Characterization of multidrug-resistant Enterobacteriaceae carrying plasmid-mediated quinolone resistance mechanisms in Spain

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Objectives: The aim of this study was to detect and characterize plasmid-mediated quinolone resistance determinants as well as genes responsible for additional resistances in Enterobacteriaceae isolates in Spain.

Methods: The resistance genes were identified by PCR and sequencing. Plasmid analysis was carried out by S1-PFGE and PCR-based replicon typing. Conjugation assays were performed to link resistance genes to plasmids. The genetic relationships among the strains were determined by XbaI-PFGE.

Results: One hundred and twenty-three isolates carried *qnr* as the only quinolone resistance determinant. One *Salmonella* Bredeney was positive for *qnrB2* harboured on a 320 kb conjugative IncHI2 plasmid. One *Salmonella* Newport was positive for *qnrB4* harboured on a 70 kb conjugative IncFIIs plasmid. Twenty-five *Salmonella* Thompson were positive for *qnrA1*. Twenty-two harboured a 220 kb non-conjugative and non-typeable plasmid, two a 220 kb conjugative IncHI2 plasmid and one a 120 kb non-conjugative IncA/C plasmid. *qnrS1* was always detected on non-conjugative ColETP plasmids of various sizes. Thus, two *Salmonella* Montevideo strains carried a 20 kb plasmid while *Salmonella* Typhimurium strains carried plasmids of 10 kb (n=91) or 30 kb (n=2). One *Escherichia coli* was positive for *qnrA1* detected on a 220 kb conjugative IncHI2 plasmid. *qnr* alleles and β-lactamases were associated in *Salmonella* Bredeney (harbouring *bla*SHV-12), *Salmonella* Newport (harbouring *bla*3,4SHV-12), *Salmonella* Montevideo (harbouring *bla*SHV-12) and *E. coli* (harbouring *bla*CTX-M-9).

Conclusions: This is the first epidemiological study of *qnr* genes in Enterobacteriaceae isolates from Spain. *Salmonella* plasmids bearing *qnr* alleles are not a localized phenomenon in Spain and wide variation in plasmids and co-resistance was detected. The presence of *qnr* determinants in *Salmonella* serotypes commonly reported in human disease is concerning.

Keywords: *Salmonella*, antimicrobial resistance surveillance, molecular identification

Introduction

Fluoroquinolones are antimicrobials that are commonly used in clinical or veterinary medicine. Their common mechanism, whereby they act as a cellular poison by binding the target enzyme–DNA complexes after strand breakage and before resealing of DNA, means that resistance to one fluoroquinolone will confer resistance to all fluoroquinolones. Quinolone resistance in Enterobacteriaceae usually originates from mutations in target enzymes (DNA gyrase and/or topoisomerase IV) or because of impaired access to the target, occurring either because of changes in porin expression or because of overexpression of efflux pumps. Recently, plasmid-mediated quinolone resistance (PMQR) has been detected conferring low-level resistance to quinolones by different mechanisms: a modifying enzyme [AAC(6’)-Ib-cr]; the QepA and OqxAB efflux pumps; or gyrase and topoisomerase IV protection proteins (*Qnr*). A unique allele has been described for the *aac(6’)-Ib-cr* and *qepA* genes, two alleles have been described for *qepA* and several *qnr* genes, named *qnrB*, *qnrS*, *qnrC* and *qnrD*, have been described in various Gram-negative bacterial species since the first description of *qnrA*. To date, 7 variants of *QnrA* (QnrA1 to QnrA7), 25 variants of *QnrB* (QnrB1 to QnrB25), 1 variant of *QnrC*, 4 variants of *QnrS* (QnrS1 to QnrS4) and 1 variant of *QnrD* are known (http://www.lahey.org/qnrStudies). Although currently *qnr*-positive strains are relatively infrequent, widespread horizontal transfer among strains could contribute to the eventual compromise of the use of fluoroquinolones. Besides, *qnr*-positive strains in the absence of other resistance mechanisms are susceptible to fluoroquinolones according to the CLSI breakpoints, making their detection
difficult. Although the clinical relevance of PMQR is unclear, linkage of PMQR to β-lactamases on plasmids has been described to contribute to the dissemination of extended-spectrum β-lactamase (ESBL) producers with reduced susceptibility to quinolones through co-selection. The aim of this study was to detect and characterize PMQR determinants as well as genes responsible for additional resistances in Enterobacteriaceae isolates in Spain.

**Materials and methods**

**Bacterial isolates and susceptibility tests**

As a routine, a third of the isolates submitted to the Spanish Reference Laboratory for Salmonella (SRLS) are screened for resistance. Susceptibility to antimicrobials was tested by the disc diffusion method according to CLSI guidelines. The original panel included the following antimicrobials: ampicillin; cefalotin; cefotaxime; amoxicillin/clavulanic acid; tetracycline; streptomycin; kanamycin; gentamicin; nalidixic acid; ciprofloxacin; chloramphenicol; trimethoprim/sulfamethoxazole; and a sulfonamide compound (sulfadiazine, sulfathiazole and sulfamerazine sodium). A synergy test was performed for all isolates with amoxicillin/clavulanic acid and cefotaxime discs placed 30 mm apart. The MICs of nalidixic acid and ciprofloxacin were determined by Etests for isolates susceptible to nalidixic acid but showing a decrease in the diameter of the inhibition halo of ciprofloxacin (≤27 mm). Results were interpreted according to the CLSI guidelines. *Escherichia coli* ATCC 25922 was used as a control strain.

The XbaI–PFGE patterns of strains were compared according to the PulseNet-Europe protocol (http://www.pulsenet-europe.org/docs.htm).

**Characterization of resistances**

PCR and sequencing of PMQR determinants [qnr, aac(6′)-Ib-cr and qepA genes] and the study of the quinolone resistance-determining regions (QRDRs) of gyrA and parC genes were performed as previously described.\(^5,6\) Detection and identification of β-lactamases and additional resistance genes were performed for the PMQR-positive isolates as previously described. Class I integrons were analysed by PCR and sequencing on both strands.\(^5,7\)

**Plasmid characterization and localization of qnr genes**

Transfer of resistance was tested by conjugation experiments using a rifampicin-resistant *E. coli* as recipient and all Qnr-producing strains as donors and rifampicin (50 mg/L) and ampicillin/streptomycin (100 mg/L) to select transconjugants.\(^6\) The presence of plasmids and plasmid sizes were assessed by S1-PFGE or plasmid extraction following the previously described protocol.\(^3,4\) Detection and identification of integrons, β-lactamases and additional resistance genes were performed for the PMQR-positive isolates as previously described. Class I integrons were analysed by PCR and sequencing on both strands.\(^5,7\)

**Results and discussion**

In total, 19010 isolates (18624 Salmonella spp., 285 *E. coli*, 68 *Shigella* spp., 29 *Klebsiella pneumoniae*, 2 *Citrobacter freundii* and 2 *Proteus mirabilis*) were tested between 2003 and 2008. Thirty-seven strains exhibited an unusual quinolone resistance phenotype, having decreased susceptibility to ciprofloxacin (MIC 0.12–0.5 mg/L) but still susceptible to nalidixic acid (MIC 8–16 mg/L). These values have been previously proposed to identify qnr-positive strains. Because of the predominance of *Salmonella* Thompson (n=6) and *Salmonella Typhimurium* DT104b (n=24) among these strains, we extended the study to all the strains with these sero-phenotypes in our collection. Thus, 19 additional *Salmonella* Thompson and 67 *Salmonella Typhimurium* DT104b strains exhibited a quinolone resistance phenotype compatible with the presence of qnr. Therefore, a total of 123 strains from the SRLS collection were included in the study. Amplification and sequencing of aac(6′)-Ib-cr, qepA and qnr genes, and analysis of the mutations in the QRDRs of gyrA and parC genes, revealed qnr genes as the only determinant responsible for the quinolone phenotype (Table 1). All of the positive strains were isolated from sporadic cases in humans, except for nine *Salmonella Typhimurium* DT104b strains, which were isolated from bathing areas (n=3) and aquarium water (n=6).

Many surveys have shown that Qnr-positive Enterobacteriaceae express plasmid-encoded β-lactamases, especially for QnrA- and QnrB-producing isolates.\(^2\) In this study 4 out of 123 qnr-positive isolates expressed an ESBL or AmpC enzyme (Table 1).

Only six transconjugants were obtained. Plasmid analysis showed that qnrB2 was transferred with *bla*<sub>S</sub><sup>SHV-12</sup> on an IncHI2 plasmid of 320 kb. Resistances to streptomycin, tetracycline, chloramphenicol and the sulfonamide compound were co-transferred. qnrB4 was transferred with *bla*<sub>DHA-1</sub> on an IncFI1s plasmid of 70 kb. IncHI2 plasmids associated with qnrB2 and *bla*<sub>S</sub><sup>SHV-12</sup> have already been described in *Salmonella* Bredeney in Spain and in *Salmonella Senftenberg* in the Netherlands.\(^9\) However, although the association of qnrB4 and *bla*<sub>S</sub><sup>SHV-12</sup> is very common in *K. pneumoniae*,\(^6\) to the best of our knowledge this is the first description of such an association in *Salmonella* and the first description of a qnrB4 gene on an IncFI1s plasmid. qnrA1 was transferred with *bla*<sub>CTX-M-9</sub> in *E. coli* on an IncH1 plasmid of 220 kb. Resistances to the sulfonamide compound, trimethoprim/sulfamethoxazole and chloramphenicol were co-transferred. The association of qnrA1 and *bla*<sub>CTX-M-9</sub> has been reported in *E. coli*, but no reference to the incompatibility group of the plasmid was made.\(^10\) One transconjugant from *Salmonella Typhimurium* harbouring *bla*<sub>S</sub><sup>SHV-12</sup> was obtained, but only an IncHI2 plasmid of 95 kb carrying the β-lactamase gene was transferred. IncHI2 plasmids harbouring *bla*<sub>CTX-M-9</sub> genes are very common in Spain in both *E. coli* and *Salmonella*. *E. coli* strains harbouring qnrA1, *bla*<sub>CTX-M-9</sub> and *bla*<sub>S</sub><sup>SHV-12</sup> have been reported in Spain.\(^11\) Finally, two transconjugants from *Salmonella Thompson* harbouring qnrA1 also harboured an IncHI2 plasmid of 220 kb. In these cases, resistances to ampicillin, amoxicillin/clavulanic acid and the sulfonamide compound were co-transferred. Hybridization assays revealed the presence of the qnr gene on the plasmid. This is the first description of a qnrA1 gene on an IncHI2 plasmid in *Salmonella Thompson*, although qnrA1 has been described in a 250–340 kb IncHI2 non-conjugative plasmid in *Salmonella Concord* in Spain.\(^12\)

Considering the strains that were negative with regard to conjugation assays, most of the *Salmonella* Thompson strains harboured a large non-typeable plasmid, although one strain carried an IncA/C plasmid, and as far as we know this is the first report of a qnrA1 gene on an IncA/C plasmid in *Salmonella*. The qnrS1 gene was detected mostly on a ColE<sub>TP</sub> plasmid of 10 kb (Table 1). qnrS1 has been widely described in *Salmonella*.
and the same plasmid identified here has been completely sequenced from Salmonella Typhimurium DT193.15

Comparison of PFGE patterns of all Salmonella Thompson strains suggested that they were clonally related (92.5% similarity), with the exception of the isolate with the larger plasmid (79.1%), which shared an identical PFGE profile.

In Spain, some variants of qnr genes have been described in Enterobacteriaceae: qnrA1 in K. pneumoniae, Enterobacter cloacae, C. freundii and E. coli, qnrB-like, qnrB6 and qnrB16 in E. coli and Citrobacter spp.; and qnrS1 in Enterobacter spp. and K. pneumoniae.2,14,15 This is the first epidemiological study of

<table>
<thead>
<tr>
<th>qnr gene</th>
<th>Species/sero-phagetype (no. of isolates)</th>
<th>Resistance pheno/genotypes (no. of isolates)</th>
<th>MIC (mg/L) NAL/CIP</th>
<th>Plasmid size (kb)/incompatibility group</th>
</tr>
</thead>
<tbody>
<tr>
<td>qnrB2</td>
<td>Salmonella Bredeney (n=1)</td>
<td>AMP, CTX, STR, GEN, KAN, SSS, SXT, CHL, TET blAox12, blAonem-1, strRAB, aphA1, aac(6′)-Ib-cr, sul1, dfrA17, cmlA, tet(D)</td>
<td>8/0.19</td>
<td>320/IncHI2</td>
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<td>qnrB4</td>
<td>Salmonella Newport (n=1)</td>
<td>AMP, CTX, AMC blAox1A-1</td>
<td>12/0.25</td>
<td>70/IncFII</td>
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<td>qnrA1</td>
<td>E. coli (n=1)</td>
<td>AMP, CEF, CTX, STR, KAN, SSS, SXT, CHL blOCTX-M-9, strRAB, aadA2, aadB, sul1, dfrA16, cat2</td>
<td>6/0.25</td>
<td>220/IncHI2</td>
</tr>
<tr>
<td></td>
<td>Salmonella Thompson (n=25)</td>
<td>AMP, AMC, SSS, CHL, TET (n=21) blAox1A-1, sul1, cmlA, tet(D) AMP, AMC, STR, KAN, SSS, CHL, TET (n=1) blAox1A-1, strRAB, aphA1, sul1, cmlA, tet(D) AMP, AMC, KAN, SSS, CHL, TET (n=1) blAox1A-1, aphA1, sul1, cmlA, tet(D) AMP, AMC, SSS, TET (n=2) blAox1A-1, sul1, tet(D)</td>
<td>8–12/0.25–0.3</td>
<td>220/NT, IncHI2</td>
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<td>Salmonella Montevideo (n=2)</td>
<td>AMP, AMC, STR, KAN, SSS, TET blAox1A-1, strRAB, aadA2, aphA1, sul1, tet(D)</td>
<td>8–12/0.25–0.3</td>
<td>220/NT, IncHI2</td>
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<td>qnrS1</td>
<td>Salmonella Typhimurium DT104b (n=91)</td>
<td>SSS, STR, TET (n=75) sul2, strRAB, tet(A) AMP, AMC, STR, SSS, CHL, TET (n=1) blAox1A-1, strRAB, sul2, cat, tet(A) AMP, AMC, STR, SSS, CHL, TET (n=1) blAox1A-1, aadA1, sul1, cat, tet(A)</td>
<td>8–12/0.25–0.3</td>
<td>220/NT, IncHI2</td>
</tr>
<tr>
<td></td>
<td>Salmonella Typhimurium DT120 (n=1)</td>
<td>fully susceptible</td>
<td>8–12/0.25–0.3</td>
<td>220/NT, IncHI2</td>
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<td>Salmonella Typhimurium DT41 (n=1)</td>
<td>fully susceptible</td>
<td>8–12/0.25–0.3</td>
<td>220/NT, IncHI2</td>
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<tr>
<td></td>
<td>Salmonella Typhimurium DT41 (n=1)</td>
<td>fully susceptible</td>
<td>8–12/0.25–0.3</td>
<td>220/NT, IncHI2</td>
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AMP, ampicillin; CEF, cefalotin; CTX, cefotaxime; AMC, amoxicillin/clavulanic acid; STR, streptomycin; GEN, gentamicin; KAN, kanamycin; SSS, sulfonamides; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol, TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; ND, not detected; NT, non-typeable.
qnr genes in Enterobacteriaceae isolates from Spain. The Salmonella plasmids bearing these genes were not a localized phenomenon. We identified these resistance determinants in five serotypes distributed across Spain. Regarding the temporal distribution of the qnr-positive serotypes most commonly identified, Salmonella Typhimurium DT104b appeared throughout all the years of the study while Salmonella Thompson appeared in the last 3 years. The presence of qnrA1 in Salmonella Thompson and qnrS1 in Salmonella Typhimurium with a high genetic similarity by PFGE (one and three bands difference, respectively), suggests that two clonal groups appeared and have persisted, increased and evolved over the years. Although their clinical implications are still unknown, the spread of PMQR in Salmonella serotypes commonly implicated in human infections in Spain is concerning since there have been reported failures in the treatment with fluoroquinolones of patients infected with Salmonella with decreased susceptibility to this compound. Furthermore, they often co-exist with other resistances (98% of the isolates showed resistance to other antimicrobial agents) and sometimes are co-transferred, enabling the propagation of resistance to antimicrobial agents commonly used to treat serious Salmonella infections, in the case of ESBLs, and co-selection by the use of either agent alone. Salmonellosis is a zoonotic disease. Therefore, vigilant surveillance and prospective studies (together with the proper use of antimicrobial agents in human and veterinary medicine) are necessary to determine changes in prevalence, and the basis for the appearance and co-selection of resistances, in order to limit the spread of PMQR.

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Transparency declarations

None to declare.

References


