

Detection of Plasmid-Mediated Quinolone Resistance Genes in Clinical Isolates of *Enterobacter* spp. in Spain[∇]

M. E. Cano,¹ J. M. Rodríguez-Martínez,² J. Agüero,^{1,3} A. Pascual,^{2,4} J. Calvo,¹ J. M. García-Lobo,³ C. Velasco,² M. V. Francia,¹ and L. Martínez-Martínez^{1,3*}

Service of Microbiology, University Hospital Marqués de Valdecilla, Santander,¹ Department of Microbiology, School of Medicine, Seville,² Department of Molecular Biology, School of Medicine, Santander,³ and Service of Microbiology, University Hospital Virgen Macarena, Seville,⁴ Spain

Received 19 November 2008/Returned for modification 20 November 2008/Accepted 14 April 2009

We have studied by PCR and DNA sequencing the presence of the *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*, *intI1*, and *ISCR1* genes in 200 clinical isolates of *Enterobacter cloacae* ($n = 153$) and *E. aerogenes* ($n = 47$) consecutively collected between January 2004 and October 2005 in two hospitals located in Santander (northern Spain) and Seville (southern Spain). Mutations in the quinolone resistance-determining region of *gyrA* and *parC* also were investigated in organisms containing plasmid-mediated quinolone resistance genes. The isolates had different resistant phenotypes, including AmpC hyperproduction, extended-spectrum β -lactamase production, resistance or decreased susceptibility to quinolones, and/or resistance to aminoglycosides. Among the 116 *E. cloacae* isolates from Santander, *qnrS1*, *qnrB5*, *qnrB2*, and *aac(6′)-Ib-cr* were detected in 22 (19%), 1 (0.9%), 1 (0.9%), and 3 (2.6%) isolates, respectively. Twenty-one, 17, and 2 *qnrS1*-positive isolates also contained *bla*_{LAP-1}, *intI1*, and *ISCR1*, respectively. A *qnrB7*-like gene was detected in one *E. aerogenes* isolate from Santander. No plasmid-mediated quinolone resistance gene was detected in the isolates from Seville. The *qnrS1*-containing isolates corresponded to four pulsed-field gel electrophoresis patterns and showed various levels of resistance to quinolones. Six isolates were susceptible to nalidixic acid and presented reduced susceptibility to ciprofloxacin. The *qnrS1* gene was contained in a conjugative plasmid of ca. 110 kb, and when the plasmid was transferred to recipient strains that did not have a specific mechanism of quinolone resistance, the ciprofloxacin MICs ranged from 0.047 to 0.125 μ g/ml.

Resistance to quinolones in enterobacteria is increasing worldwide (8, 24). Previous studies have demonstrated that in this group of organisms, multiple mechanisms are involved in this problem, including altered type II topoisomerases, decreased permeability, increased active efflux, target protection, and drug modification (8, 18, 33). Most of the genes coding for these mechanisms are of chromosomal origin, but since 1998, when *qnrA1* was discovered in a clinical isolate of *K. pneumoniae* from the United States (18), several plasmid-mediated quinolone resistance (PMQR) genes (9) have been reported from clinical isolates, including new *qnrA* variants, multiple *qnrB* and *qnrS* alleles, *qnrC*, *qnrD*, *aac(6′)-Ib-cr*, and two *qepA* alleles (4, 8, 17, 44).

Qnr proteins protect type II topoisomerases from quinolone attack (38, 39). *Aac(6′)-Ib-cr* modifies quinolones containing a piperazinyl group (31), and *QepA* proteins are involved in active efflux (25). Plasmid-borne genes involved in quinolone resistance cause low-level resistance to quinolones (18, 29) and have an additive effect on the level of resistance caused by other mechanisms (19, 28).

qnrA has been identified in complex *sulI*-type integrons of the *In4* family (39). These integrons contain the *ISCR1* sequence (37), which codes for a recombinase involved in the mobilization of antibiotic resistance genes in its proximity. The

qnrB gene also is associated with *ISCR1* as well as with another presumed recombinase, Orf1005 (11). *qnrS* is not part of an integron, but it often is bracketed by inverted repeats with insertion sequence-like structures (7).

PMQR genes have been found worldwide in multiple species of enterobacteria (18, 29), being particularly frequent in *Enterobacter* (more often *E. cloacae*) (10, 12, 16, 22, 23, 30, 32, 35, 42), *Klebsiella pneumoniae* (10, 16, 25, 30, 32, 35, 43), and *Escherichia coli* (16, 25, 30, 32). Interestingly, on some occasions more than one PMQR gene has been identified in the same organism (18, 29).

In a previous study by our group (unpublished data), we did not detect PMQR genes in *K. pneumoniae* or in *E. coli*, but we identified the *qnrA1* gene in one isolate of *E. cloacae* and one of *Citrobacter freundii* from a single patient in Santander.

In the study reported here, we focused on the detection of *qnrA*, *qnrS*, *qnrB*, *aac(6′)-Ib-cr*, or *qepA* in *E. cloacae* and *E. aerogenes*, the two more clinically relevant species in the genus *Enterobacter*. The recently described *qnrC* (43) and *qnrD* (4) genes were not included in this study, as these descriptions were not known when the study was planned. The study also aimed to compare the incidence of these genes in two different geographical locations: Santander (northern Spain) and Seville (southern Spain).

MATERIALS AND METHODS

Bacteria. We evaluated 200 clinical isolates (1 per patient) of *E. cloacae* and *E. aerogenes* collected at the University Hospital Marqués de Valdecilla in Santander (138 isolates: 116 *E. cloacae* and 22 *E. aerogenes*) and the University Hospital Virgen Macarena in Seville (62 isolates: 37 *E. cloacae* and 25 *E. aero-*

* Corresponding author. Mailing address: Service of Microbiology, University Hospital Marqués de Valdecilla, Av. de Valdecilla s/n, 39008 Santander, Spain. Phone: 34 942 202580. Fax: 34 942 203462. E-mail: mecano@humv.es.

[∇] Published ahead of print on 22 April 2009.

TABLE 1. Primers used for PCR and expected amplicon sizes

| Gene or assay | Primer | Amplicon size (bp) | Reference or source |
|----------------------------|---|--------------------|---------------------|
| <i>qnrA</i> | 5'-GAT AAA GTT TTT CAG CAA GAG G-3' 5'-ATC CAG ATC GGC AAA GGT TA-3' | 543 | 10 |
| <i>qnrB</i> | 5'-GGC ATT GAA ATT CGC CAC TG-3' 5'-TTT GCT GCT CGC CAG TCG A-3' | 360 | This study |
| <i>qnrS</i> | 5'-TGG AAA CCT ACA ATC ATA CA-3' 5'-TGC AAT TTT GAT ACC TGA TG-3' | 599 | This study |
| <i>aac(6')-Ib-cr</i> | 5'-ATG ACT GAG CAT GAC CTT GC-3' 5'-TTA GGC ATC ACT GCG TGT TC-3' | 519 | 13 |
| <i>qepA</i> | 5'-AAC TGC TTG AGC CCG TAG AT-3' 5'-CGT GTT GCT GGA GTT CTT CC-3' | 189 | This study |
| <i>intI1</i> | 5'-GCG AAG TCG AGG CAT TTC TGT C-3' 5'-ATG CGT GTA AAT CAT CGT CGT AGA GA-3' | 767 | This study |
| <i>ISCR1</i> | 5'-CGC CCA CTC AAA CAA ACG-3' 5'-GAG GCT TTG GTG TAA CCG-3' | 469 | 34 |
| <i>gyrA</i> | 5'-AAA TCT GCC CGT GTC GTT GGT-3' 5'-GCC ATA CCT ACG GCG ATA CC-3' | 343 | 41 |
| <i>parC</i> | 5'-ATG TAC GTG ATC ATG GAC CG-3' 5'-ATT CGG TGT AAC GCA TCG CC-3' | 300 | This study |
| <i>bla_{LAP-1}</i> | 5'-CAA TAC AAA GCA CAG AAG ACC-3' 5'-CCG ATC CCT GCA ATA TGC TC-3' | 748 | 26 |
| REP-PCR | 5'-III-GCG CCG ICA TCA GGC-3' 5'-ACG TCT TAT CAG GCC TAC-3' | Variable | 40 |

genes) between January 2004 and October 2005. The organisms were selected as consecutive isolates presenting resistance to β -lactams (including AmpC hyperproduction or extended-spectrum β -lactamase production), resistance or decreased susceptibility to quinolones, and/or resistance to aminoglycosides. Identification and preliminary susceptibility testing were performed by the MicroScan WalkAway 96 system (Dade Behring, West Sacramento, CA) in Santander and by the Vitek 2 system (bioMérieux, Hazelwood, MO) in Seville. *E. coli* ATCC 25922 and ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were routinely used in the two centers for quality control purposes. Results obtained with Etest strips of ciprofloxacin were controlled using *E. coli* ATCC 25922 and *P. aeruginosa* 27853, those of nalidixic acid with *E. coli* ATCC 25922, and those of ticarcillin-clavulanate with *E. coli* ATCC 35218, by testing them in parallel to clinical isolates.

PCR amplification and DNA sequencing. All 200 isolates were screened by PCR. The corresponding primers and expected amplicon sizes are presented in Table 1. Genomic DNA was extracted using the InstaGene matrix kit (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions, and 1 μ l was added to a reaction mixture containing 1 \times PCR buffer, 1.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphate, 0.5 μ M each primer, and 1 U of AmpliTaq Gold (Applied Biosystems, Madrid, Spain). The amplification conditions were 94°C for 10 min, and then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final elongation at 72°C for 10 min. A multiplex PCR was developed for detecting *intI1*, *ISCR1*, and *qnrA*, and independent PCRs were used for detecting *qnrB*, *qnrS*, *qepA*, and *aac(6')-Ib-cr*. Positive controls for the presence of *qnrA1* (20), *qnrB1* (11), *qnrS1* (7), *aac(6')-Ib-cr* (17), and *qepA1* (25) were included.

The quinolone resistance-determining regions (QRDR) of *gyrA* and *parC* and the presence of *bla_{LAP-1}* also were screened by PCR in strains containing PMQR genes by following previously described conditions (26, 41) and using the primers shown in Table 1.

Amplicons were purified with a Qiaquick PCR purification kit (Qiagen, Izasa, Barcelona, Spain). The sequencing of both strands was performed at the Molecular Genetics Unit of the University Hospital Marqués de Valdecilla using a CEQ 2000 Dye Terminator for cycle sequencing with the Quick Start kit (Beckman Coulter, Inc.) or at the SCAI laboratory (Cordoba, Spain) using the Big Dye Terminator v3.0 sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The BLAST program was used to compare the nucleotide and protein sequences to those available on the internet at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Antimicrobial susceptibility testing. The MICs of ciprofloxacin, nalidixic acid, ticarcillin, and ticarcillin-clavulanate against isolates containing PMQR genes were determined with Etest strips (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. For other antimicrobial agents, MICs were determined with the MicroScan WalkAway 96 system in Santander and by the Vitek 2 system in Seville.

Molecular fingerprinting. Repetitive extragenic palindromic PCR (REP-PCR) typing was performed on *E. cloacae* isolates containing PMQR genes (Table 2). Amplicons were run in a 1.5% agarose gel for 100 min, stained with ethidium bromide, and photographed. After visual inspection, two isolates were considered to be clonally unrelated when two or more different bands were observed.

Additionally, pulsed-field gel electrophoresis (PFGE) was performed on all *qnrS1*-containing isolates with a CHEF-DR-II system (Bio-Rad, Hemel Hempstead, United Kingdom). DNA in agarose plugs was digested overnight with XbaI at 37°C. DNA was electrophoresed for 7 h (1- to 15-s pulse ramp) and 16 h (15- to 35-s pulse ramp) at 14°C in a 1% agarose gel at 6 V/cm. The interpretation of PFGE patterns was based on the criteria of Tenover et al. (36).

Conjugation experiments. After results of PMQR genes detection were obtained (see below), one isolate of *qnrS1*-producing *E. cloacae* that was representative of each REP-PCR pattern was selected for conjugation experiments. Matings were performed on 0.22- μ m nitrocellulose membranes (Millipore Corporation, Billerica, MA) with *E. coli* J53, which is resistant to rifampin (rifampicin), as the recipient. After 2 h of incubation, mating mixtures were plated onto agar containing rifampin (100 mg/liter) and ciprofloxacin (0.03 mg/liter). The presence of *qnrS1* was confirmed in the transconjugants by PCR, as described above.

Plasmid analysis. Plasmids were detected in *qnrS1*-containing isolates and derived transconjugants using DNA S1 nuclease treatment followed by PFGE as described by Barton et al. (1). Lambda Ladder PFGE Marker from New England Biolabs (IZASA SA, Barcelona, Spain) was used as the size marker.

Plasmid DNA of *qnrS1*-positive isolates and transconjugants separated in PFGE gels was transferred onto a nylon membrane and probed for the presence of *qnrS1*. The probe specific for the *qnrS1* gene consisted of a 599-bp PCR fragment amplified from whole-cell DNA of *E. coli* strain DH5 α containing the plasmid pBC-H2.6. The labeling of the probe and signal detection were carried out using a DIG-High-Prime labeling and luminiscent detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

RESULTS

The *qnrS1* gene was detected in 22 out of the 116 *E. cloacae* isolates from Santander (19%) and was not detected in any isolate from Seville. *qnrB5* and *qnrB2* were identified in two other *E. cloacae* isolates. Finally, the sequencing of one of the amplicons allowed us to identify a *qnrB7*-like gene (coding for an Asp197Gly change in the corresponding QnrB7 sequence)

TABLE 2. MICs, REP-PCR patterns, resistance genes, and changes in topoisomerases in *Enterobacter* spp.^a

| Isolate | Organism | Sample source | Ward | MIC ($\mu\text{g/ml}$) | | | | | | REP-PCR pattern | PGE pattern | PMOR | Presence of: | | | Changes in: | |
|---------|---------------------|--------------------|------------------------|--------------------------|------|-------|------|-----|-----|-----------------|-------------|--------------------|------------------------------|-------------|-------|----------------|---------------------|
| | | | | CAZ | T-CL | CIP | NAL | AMK | TOB | | | | <i>bla</i> _{1-AP-1} | <i>int1</i> | ISCR1 | DNA- gyrase | Topoisomerase IV |
| 1599 | <i>E. cloacae</i> | Abdominal drainage | Internal medicine | >16 | 96 | 0.38 | 8 | <8 | <4 | A | A1 | <i>qnrS1</i> | + | - | - | WT | WT |
| 2531 | <i>E. cloacae</i> | Blood | Hematology | >16 | 96 | 0.38 | 8 | <8 | <4 | A | A1 | <i>qnrS1</i> | + | - | - | WT | WT |
| 1480 | <i>E. cloacae</i> | Wound | Nephrology | >16 | 128 | 0.38 | 8 | <8 | <4 | A | A1 | <i>qnrS1</i> | + | - | - | WT | WT |
| 3249 | <i>E. cloacae</i> | Wound | Domicil. hospital | >16 | 128 | 0.38 | 8 | <8 | <4 | A | A1 | <i>qnrS1</i> | + | - | - | WT | WT |
| 3174 | <i>E. cloacae</i> | Wound | Domicil. hospital | >16 | >256 | 1.5 | 32 | <8 | <4 | B | B | <i>qnrS1</i> | - | + | - | WT | WT |
| 3210 | <i>E. cloacae</i> | Urine | Urology | <1 | 48 | 2 | 8 | <8 | 8 | C | A2 | <i>qnrS1</i> | + | + | + | WT | WT |
| 1640 | <i>E. cloacae</i> | Catheter tip | Internal medicine | >16 | 128 | 3 | >256 | <8 | <4 | D | C | <i>qnrS1</i> | + | - | - | S831 | WT |
| 3049 | <i>E. cloacae</i> | Catheter tip | Urology | <1 | 16 | 4 | 16 | <8 | 8 | C | A2 | <i>qnrS1</i> | + | + | + | WT | WT |
| 3138 | <i>E. cloacae</i> | Urine | Cardiovascular surgery | >16 | 12 | 32 | >256 | <8 | 8 | E | D1 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 248 | <i>E. cloacae</i> | Tracheal aspirate | ICU | <1 | 12 | >32 | >256 | <8 | <4 | E | D2 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 366 | <i>E. cloacae</i> | Tracheal aspirate | ICU | <1 | 6 | >32 | >256 | <8 | <4 | E | D2 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 437 | <i>E. cloacae</i> | Sputum | Internal medicine | <1 | 12 | >32 | >256 | <8 | <4 | E | D2 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 442 | <i>E. cloacae</i> | Tracheal aspirate | ICU | <1 | 12 | >32 | >256 | <8 | <4 | E | D2 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 533 | <i>E. cloacae</i> | Sputum | ICU | <1 | 16 | >32 | >256 | <8 | <4 | E | D2 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 812 | <i>E. cloacae</i> | Sputum | ICU | <1 | 16 | >32 | >256 | <8 | <4 | E | D3 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 930 | <i>E. cloacae</i> | Sputum | ICU | <1 | 16 | >32 | >256 | <8 | <4 | E | D3 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 1034 | <i>E. cloacae</i> | Urine | Internal medicine | <1 | 48 | >32 | >256 | <16 | <4 | E | D4 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 1137 | <i>E. cloacae</i> | Wound | Pneumology | <1 | 3 | >32 | >256 | <8 | <4 | E | D5 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 1619 | <i>E. cloacae</i> | Wound | Internal medicine | >16 | >256 | >32 | >256 | <8 | <4 | E | D6 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 2376 | <i>E. cloacae</i> | Abdominal drainage | Internal medicine | <1 | 8 | >32 | >256 | <8 | <4 | E | D7 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 3164 | <i>E. cloacae</i> | Urine | Gastroenterology | <1 | 12 | >32 | >256 | <8 | <4 | E | D8 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 469 | <i>E. cloacae</i> | Urine | Outpatient | <1 | 1.5 | >32 | >256 | <16 | <4 | E | D9 | <i>qnrS1</i> | + | + | - | S831 | S801 |
| 258 | <i>E. cloacae</i> | Urine | Outpatient | <1 | 256 | 0.032 | 2 | <16 | >8 | F | ND | <i>aac(6')</i> | + | + | - | ND | ND |
| 3157 | <i>E. cloacae</i> | Blood | Gastroenterology | <1 | 64 | 1 | 16 | <8 | 8 | G | ND | <i>aac(6')</i> | + | + | - | ND | ND |
| 2903 | <i>E. cloacae</i> | Wound | Oncology | 16 | >256 | 6 | >256 | <8 | 8 | H | ND | <i>aac(6')</i> | + | + | - | ND | ND |
| 1895 | <i>E. cloacae</i> | Blood | Urology | <1 | 32 | 1 | 32 | <8 | >8 | I | ND | <i>qnrB5</i> | - | - | - | ND | ND |
| 3338 | <i>E. cloacae</i> | Urine | Cardiology | >16 | 48 | 1.5 | >256 | <8 | >8 | J | ND | <i>qnrB2</i> | + | + | + | ND | ND |
| 3383 | <i>E. aerogenes</i> | Urine | Outpatient | >16 | >256 | >32 | >256 | <8 | <4 | NA | NA | <i>qnrB7</i> -like | ND | - | - | ND | ND |

^a Domicil.: domiciliary; ICU, intensive care unit; ND, not determined; NA, not applicable (one single isolate). Drug abbreviations: CAZ, ceftazidime; T-CL, ticarcillin-clavulanic acid; CIP, ciprofloxacin; NAL, nalidixic acid; AMK, amikacin; and TOB, tobramycin.

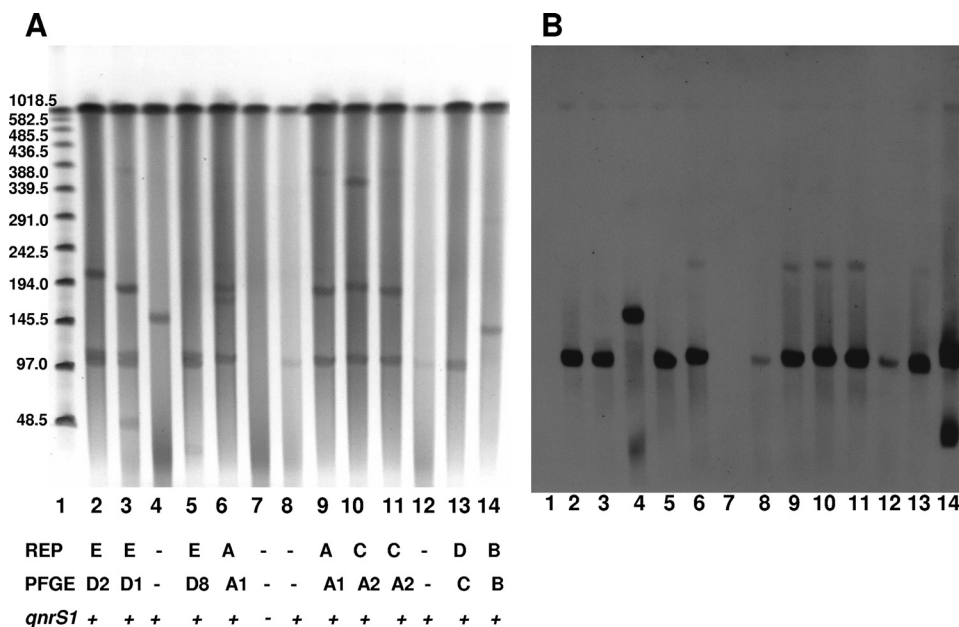


FIG. 1. Analysis of plasmid DNA from *E. cloacae* parental isolates and derived transconjugants by pulsed-field agarose gel electrophoresis (A) and Southern blotting (B). Lanes: 1, molecular marker; 2, *E. cloacae* 248; 3, *E. cloacae* 3138; 4, *qnrS1*-positive transconjugant from *E. cloacae* 3138; 5, *E. cloacae* 3164; 6, *E. cloacae* 2531; 7, transconjugant from *E. cloacae* 2531 lacking *qnrS1*; 8, *qnrS1*-positive transconjugant from *E. cloacae* 2531; 9, *E. cloacae* 3249; 10, *E. cloacae* 3049; 11, *E. cloacae* 3210; 12, transconjugant from *E. cloacae* 3210; 13, *E. cloacae* 1640; 14, *E. cloacae* 3714.

in *E. aerogenes*. The latter three isolates also were from Santander. *aac(6')-Ib-cr* was detected in three *E. cloacae* isolates (lacking *qnr* genes) from Santander, and an additional isolate of *E. cloacae* also carried the *aac(6')-Ib* gene (lacking the mutations involved in quinolone resistance). Neither *qepA* nor *qnrA* alleles were found in any of the tested isolates from Santander or from Seville.

The 22 organisms containing *qnrS* were cultured from tracheal aspirates ($n = 3$), sputa ($n = 4$), urine ($n = 5$), blood ($n = 1$), wound exudates ($n = 5$), catheter tips ($n = 2$), or abdominal drainages ($n = 2$). The patients from whom *E. cloacae* with *qnrS1* were cultured had been admitted to 12 different wards in two different buildings of our institution. Three patients were not hospitalized when the *qnrS1*-containing *E. cloacae* strain was isolated, including two patients on a home health care program and one outpatient. The three isolates with *aac(6')-Ib-cr* were cultured from urine ($n = 2$) and blood ($n = 1$), while the three *qnrB*-containing isolates were obtained from urine ($n = 1$), blood ($n = 1$), and wound exudates ($n = 1$).

Five REP-PCR patterns were observed among the 22 *E. cloacae* isolates containing *qnrS1*, including 4 type A, 1 type B, 2 type C, 1 type D, and 14 type E isolates (Table 2). Types A and C were considered possibly related by PFGE according to Tenover's criteria. The three *E. cloacae* isolates containing *aac(6')-Ib-cr* were clonally unrelated, as were the two isolates with *qnrB* genes. The latter two groups of organisms also were unrelated to any of the *qnrS1*-containing isolates.

Data of susceptibility to quinolones of *qnrS1*-containing *E. cloacae* isolates are presented in Table 2. A strong correlation between the phenotype of resistance to quinolone and the PFGE type was detected. All 14 *qnrS1*-positive isolates of REP-PCR pattern E were highly resistant to both nalidixic acid

(MIC > 256 $\mu\text{g/ml}$) and ciprofloxacin (MIC > 32 $\mu\text{g/ml}$) and contained mutations at the *gyrA* and *parC* genes that were responsible for the changes Ser83Ile (DNA-gyrase) and Ser80Ile (topoisomerase IV), respectively. In addition, one of these isolates also contained a *gyrA* mutation that was responsible for the change Asp87Asn.

The four isolates with REP-PCR pattern A were susceptible to nalidixic acid but presented decreased susceptibility to ciprofloxacin. They did not contain any change at the QRDR of *gyrA* and *parC* in comparison to the reported wild-type sequence of *E. cloacae*. In the single isolate of REP-PCR pattern D, a mutation in *gyrA* (causing a Ser83Phe change) was observed that correlated with resistance to nalidixic acid. The remaining three isolates (patterns B and C) lacked any mutation in the QRDR of *gyrA* and *parC* and were either susceptible or resistant to nalidixic acid and intermediate or resistant to ciprofloxacin (Table 2).

Transconjugants were obtained from isolates of REP-PCR types A, C, and E. The MICs of nalidixic acid and ciprofloxacin for the transconjugants ranged from 4 to 32 and 0.047 to 0.125 $\mu\text{g/ml}$, respectively.

Plasmid analysis showed that the studied isolates possessed one to three large plasmids, with sizes ranging from ca. 110 to ca. 350 kb. The *qnrS1* gene was associated to the 110-kb plasmid, although in one of three transconjugants studied, the gene was associated with a plasmid of larger size (ca. 150 kb). As shown in the Fig. 1, some of the transconjugants from *E. cloacae* 2531 (REP-PCR pattern A) lacked the plasmid observed in true transconjugants with *qnrS1* and did not contain the *qnrS1* gene. As these organisms were proven by PCR to contain *qnrS1* when first isolated in the selection plate of the conjugation assay, they may represent transconjugants that

have lost the plasmid upon subculture, but further studies were not done with these organisms.

All *qnrS1*-positive isolates, except the one with REP-PCR pattern B (see below), contained the *bla*_{LAP-1} gene. However, none of the transconjugants obtained from them was resistant to ticarcillin (MIC, 1.5 to 3 µg/ml).

Seventeen of these isolates contained the *intI1* gene, two of which also carried *ISCRI*. Among the 200 isolates tested, *intI1* was detected in 61 isolates from Santander (58 *E. cloacae* and 3 *E. aerogenes*) and in 10 isolates from Seville (8 *E. cloacae* and 2 *E. aerogenes*). *ISCRI* was detected only in *E. cloacae* (29 and 2 isolates in Santander and Seville, respectively).

DISCUSSION

Several previous reports indicate that PMQR genes are particularly common in *E. cloacae* and *E. aerogenes* (for a review, see reference 18). To expand our knowledge about the distribution of PMQR determinants, we screened a collection of *Enterobacter* spp. from two hospitals from northern and southern Spain.

There are no phenotypic markers that indicate the presence of PMQR genes in a concrete organism. Several of the primers we used (Table 1) were obtained from previous reports on PMQR genes in enterobacteria. Similarly, the other primers in that table will be helpful for a more complete study of genes directly or indirectly related to quinolone resistance in enterobacteria. As the number of *qnr* families is increasing, a multiplex PCR may be used in epidemiological surveys on PMQR genes. Previous studies indicate that strains containing PMQR genes usually express other mechanisms of resistance; for this reason, we included isolates that are resistant to any of the commonly used antimicrobial agents. On the other hand, as some *qnr* genes are located within a complex type 1 integron containing *intI1* and *ISCRI*, we additionally looked for these two genes.

Although *qnrA1* was the first PMQR gene discovered, several studies have indicated that other genes [particularly *qnrS1*, some *qnrB* alleles, and *aac(6')-Ib-cr*] are more common (reviewed in reference 18). This was the case in this study, where *qnrS1* has been the more frequent PMQR gene, being present in 19% of the *E. cloacae* isolates from Santander. In this area, *qnrB* alleles and the *aac(6')-Ib-cr* gene also were found, but less commonly. On the other hand, none of these genes was detected in the *Enterobacter* isolates from Seville, indicating that relevant differences in the prevalence of PMQR genes can occur within the same country. These results may represent differences in local resistance rates of *Enterobacter* (likely related to antibiotic use or infection control policies) in the two participating centers. Because the organisms included in this study were selected as being resistant to commonly used antimicrobial agents, we cannot yet make clear conclusions about the current prevalence of PMQR genes in the two regions considered in this study. We currently are studying enterobacteria isolated consecutively from clinical samples (without taking into consideration their resistance profile) to obtain information on this issue.

E. cloacae isolates containing *qnrS1* were susceptible, intermediate, or resistant to quinolones. Hata et al. reported (7) that a *qnrS*-containing *Shigella flexneri* strain has changes at the

QRDR of both *GyrA* (Ser83Leu) and *ParC* (Ser80Ile). In our case, the 14 isolates of REP-PCR pattern A presented changes in both DNA-gyrase and topoisomerase IV, which are known to be associated by themselves with quinolone resistance. This is similar to what has been described for organisms containing other PMQR genes, in which mutations in topoisomerase-coding genes also are frequent.

On the other hand, several *E. cloacae* isolates (of three different REP-PCR patterns; Table 2) lacking mutations at the QRDR of *gyrA* and *parC* were susceptible to nalidixic acid and presented decreased susceptibility or showed intermediate or low-level resistance to ciprofloxacin. This pattern of resistance is unexpected, as multiple studies previously have indicated that enterobacteria with decreased susceptibility or intermediate resistance to fluoroquinolones are highly resistant to nalidixic acid. Hakanen et al. (6) described *Salmonella enterica* from southeast Asia that is susceptible to nalidixic acid but exhibits reduced susceptibility to ciprofloxacin; new studies of this organism indicate that it contains the *qnrS1* gene (2, 21). We also have observed (3) that PMQR genes are found rather frequently in strains susceptible to nalidixic acid and with decreased susceptibility to fluoroquinolones. The various levels of resistance to ciprofloxacin in the studied isolates anticipates that other mechanisms of resistance (such as mutations outside the QRDR of *gyrA/parC*, mutations in *gyrB/parE*, active efflux, or decreased permeability) or differences in gene expression may be involved in individual isolates.

In addition to *qnrS1*, two *E. cloacae* isolates contained *qnrB* alleles (*qnrB5* and *qnrB2*), and three other isolates contained the gene coding for the fluoroquinolone-modifying acetyltransferase *Aac(6')-Ib-cr*. One strain of *E. aerogenes* expressed a *qnrB7*-like gene that coded for a protein differing from *QnrB7* by (at least) a single change (Asp197Gly). Studies for determining the complete sequence of this new variant are in progress.

As expected from our inclusion criteria, *E. cloacae* strains containing *qnrS1* presented different phenotypes of susceptibility to antimicrobial agents other than quinolones. All *intI1*-containing strains were resistant to cotrimoxazole, and some strains were resistant to oxyminocephalosporins (10 isolates were presumably AmpC hyperproducers, and 2 isolates presented a phenotype compatible with extended-spectrum β-lactamase production). The LAP-1 enzyme was detected in all except one isolate (that with REP-PCR pattern B). The association of *qnrS1* and *bla*_{LAP-1} already has been described (27). Twenty-one of the 22 *E. cloacae* isolates with *qnrS1* were resistant to ticarcillin, of which 13 isolates were susceptible to ticarcillin-clavulanic acid, a phenotype compatible with the presence of this narrow-spectrum class A β-lactamase (26). Resistance to ticarcillin-clavulanate in the remaining nine isolates should be related to other unexplored mechanisms.

The fact that *qnrS1* has been found in several clonally unrelated isolates of *E. cloacae* is epidemiologically relevant and indicates the potential for the spreading of this gene. Although preliminary information suggests that the plasmid containing *qnrS1* in our isolates has disseminated to different *E. cloacae* strains, it also would be possible that the plasmid was acquired by the different strains from another organism. Two plasmids containing *qnrS1*, pTPqnrS-1a (10 kb) and pK245 (98 kb), have been completely sequenced (5, 15). The first one presents a

region highly homologous to ColE plasmid pEC278 (from *E. coli* [accession no. AY589571]) and to the *qnrS1*-containing plasmid pINF5 (from *Salmonella enterica* serovar Infantis) (14). The region containing *qnrS1* in plasmid pK245 is >99% identical to those of plasmids pAH0376 from *S. flexneri* (6) and pINF5; pK245 also contains genes coding for SHV-2, for resistance to aminoglycosides (*aacC2*, *strA*, and *strB*), chloramphenicol (*catA2*), sulfonamides (*sul2*), tetracycline (*tetD*), and trimethoprim (*dfrA14*, within a type I integron), as well as multiple insertion sequence elements that may facilitate the dissemination of these resistance determinants. Many of our *E. cloacae* isolates with *qnrS1* contain type 1 integrons, but in the previously characterized *S. flexneri* strain containing *qnrS1* (7), this gene was not contained in a type 1 integron but was in proximity to a Tn3-like structure downstream of complete or truncated *ISEc1* (26). Additional studies are planned to characterize the involved plasmid(s) and to define the genetic background of *qnrS1* in our isolates.

It also seems relevant to us that three isolates containing *qnrS1* came from nonhospitalized patients. At this moment it is not possible to determine if these organisms had originated in the hospital and disseminated into the community or whether they came from the community and were imported into the hospital. In any case, this finding reinforces the idea of the global relationship among different compartments of the health care system and clearly indicates a need for additional studies looking for potential niches of bacteria containing PMQR genes.

ACKNOWLEDGMENTS

pBC-H2.6, which was deposited by M. Hata, was purchased from the DNA Bank, RIKEN BioResource Center (Tsukuba, Japan), with the support of the National Bio-Resources Project of Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT).

We thank George A. Jacoby, Patrice Nordmann, Luisa Peixe, and Marc Gallimand for providing control strains.

This work was supported by the Ministerio de Sanidad y Consumo, the Instituto de Salud Carlos III, Spain (projects PI050690 to L.M.M. and PI060580 to A.P.), the Consejería de Innovación Ciencia y Empresa, Junta de Andalucía, Spain (P07-CTS-02908), and the Spanish Network for Research in Infectious Diseases, Spain (REIPI RD06/0008).

REFERENCES

- Barton, B. M., G. P. Harding, and A. J. Zuccarelli. 1995. A general method for detecting and sizing large plasmids. *Anal. Biochem.* **226**:235–240.
- Caddick, J. M., M. Lindgren, M. A. Webber, P. Kotilainen, A. Siitonen, A. J. Hakanen, and L. J. V. Piddock. 2008. Mechanisms of resistance in nontyphoidal *Salmonella enterica* exhibiting a novel quinolone resistance phenotype, abstr. P-1536. Abstr. 18th Eur. Cong. Clin. Microbiol. Infect. Dis., Barcelona, Spain.
- Cano, M. E., J. Calvo, J. Agüero, J. M. Rodríguez-Martínez, A. Pascual, and L. Martínez-Martínez. 2007. Plasmid-mediated quinolone resistance among enterobacteria with reduced susceptibility or resistant to ciprofloxacin but susceptible to nalidixic acid. Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother. Chicago, IL.
- Cavaco, L. M., H. Hasman, S. Xia, and F. M. Aarestrup. 2009. *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar Kentucky and Bovismorbificans strains of human origin. *Antimicrob. Agents Chemother.* **53**:603–608.
- Chen, Y. T., H. Y. Shu, L. H. Li, T. L. Liao, K. M. Wu, Y. R. Shiau, J. J. Yan, I. J. Su, S. F. Tsai, and T. L. Lauderdale. 2006. Complete nucleotide sequence of pK245, a 98-kilobase plasmid conferring quinolone resistance and extended-spectrum-beta-lactamase activity in a clinical *Klebsiella pneumoniae* isolate. *Antimicrob. Agents Chemother.* **50**:3861–3866.
- Hakanen, A. J., M. Lindgren, P. Huovinen, J. Jalava, A. Siitonen, and P. Kotilainen. 2005. New quinolone resistance phenomenon in *Salmonella enterica*: nalidixic acid-susceptible isolates with reduced fluoroquinolone susceptibility. *J. Clin. Microbiol.* **43**:5775–5778.
- Hata, M., M. Suzuki, Matsumoto, M. Takahashi, K. Sato, S. Ibe, and K. Sakae. 2005. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. *Antimicrob. Agents Chemother.* **49**:801–803.
- Hooper, D. C. 2001. Emerging mechanisms of fluoroquinolone resistance. *Emerg. Infect. Dis.* **7**:337–341.
- Jacoby, G., V. Cattoir, D. Hooper, L. Martínez-Martínez, P. Nordmann, A. Pascual, L. Poirel, and M. Wang. 2008. *qnr* gene nomenclature. *Antimicrob. Agents Chemother.* **52**:2297–2299.
- Jacoby, G. A., N. Chow, and K. B. Waites. 2003. Prevalence of plasmid-mediated quinolone resistance. *Antimicrob. Agents Chemother.* **47**:559–562.
- Jacoby, G. A., K. E. Walsh, D. M. Mills, V. J. Walker, H. Oh, A. Robicsek, and D. C. Hooper. 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. *Antimicrob. Agents Chemother.* **50**:1178–1182.
- Jun, J., Y. Kwak, S. Kim, J. Lee, S. Choi, J. Jeong, Y. Kim, and J. Woo. 2005. Prevalence of plasmid-mediated quinolone resistance in clinical isolates of *Enterobacter cloacae* from Korea, abstr. C2-787. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., Washington, DC.
- Karisik, E., M. J. Ellington, R. Pike, R. E. Warren, D. M. Livermore, and N. Woodford. 2006. Wide occurrence of the fluoroquinolone-modifying acetyltransferase gene *aac(6′)-Ib-cr* in CTX-M-positive *Escherichia coli* from the United Kingdom. 2513. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., Washington, DC.
- Kehrenberg, C., S. Friederichs, A. de Jong, G. B. Michael, and S. Schwarz. 2006. Identification of the plasmid-borne quinolone resistance gene *qnrS* in *Salmonella enterica* serovar Infantis. *J. Antimicrob. Chemother.* **58**:18–22.
- Kehrenberg, C., K. L. Hopkins, E. J. Threlfall, and S. Schwarz. 2007. Complete nucleotide sequence of a small *qnrS1*-carrying plasmid from *Salmonella enterica* subsp. *enterica* Typhimurium DT193. *J. Antimicrob. Chemother.* **60**:903–905.
- Lu, X., X. Ma, and R. Yu. 2007. Study of *qnrB* among Cephalosporin-Resistant *Enterobacteriaceae* species in West China. Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother., Chicago, IL.
- Machado, E., T. M. Coque, R. Cantón, F. Baquero, J. C. Sousa, L. Peixe, et al. 2006. Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing *Enterobacteriaceae* strains containing the *aac(6′)-Ib-cr* gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. *Antimicrob. Agents Chemother.* **50**:3220–3221.
- Martínez-Martínez, L., M. E. Cano, J. M. Rodríguez-Martínez, J. Calvo, and A. Pascual. 2008. Plasmid-mediated quinolone resistance. *Expert. Rev. Anti-Infect. Ther.* **6**:685–711.
- Martínez-Martínez, L., A. Pascual, I. García, J. Tran, and G. A. Jacoby. 2003. Interaction of plasmid and host quinolone resistance. *J. Antimicrob. Chemother.* **51**:1037–1039.
- Martínez-Martínez, L. A. Pascual, and G. A. Jacoby. 1998. Quinolone resistance from a transferable plasmid. *Lancet* **351**:797–799.
- Murray, A., H. Mather, J. E. Coia, and D. J. Brown. 2008. Plasmid-mediated quinolone resistance in nalidixic-acid-susceptible strains of *Salmonella enterica* isolated in Scotland. *J. Antimicrob. Chemother.* **62**:1153–1155.
- Paauw, A. A. C. Fluit, J. Verhoef, and M. A. Leverstein-van Hall. 2006. *Enterobacter cloacae* outbreak and emergence of quinolone resistance gene in Dutch hospital. *Emerg. Infect. Dis.* **12**:807–812.
- Park, C. H., A. Robicsek, G. A. Jacoby, D. Sahn, and D. C. Hooper. 2006. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob. Agents Chemother.* **50**:3953–3955.
- Paterson, D. L. 2006. Resistance in gram-negative bacteria: enterobacteriaceae. *Am. J. Med.* **119**(Suppl. 1):S20–S28.
- Périchon, B., P. Courvalin, and M. Gallimand. 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. *Antimicrob. Agents Chemother.* **51**:2464–2469.
- Poirel, L., V. Cattoir, A. Soares, C. J. Soussy, and P. Nordmann. 2007. Novel Ambler class A beta-lactamase LAP-1 and its association with the plasmid-mediated quinolone resistance determinant QnrS1. *Antimicrob. Agents Chemother.* **51**:631–637.
- Poirel, L., C. Leviandier, and P. Nordmann. 2006. Prevalence and genetic analysis of plasmid-mediated quinolone resistance determinants QnrA and QnrS in *Enterobacteriaceae* isolates from a French university hospital. *Antimicrob. Agents Chemother.* **50**:3992–3997.
- Poirel, L., J. D. Pitout, L. Calvo, J. M. Rodríguez-Martínez, D. Church, and P. Nordmann. 2006. In vivo selection of fluoroquinolone-resistant *Escherichia coli* isolates expressing plasmid-mediated quinolone resistance and expanded-spectrum beta-lactamase. *Antimicrob. Agents Chemother.* **50**:1525–1527.
- Robicsek, A., G. A. Jacoby, and D. C. Hooper. 2006. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect. Dis.* **6**:629–640.
- Robicsek, A., D. F. Sahn, J. Strahilevitz, G. A. Jacoby, and D. C. Hooper. 2005. Broader distribution of plasmid-mediated quinolone resistance in the United States. *Antimicrob. Agents Chemother.* **49**:3001–3003.
- Robicsek, A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. H. Park, K. Bush, and D. C. Hooper. 2006. Fluoroquinolone-modifying enzyme:

- a new adaptation of a common aminoglycoside acetyltransferase. *Nat. Med.* **12**:83–88.
32. **Robicsek, A., J. Strahilevitz, D. F. Sahn, G. A. Jacoby, and D. C. Hooper.** 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. *Antimicrob. Agents Chemother.* **50**:2872–2874.
 33. **Ruiz, J.** 2003. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *J. Antimicrob. Chemother.* **51**:1109–1117.
 34. **Sabaté, M., F. Navarro, E. Miró, S. Campoy, B. Mirelis, J. Barbé, and G. Prats.** 2002. Novel complex sul1-type integron in *Escherichia coli* carrying *bla*_{CTX-M-9}. *Antimicrob. Agents Chemother.* **46**:2656–2661.
 35. **Strahilevitz, J., D. Engelstein, A. Adler, V. Temper, A. E. Moses, C. Block, and A. Robicsek.** 2007. Changes in *qnr* prevalence and fluoroquinolone resistance in clinical isolates of *Klebsiella pneumoniae* and *Enterobacter* spp. collected from 1990 to 2005. *Antimicrob. Agents Chemother.* **51**:3001–3003.
 36. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**:2233–2239.
 37. **Toleman, M. A., P. M. Bennett, and T. R. Walsh.** 2006. ISCR elements: novel gene-capturing systems of the 21st century? *Microbiol. Mol. Biol. Rev.* **70**:296–316.
 38. **Tran, J. H., G. A. Jacoby, and D. C. Hooper.** 2005. Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. *Antimicrob. Agents Chemother.* **49**:118–125.
 39. **Tran, J. H., and G. A. Jacoby.** 2002. Mechanism of plasmid-mediated quinolone resistance. *Proc. Natl. Acad. Sci. USA* **99**:5638–5642.
 40. **Vila, J., M. A. Marcos, and M. T. Jiménez de Anta.** 1996. A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus-A. baumannii* complex. *J. Med. Microbiol.* **44**:482–489.
 41. **Vila, J., J. Ruiz, P. Goñi, M. A. Marcos, and M. T. Jiménez de Anta.** 1995. Mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **39**:1201–1203.
 42. **Wang, C. X., P. Q. Cai, Y. Guo, Z. M. Huang, and Z. H. Mi.** 2006. Emerging plasmid-mediated quinolone resistance associated with the *qnrA* gene in *Enterobacter cloacae* clinical isolates in China. *J. Hosp. Infect.* **63**:349–350.
 43. **Wang, M., D. F. Sahn, G. A. Jacoby, and D. C. Hooper.** 2004. Emerging plasmid-mediated quinolone resistance associated with the *qnr* gene in *Klebsiella pneumoniae* clinical isolates in the United States. *Antimicrob. Agents Chemother.* **48**:1295–1299.
 44. **Wang, M. H., X. Xu, S. Wu, D. Zhu, and M. Wang.** 2008. A new plasmid-mediated gene for quinolone resistance, *qnrC*, abstr. O207. Abstr. 18th Eur. Cong. Clin. Microbiol. Infect. Dis., Barcelona, Spain.