determinants, such as the qnrA, qnrB, and qnrS genes, in different water samples and chicken feces. The results indicate that the developed assays are specific and sensitive for the quantification of qnr genes in complex samples.

Quinolones are broad-spectrum antibiotics of considerable importance in both human and veterinary medicine, but their extensive use has resulted in the development of antibiotic resistance. Moreover, several studies have suggested that clinical resistance may be intimately associated with antibiotic resistance genes that may have their origin in environmental bacteria (1, 2). It is important, hence, to study the antibiotic resistance in the environment because it could be a reservoir for antibiotic resistance genes that can be transferred to human pathogens. The main mechanisms of quinolone resistance are associated with mutations in the gyrA and parC genes encoding the A subunits of the DNA gyrase and topoisomerase IV, respectively (3). A number of plasmid-mediated quinolone resistance (PMQR) determinants have also been described, including the qnr genes encoding pentapeptide repeat proteins, which block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV, the AAC(6’)-Ib-cr aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine, and the quinolone efflux pump QepA (4). Plasmid-mediated resistance encoded by qnr genes has been reported in Enterobacteriaceae and, to a lesser extent, in Aeromonadaceae and Vibrioaceae (1, 4, 5). In addition, there are currently different qnr genes described (qnrA, qnrS, qnrB, qnrC, qnrD, and qnrVC), but here we focus on qnrA, qnrB, and qnrS due to the fact that they are more prevalent (4). Since the culture-based methods cannot help us to distinguish the presence of specific resistance genes, it is necessary to turn to molecular techniques. Therefore, the aim of the present study was to develop real-time PCR assays for the rapid and specific quantification of qnr genes in environmental samples in order to understand the environmental distribution of such genes and how anthropogenic inputs affect their spread.

This study was conducted on the following types of samples: water and chicken feces. Water was obtained from four different sources, representing different grades of pollution, such as human and veterinary hospital wastewater effluents, subterranean water, and the Ter River, all of them located in the Autonomous Community of Catalonia. One-liter samples of subterranean and river water were collected and filtered through 0.45-μm-pore-size membranes. In the cases of human and veterinary hospital wastewater samples, only 50 ml was filtered due to the high particulate matter concentrations in the sample. The membranes were then resuspended in 1 ml of lysis buffer (1.2% Triton X-100, 20 mM Tris-Cl, 2 mM EDTA, and 20 mg/ml lysozyme) (6). Concerning the prevalence of qnr in poultry animals (7, 8), feces samples of chicken were also taken. These samples were weighted (around 25 mg each) and diluted directly with 1 ml of lysis buffer. In all cases, the samples were collected and analyzed in triplicate and DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions.

The plasmid-mediated quinolone resistance genes qnrA, qnrB, and qnrS were quantified in all environmental DNA samples using real-time PCR assays. Primers used (Table 1) were adapted from prior studies or designed using the Primer3Plus software (11), on the basis of the alignments of available sequences (http://www.lahey.org/qnrStudies). Primer pairs were designed to amplify all alleles of each known qnr gene until use, and the specificities and sensitivities were verified using the BLAST alignment tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (see Fig. S1 in the supplemental material). For the qnrB gene, the primers selected were degenerated due to the existence of a huge quantity of alleles. Real-time PCR assays were initially performed with various concentrations of primers and also the annealing temperature. Moreover, the DNA extracted from samples was diluted several times to determine which concentration was better and positive controls were spiked with our DNA samples in order to screen for PCR inhibition. The absence of PCR inhibitors was confirmed, except in the case of DNA from chicken feces, which presented some inhibitors that interfered with the quantification of qnr genes. However, this effect was avoided by doing serial dilutions (1:10) of the extracted DNA.

Once real-time PCR assays were optimized, analyses were performed to make an absolute quantification of selected genes in the samples. First, the copy number of the 16S rRNA gene was quantified to confirm the presence of bacteria in the samples and for normalization of the data. In this case, the real-time PCR was performed according to the conditions described previously (12). All real-time PCR assays were amplified in duplicate using SYBR green on a MX3005P system (Agilent Technologies, Santa Clara, CA). The optimal reaction component concentrations to amplify qnr genes were as follows: in a total volume of 30 μl, each reaction...
This study, a dissociation curve (melting curve) was constructed in the range of 60°C to 95°C to verify the specificity of the amplified products.

*Escherichia coli* strain 226 (*qnr*A1), *E. coli* strain J53 pMG301 (*qnr*B2), and *E. coli* strain J53 pMG306 (*qnr*S1) were used as positive controls to test the sensitivity of each real-time PCR, which included the above-mentioned strains, *E. coli* strain J53 pMG289 (*qnr*B1), and *Aeromonas* sp. strain pP2G1 (*qnr*S2). The efficiency and sensitivity of the real-time PCRs were determined by the amplification of standard serial dilutions. Efficiencies were calculated using the resulting standard curves, by the formula $E = 10^{(-1/slope)} - 1$, and the analytical sensitivity of the real-time PCRs was determined as the smallest DNA quantity detected for each assay. The obtained efficiency indicated that all real-time PCR assays were suitable (ranging from 95.3 to 101.7%). Moreover, the analytical sensitivity indicated that the reaction for *qnr*A, *qnr*S, and *qnr*B was able to detect 17, 18, and 27 gene copy numbers per reaction, respectively (Table 1). The correlation coefficient ($R^2$) obtained from the regression line of real-time PCR assays varied from 0.994 to 0.996, demonstrating that the quantification method was linear over a minimum range of 5 logs (see Fig. S2 in the supplemental material). Therefore, all these parameters indicate the validity of the three real-time PCR assays developed for the quantification of *qnr* genes.

A threshold cycle ($C_T$) average from the replicate samples was used for data analysis. To obtain the final result in gene copy numbers per sample volume, calculated gene copy numbers per reaction were divided by the template volume, multiplied by the total DNA elution volume, and the total divided by the sample volume (13). Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL). The quantification results for the samples are summarized in Table 2. Briefly, the presence of bacterial DNA in all samples was confirmed by amplification of bacterial 16S rRNA gene, which ranged from $4.40 \times 10^{10}$ copy numbers/mg in chicken feces samples to $1.20 \times 10^{10}$ copy numbers/ml in water from river samples. Regarding antibiotic resistance genes, not all *qnr* genes were detected in the samples, but we quantified a high copy number for *qnr*A ($1.19 \times 10^{5}$ copies/mg) and *qnr*B ($2.60 \times 10^{4}$ copies/mg) in chicken feces and for *qnr*S in hospital ($5.29 \times 10^{4}$ copies/ml) and veterinary hospital ($4.01 \times 10^{4}$ copies/ml) wastewater effluents. However, when these quantifications were normalized to 16S rRNA gene copies (see Table S1 in the supplemental material), the results showed that the resistance gene with the highest relative copy number was *qnr*S in the river

### TABLE 1 Description of the primers and protocols used in developed real-time PCR assays

<table>
<thead>
<tr>
<th>Target gene (gene)</th>
<th>Primer (Primer)</th>
<th>Sequence (Sequence)</th>
<th>Source or reference (Source)</th>
<th>Thermal cycling (Thermal cycling)</th>
<th>Calibration curve (Calibration curve)</th>
<th>% efficiency (Efficiency)</th>
<th>Analytical sensitivity (Copies/µL of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>qnr</em>A</td>
<td>qnrAF-RT</td>
<td>ATTTCCTGACGCCAGGATT</td>
<td>This study</td>
<td>95°C for 3 min (1 cycle); 95°C for 15 s and 62°C for 20 s (40 cycles)</td>
<td>$y = -3.439 \log(x) + 39.39$; $R^2 = 0.994$</td>
<td>95.3</td>
<td>17</td>
</tr>
<tr>
<td><em>qnr</em>B</td>
<td>qnrBmF</td>
<td>GGMATHGAAATTCGCCAGCT</td>
<td>Modified from reference 9</td>
<td>95°C for 3 min (1 cycle); 95°C for 15 s and 62°C for 20 s (40 cycles)</td>
<td>$y = -3.379 \log(x) + 43.62$; $R^2 = 0.994$</td>
<td>97.7</td>
<td>27</td>
</tr>
<tr>
<td><em>qnr</em>S</td>
<td>qnrSrF11</td>
<td>GACGTGCTAACTTGCGGT</td>
<td>This study</td>
<td>95°C for 3 min (1 cycle); 95°C for 15 s and 62°C for 20 s (40 cycles)</td>
<td>$y = -3.328 \log(x) + 39.56$; $R^2 = 0.996$</td>
<td>101.7</td>
<td>18</td>
</tr>
</tbody>
</table>

- **Source or reference:** Modified from reference
- **Analytical sensitivity:** (copies/µL of DNA)

### TABLE 2 Quantification of 16S rRNA, *qnr*S, *qnr*B, and *qnr*A in different analyzed samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA (Gene copy no./µL)</th>
<th><em>qnr</em>S (Gene copy no./µL)</th>
<th><em>qnr</em>B (Gene copy no./µL)</th>
<th><em>qnr</em>A (Gene copy no./µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital effluent</td>
<td>$1.85 \times 10^{9}$ (±6.97 × 10^6)</td>
<td>$5.29 \times 10^{7}$ (±2.01 × 10^7)</td>
<td>$2.50 \times 10^{7}$ (±2.55 × 10^7)</td>
<td>$9.25 \times 10^{5}$ (±4.23 × 10^5)</td>
</tr>
<tr>
<td>River</td>
<td>$1.20 \times 10^{9}$ (±2.73 × 10^6)</td>
<td>$1.46 \times 10^{7}$ (±2.59 × 10^7)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Veterinary hospital effluent</td>
<td>$5.23 \times 10^{7}$ (±5.84 × 10^5)</td>
<td>$4.01 \times 10^{7}$ (±6.33 × 10^7)</td>
<td>$2.05 \times 10^{7}$ (±1.08 × 10^7)</td>
<td>ND</td>
</tr>
<tr>
<td>Subterranean water</td>
<td>$3.27 \times 10^{7}$ (±2.53 × 10^6)</td>
<td>ND</td>
<td>$2.45 \times 10^{7}$ (±1.16 × 10^7)</td>
<td>ND</td>
</tr>
<tr>
<td>Chicken feces</td>
<td>$4.40 \times 10^{8}$ (±4.06 × 10^6)</td>
<td>ND</td>
<td>$2.60 \times 10^{8}$ (±2.42 × 10^8)</td>
<td>$1.19 \times 10^{8}$ (±7.42 × 10^6)</td>
</tr>
</tbody>
</table>

* Mean values and standard deviations of the means (in parentheses) are shown. Gene copy number/mg of sample for chicken feces. ND, not detected.
earlier assays were carried out only for the detection of specifically quantify the most prevalent to our knowledge, this is the first real-time PCR targeted to specifically quantify the most prevalent qnr genes, because all the earlier assays were carried out only for the detection of qnr.

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REFERENCES