

Prevalence of Plasmid-Mediated Quinolone Resistance Genes in Clinical Enterobacteria from Argentina

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This first nationwide study was conducted to analyze the prevalence of plasmid-mediated quinolone resistance (PMQR) genes in phenotypically unselected (consecutive) clinical enterobacteria. We studied 1,058 isolates that had been consecutively collected in 66 hospitals of the WHONET-Argentina Resistance Surveillance Network. Overall, 26% of isolates were nonsusceptible to at least one of the three quinolones tested (nalidixic acid, ciprofloxacin, and levofloxacin). The overall prevalence of PMQR genes was 8.1% (4.6% for *aac(6′)-Ib-cr*; 3.9% for *qnr* genes; and 0.4% for *oqxA* and *oqxB*, which were not previously reported in enterobacteria other than *Klebsiella* spp. from Argentina). The PMQR prevalence was highly variable among the enterobacterial species or when the different genes were considered. The prevalent PMQR genes were located in class 1 integrons [*qnrB2*, *qnrB10*, and *aac(6′)-Ib-cr*]; in the ColE1-type plasmid pPAB19-1 or Tn2012-like transposons (*qnrB19*); and in Tn6238 or bracketed by IS26 and *bla*_{OXA-1} [*aac(6′)-Ib-cr*]. The mutations associated with quinolone resistance that were located in the quinolone resistance-determining region (QRDR mutations) of *gyrA*, *parC*, and *gyrB* were also investigated. The occurrence of QRDR mutations was significantly associated with the presence of PMQR genes: At least one QRDR mutation was present in 82% of the PMQR-harboring isolates but in only 23% of those without PMQR genes ($p < 0.0001$, Fisher's Test). To the best of our knowledge, this is the first report on the prevalence of PMQR genes in consecutive clinical enterobacteria where all the genes currently known have been screened.

Keywords: quinolone resistance, PMQR, S83A, D87G, D87Y

Introduction

THE MAJOR MECHANISM of quinolone resistance is based on mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding the targets of these drugs, namely the type II topoisomerases DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*).¹ The acquisition of resistance is a stepwise process, and several mutations are needed to produce high-level resistance to all quinolones.² In clinical enterobacteria, the main type II topoisomerase-encoding genes involved in the development of resistance are, by far, *gyrA* and, to a lesser extent, *parC*.^{2,3} The contribution of mutations in *gyrB* seems to be

limited to a few species,⁴⁻⁶ whereas *parE* mutations are very uncommon and their role appears to be irrelevant.^{2,3}

Besides the chromosomal-driven mechanism described above, three kinds of plasmid-mediated quinolone resistance (PMQR) mechanisms have been described. These constitute the second most relevant group of quinolone resistance mechanisms and are based on: (i) Qnr proteins, which protect the quinolone targets; (ii) the enzyme *Aac(6′)-Ib-cr*, which acetylates aminoglycosides and also ciprofloxacin and norfloxacin; and (iii) efflux pumps, which either specifically (*QepA*) or unspecifically (*OqxAB*) decrease the intracellular concentration of quinolones.⁷ Five classes of *qnr* genes are currently known, namely *qnrA*, *qnrB*, *qnrS*, *qnrC*, and *qnrD*, with the

Preliminary data were presented in abstract form at the 52nd, 53rd, and 54th Interscience Conferences on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, 2012, 2013, and 2014, respectively.

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first three including several alleles (www.lahey.org/qnrStudies). PMQR genes and QRDR mutations are often found together in enterobacteria with high-level quinolone resistance, highlighting the interplay and evolution of both mechanisms in resistant clinical isolates.⁷

In the previous decade, many studies on PMQR prevalence were conducted in clinical enterobacteria but the vast majority of them focused on isolates with specific resistance phenotypes, such as resistance/decreased susceptibility to quinolones; resistance to aminoglycosides or expanded-spectrum cephalosporins (ESC); and production of extended-spectrum β -lactamases, which are commonly associated with PMQR genes. These selection criteria greatly overestimate the prevalence of these genes in unselected populations of clinical enterobacteria.⁷ Only few PMQR surveys were based on phenotypically unselected isolates, that is, those that were consecutively isolated from clinical specimens in the microbiology laboratory of the hospital.^{8–22} Most of these studies analyzed a single^{8–15} or a few^{16–20} of the enterobacterial species found during the study period, whereas only two of them included all the species found.^{21,22} In regard to Argentina, only two surveys were reported and they addressed PMQR prevalence in clinical enterobacteria with resistance/decreased susceptibility to quinolones²³ or ESC resistance.²⁴

Here, we report a large nationwide study based on a point prevalence collection of 1058 unselected clinical enterobacteria that we conducted to estimate the prevalence of all the PMQR genes currently known.

Materials and Methods

Bacterial isolates and antimicrobial susceptibility testing

We used a collection of 1,058 unselected (consecutive), nonduplicated enterobacterial isolates. These strains were specifically collected for this prevalence study during the same 5 consecutive days in 2007, in the 66 hospitals of the WHONET-Argentina Resistance Surveillance Network, which were located in Buenos Aires City, and the 23 provinces of Argentina (Supplementary Fig. S1). The bacterial collection comprised 673 *Escherichia coli*; 133 *Klebsiella pneumoniae*; 10 *Klebsiella oxytoca*; 66 *Shigella* spp.; 58 *Proteus mirabilis*; 7 *Proteus vulgaris*; 38 *Enterobacter cloacae*; 14 *Enterobacter aerogenes*; 18 *Serratia marcescens*; 1 *Serratia liquefaciens*; 8 *Citrobacter freundii*; 7 *Citrobacter koseri*; 12 *Morganella morganii*; 9 *Salmonella* spp., and 4 *Providencia stuartii* (see epidemiological data in Supplementary Table S1). The 81 isolates of the tribe *Proteeae* (*Proteus*, *Morganella*, and *Providencia*) were analyzed in a preliminary paper,⁶ but we included in this work some results reported therein to facilitate the analysis of the complete bacterial collection.

Antimicrobial susceptibility tests were performed by disk diffusion (DD) and agar dilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).²⁵ DD was used to determine the susceptibility profile to three quinolones [nalidixic acid (NAL), ciprofloxacin, and levofloxacin] and 19 antibiotics of other families (ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cephalothin, cefoxitin, cefotaxime, ceftazidime, ertapenem, imipenem, meropenem, amikacin, gentamicin, kanamycin, tobramycin,

chloramphenicol, colistin, tigecycline, trimethoprim, and trimethoprim-sulfamethoxazole). Agar dilution was used to determine the MICs of NAL, ciprofloxacin, and levofloxacin.

PCR and DNA sequencing

All the primers used for PCR and DNA sequencing are listed in Supplementary Table S2. DNA sequencing was performed by using BigDye terminators with an ABI 3500 Genetic Analyzer (Applied Biosystems/Perkin Elmer, Foster City, CA). Sequences were edited and analyzed with BioEdit, v7.2.5 (www.mbio.ncsu.edu/bioedit/bioedit.html),²⁶ ClustalX2, v2.1 (www.clustal.org/clustal2),²⁷ and the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

Detection of PMQR genes

The presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *oqxA*, and *oqxB* (only in *oqxA*-positive isolates) was tested by PCR as described.²³ The different *qnrB* or *qnrS* alleles were identified by PCR and DNA sequencing of complete genes. The presence of *aac(6′)-Ib-cr* and/or *aac(6′)-Ib* was only determined in the subset of isolates that were non-susceptible to kanamycin (DD inhibition zones ≤ 17 mm) by using separate allele-specific PCRs (ASPCRs) with two primer sets directed against each variant.²³

Analysis of the QRDRs of *gyrA*, *parC*, and *gyrB*

Only the mutations located in QRDRs that were previously associated with quinolone resistance^{3,28} were considered (named onward as QRDR mutations). The QRDR of *gyrA* was analyzed by PCR and DNA sequencing as described.²³ The QRDR of *parC* was additionally analyzed²³ in those isolates where at least one QRDR mutation was detected in *gyrA*. The QRDR of *gyrB* from some isolates was also analyzed as described.⁶ To determine the presence of QRDR mutations, the obtained *gyrA*, *parC*, or *gyrB* sequences were compared with reference sequences (Supplementary Table S2).

Genetic environments of PMQR genes

The genetic environments of *qnrB2* and *qnrB10* were analyzed by PCR cartography as described.²³ The *qnrB19*-harboring plasmid pPAB19-1 was identified by PCR with intragenic divergent primers and restriction fragment length polymorphisms with *ApoI* and *RsaI* as described.²³ The *ISEcp1C*-based genetic support of *qnrB19*²⁹ was identified by PCR and DNA sequencing (Supplementary Table S2).

The genetic environments of *aac(6′)-Ib-cr* were analyzed by PCR reactions that were designed to amplify the 5′- and 3′-genetic surroundings that corresponded to the most common locations of this gene, namely class 1 integrons, Tn6238, and those bracketed by IS26 and *bla*_{OXA-1}.^{30,31} Briefly, the 5′-reactions were done by using alternative forward primers that were directed against *intI1*, the *tnpA* of Tn6238, or the *tnpA* of IS26, and the reverse primer used in the ASPCR for *aac(6′)-Ib-cr*; whereas the 3′-reactions were performed with the forward primer used in the ASPCR for *aac(6′)-Ib-cr* and alternative reverse primers directed against the 3′-conserved segment of class 1 integrons, *bla*_{OXA-9} or *bla*_{OXA-1}. All the obtained amplicons were sequenced.

Results and Discussion

Analysis of quinolone susceptibility

Overall, 26% (279/1058) of the isolates were non-susceptible (resistant plus intermediate CLSI categories²⁵) to at least one of the three quinolones tested. The distributions of quinolone susceptibility according to species are summarized in Figure 1. Excluding the particular cases of *Shigella* spp. and *P. stuartii*, for which almost all of the isolates were fully susceptible or fully resistant to quinolones, respectively, the levels of nonsusceptibility ranged from 11% (*Salmonella* spp.) to 42% (*M. morgani*), for NAL; from 7.1% (*E. aerogenes*) to 37% (*E. cloacae*), for ciprofloxacin; and from 0% (*K. oxytoca*) to 36% (*P. mirabilis*), for levofloxacin (Fig. 1).

We also examined the level of decreased ciprofloxacin susceptibility (DCS, defined as a ciprofloxacin MIC of 0.12–1 µg/ml) in all the species except *Salmonella* spp., for which DCS is currently included in the CLSI categories of non-susceptibility.²⁵ DCS was observed in 64 of 1,049 (6.1%) isolates (*Salmonella* was excluded), of which 48 (75%) were nonsusceptible to NAL. Interestingly, *S. marcescens* showed the highest DCS level (44%) observed in our collection, being two-times higher than the corresponding percentage of ciprofloxacin nonsusceptibility (Fig. 1). This might be due to the presence of the chromosomal *qnr* homolog (*smaqnr*) described in *S. marcescens*.³²

In the *E. coli* subset of the collection, 499 isolates were fully susceptible to NAL (MIC ≤4 µg/ml) and also to ciprofloxacin and levofloxacin; 2 isolates displayed susceptibility to NAL (MIC of 8–16 µg/ml) with DCS (NAL^S-DCS);

7 isolates showed intermediate or low resistance to NAL [(NAL^{I-LR}), MIC of 32–64 µg/ml, DD inhibition zones of 9–17 mm], and 165 isolates were highly resistant to NAL (MIC ≥128 µg/ml, DD inhibition zones ≤8 mm). To reduce the number of isolates to be analyzed, a sample was taken from the *E. coli* subset as follows: 10% of the isolates fully susceptible to quinolones (50 out of 499) and 25% of those highly resistant to NAL (41 out of 165) were randomly selected, whereas all the 2 NAL^S-DCS and the 7 NAL^{I-LR} isolates were included (Supplementary Fig. S2). The obtained sample of 100 isolates was used in the subsequent analyses.

Occurrence and prevalence of PMQR genes

The PMQR genes were found in 29 out of 66 hospitals, located in Buenos Aires City and 15 of the 23 provinces of Argentina. The *qnr* genes were detected in 30 isolates: *qnrB2*, 9 isolates; *qnrB10* and *qnrB19*, 7 isolates each; *qnrB9*, 2 isolates; *qnrB28* (first report in Argentina) and *qnrB6*, 1 isolate each; and *qnrS1*, 3 isolates. In addition, we had previously found *qnrD1* in 1 *P. mirabilis* and 1 *P. vulgaris*.⁶

Thirty isolates harbored *aac(6′)-Ib-cr*. In 22 of them, it was the unique PMQR gene (14 *Klebsiella* spp., 6 *E. coli*, and 2 *E. cloacae*). The remaining eight isolates were the only ones that harbored more than one PMQR gene and harbored *aac(6′)-Ib-cr* combined with *qnrB10* (2 *K. pneumoniae*, 1 *E. cloacae*, and 1 *C. freundii*), *qnrB2* (3 *K. pneumoniae*), or *qnrS1* (1 *K. pneumoniae*). *qnrB2* and *qnrB10* were significantly associated with *aac(6′)-Ib-cr* ($p=0.014$ and $p<0.001$, Fisher's Test, respectively), whereas *qnrS1* was not ($p=0.175$, Fisher's Test).

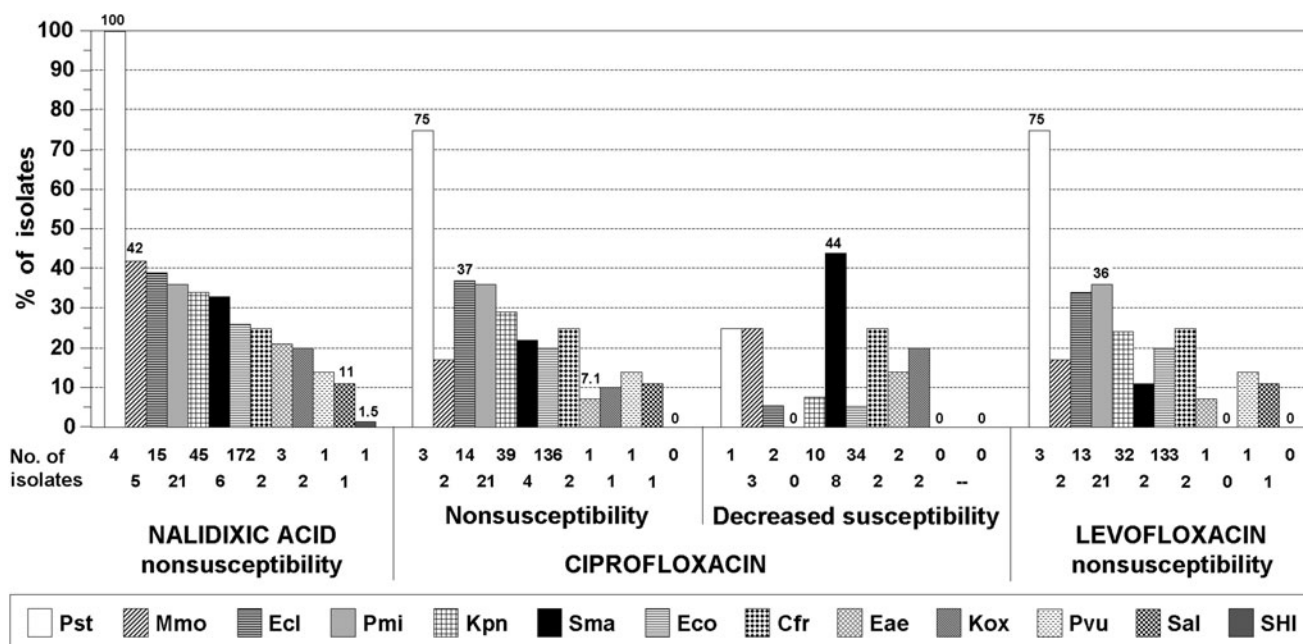


FIG. 1. Quinolone susceptibility in the enterobacterial collection. For each indicated species (Pst, *Providencia stuartii*; Mmo, *Morganella morgani*; Ecl, *Enterobacter cloacae*; Pmi, *Proteus mirabilis*; Kpn, *Klebsiella pneumoniae*; Sma, *Serratia marcescens*; Eco, *Escherichia coli*; Cfr, *Citrobacter freundii*; Eae, *Enterobacter aerogenes*; Kox, *Klebsiella oxytoca*; Pvu, *Proteus vulgaris*; Sal, *Salmonella* spp.; and SHI, *Shigella* spp.), the percentages of nonsusceptibility (resistant plus intermediate categories) and decreased ciprofloxacin susceptibility (MIC of 0.12–1 µg/ml, *Salmonella* is excluded) are shown (highest and lowest values are indicated above the bars). Data for the *Proteaceae* species are from our preliminary work.⁶ All the *Citrobacter koseri* and the unique isolate of *Serratia liquefaciens* were susceptible to all quinolones.

The *qepA* gene was not found, and *oqxA* and *oqxB* were detected in 1 *E. coli* (isolate Q4160 from a urine specimen) and in 92% of the *Klebsiella* sp. isolates (93% of *K. pneumoniae*, 90% of *K. oxytoca*). However, it was reported that in *Klebsiella* spp. these genes were mostly located on the chromosome and their presence did not correlate with the ciprofloxacin MIC, probably due to different expression levels of the *oqxAB* operon.^{23,33–35} Consistently, considering the *Klebsiella* sp. isolates of this work that did not harbor other PMQR genes nor QRDR mutations in *gyrA* or *parC*, the ciprofloxacin MICs of the isolates with and without *oqxA* and *oqxB* were almost identical (MIC₅₀ of 0.03 µg/ml, range 0.008–0.06 µg/ml and MIC₅₀ of 0.03 µg/ml, range 0.015–0.06 µg/ml, respectively). Therefore, the subsequent analyses of PMQR genes were carried out regardless of the presence of *oqxA* and *oqxB* in *Klebsiella* spp.

E. coli Q4160 showed intermediate resistance to chloramphenicol and trimethoprim, as expected from the expression of OqxAB.³⁶ The sequences of the amplified fragments of *oqxA* and *oqxB* from Q4160 were identical to those of pOLA52, the representative *oqxA-loxB*-harboring plasmid of *E. coli*.⁷ This is the first report of *oqxA* and *oqxB* in enterobacteria other than *Klebsiella* spp. from Argentina. The mobilization of *oqxA* and *oqxB* was evaluated by bi-

parental conjugation of Q4160 and *E. coli* J53 azide-resistant, as described²⁵ (150 µg/ml of sodium azide and 8 µg/ml of chloramphenicol were used for transconjugant selection). Although several attempts were made, no transconjugants were obtained.

Interestingly, 45 of the 55 PMQR-harboring isolates showed high resistance to NAL with variable ciprofloxacin susceptibility, from DCS to high resistance (Table 1). In particular, all the 10 PMQR-harboring *E. coli* were included in the sample subgroup that was highly resistant to NAL (41 isolates), which was the 25% of the corresponding one (165 isolates) in the complete *E. coli* subset (see section Analysis of Quinolone Susceptibility). Therefore, to calculate prevalence, the number of *E. coli* isolates in the enterobacterial collection that contained a given PMQR gene was estimated by multiplying by four the number of isolates with this gene in the sample set. This resulted in 85 out of 1,058 isolates with PMQR genes giving an overall prevalence of 8.1%, with disaggregated values of 4.6% for *aac(6′)-Ib-cr*, 3.9% for the *qnr* genes (mostly *qnrB* alleles, 3.1%), and 0.4% for *oqxA/oqxB*. However, the PMQR prevalence was highly variable among the species of the collection and ranged from 3.1% in *Proteus* spp.⁶ to 22% in *Klebsiella* spp., with an outlier value of 63% for *C. freundii* (Fig. 2A). The

TABLE 1. QUINOLONE SUSCEPTIBILITY OF PMQR-HARBORING ISOLATES

Isolates	NAL		Ciprofloxacin		Levofloxacin		PMQR genes ^a
	MIC (µg/ml)	DD (mm)	MIC (µg/ml)	DD (mm)	MIC (µg/ml)	DD (mm)	
With QRDR mutations (n=45) ^b							
20 <i>Klebsiella</i> spp.	≥256	6	64–≥128	6	8–≥128	6–11	<i>qnrB2</i> , <i>qnrB10</i> , <i>qnrS1</i> all alone, or combined with <i>aac(6′)-Ib-cr</i> ; <i>qnrB19</i> ; <i>aac(6′)-Ib-cr</i>
7 <i>Escherichia coli</i>	≥256	6	64–≥128	6	8–≥128	6–10	<i>qnrS1</i> ; <i>aac(6′)-Ib-cr</i>
3 <i>Enterobacter cloacae</i>	≥256	6	≥128	6	8–64	6–10	<i>qnrB10</i> ; <i>aac(6′)-Ib-cr</i>
1 <i>Citrobacter freundii</i>	≥256	6	32	6	16	6	<i>qnrB10</i> combined with <i>aac(6′)-Ib-cr</i>
4 <i>Klebsiella</i> spp.	≥256	6	4–16	9–13	1–2	17–20	<i>aac(6′)-Ib-cr</i>
2 <i>E. coli</i>	≥256	6	4–8	14–15	4–8	14	<i>qnrB19</i>
2 <i>E. cloacae</i>	≥256	6	2–8	14–16	1–2	18–21	<i>qnrB10</i> combined with <i>aac(6′)-Ib-cr</i> ; <i>qnrB2</i>
2 <i>Proteus</i> spp. ^c	≥256	6	4	12–16	4–8	9–16	<i>qnrD1</i>
1 <i>C. freundii</i>	≥256	6	2	17	4	16	<i>qnrB9</i>
2 <i>Klebsiella</i> spp.	≥256	6	1	18–19	0.5–1	24	<i>aac(6′)-Ib-cr</i>
1 <i>E. coli</i>	≥256	6	1	24	2	21	<i>oqxA</i> and <i>oqxB</i>
Without QRDR mutations (n=10) ^b							
3 <i>Klebsiella</i> spp.	16–32	12–16	0.5–2	19–23	0.5–1	20–25	<i>qnrB2</i> alone, or combined with <i>aac(6′)-Ib-cr</i> ; <i>qnrB19</i>
1 <i>Salmonella</i> spp.	16	13	0.25	26	1	24	<i>qnrB19</i>
3 <i>Klebsiella</i> spp.	4–8	19–25	0.12–2	20–28	0.06–0.5	25–35	<i>qnrB10</i> combined with <i>aac(6′)-Ib-cr</i> ; <i>qnrB2</i> ; <i>aac(6′)-Ib-cr</i>
2 <i>C. freundii</i>	4–8	23–25	0.12	27–28	0.25	28–31	<i>qnrB6</i> ; <i>qnrB9</i>
1 <i>C. freundii</i>	8	19	0.06	27	0.25	27	<i>qnrB28</i>

The ranges of MIC and DD for each group are shown. PMQR genes were not detected in *Enterobacter aerogenes*, *Citrobacter koseri*, *Serratia* spp., and *Shigella* spp., nor in *Morganella morganii* and *Providencia stuartii*.⁶

^aThe different PMQR genes and combinations of them found in each group are indicated.

^bNumber of isolates with or without at least one QRDR mutation in *gyrA*.

^c*Proteus mirabilis* and *Proteus vulgaris*, data from our preliminary work.⁶

DD, disk diffusion inhibition zone; MIC, minimum inhibitory concentration; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance-determining region.

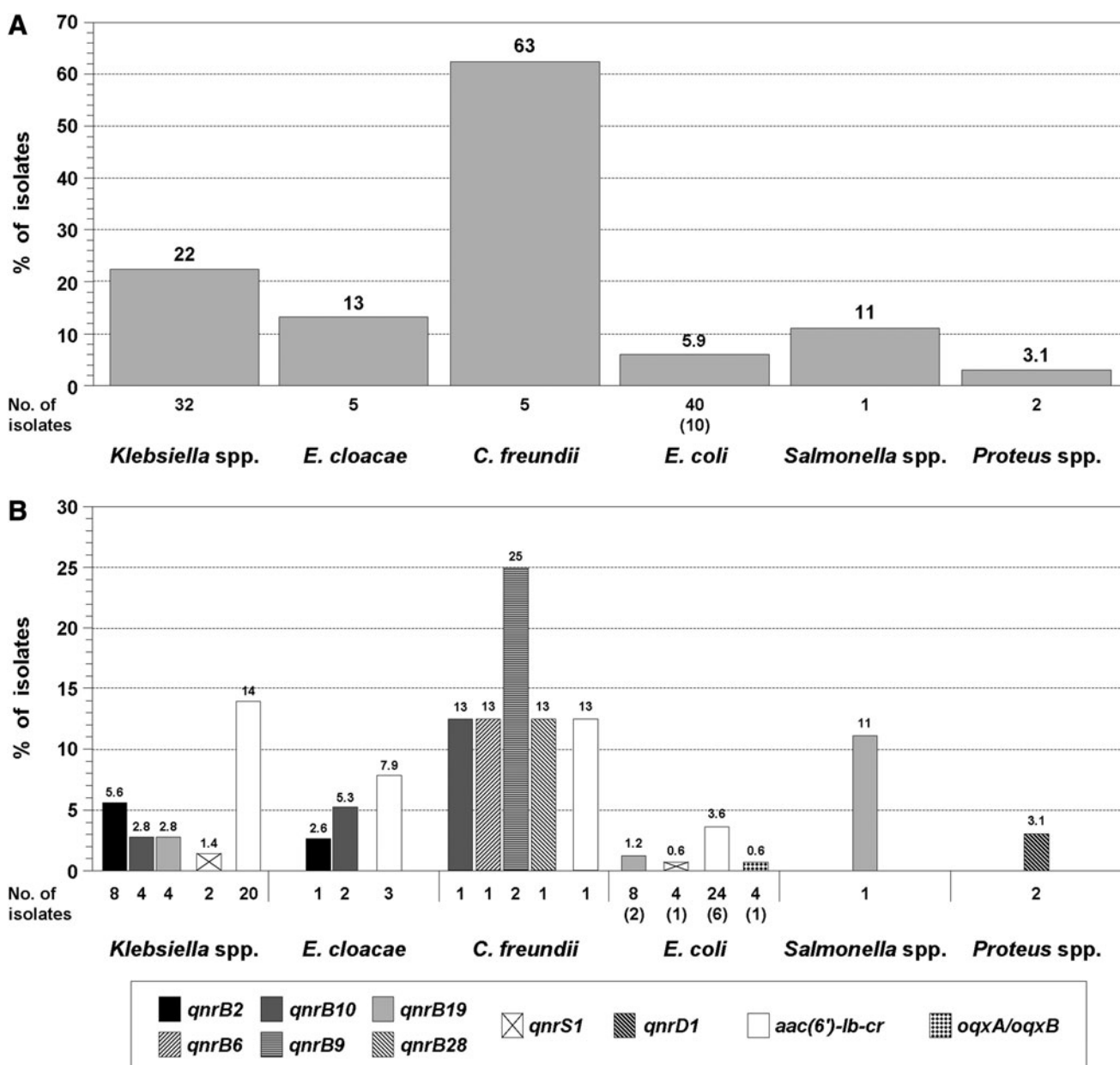


FIG. 2. Prevalence of PMQR genes. In the collection of 1,058 isolates, the number of *E. coli* that harbored a given PMQR gene was estimated by multiplying by four the number of isolates with this gene found in the sample set of 100 *E. coli*, which is indicated between brackets. For simplicity, *K. pneumoniae* and *K. oxytoca* are grouped together, as well as *P. mirabilis* and *P. vulgaris* (data are from our preliminary work⁶). (A) Overall prevalence of PMQR genes in different species. For each species, the percentage, or the number, of isolates harboring at least one PMQR gene is shown above or below the bar, respectively. (B) Prevalence of different PMQR genes in different species. For each species, the percentage of isolates harboring each indicated PMQR gene was calculated regardless of whether they had other PMQR genes or not. Percentage values and number of isolates are indicated above or below the bars, respectively. PMQR, plasmid-mediated quinolone resistance.

overall PMQR prevalence found here was similar to that observed in phenotypically unselected clinical enterobacteria from Spain (6.4%)²² and was well below those found in the two earlier surveys from Argentina, namely 57%²³ and 60%,²⁴ because both studies were based on selected isolates with resistance/decreased susceptibility to quinolones and ESC resistance, respectively.

Besides the interspecies variability in the overall PMQR prevalence, there were differences among the species when

the different PMQR genes were considered. First, *Salmonella* spp. and *Proteus* spp. only harbored a unique gene (*qnrB19* and *qnrD1*, respectively), whereas three to five different genes were found in the other species. Moreover, *qnrD1* was only found in *Proteus* spp. (Fig. 2B). These findings agree well with a previous work with another enterobacterial collection from Argentina, where it was observed that *Salmonella* spp. contained almost exclusively *qnrB19*.²³ Similarly, although *qnrD1* has been occasionally

reported in other enterobacteria, it was especially found in some species of the tribe *Proteeae*, such as *P. mirabilis*, *P. vulgaris*, and *Providencia rettgeri*, and could have originated there.⁷ Second, *qnrB6*, *qnrB9*, and *qnrB28* were only found in *C. freundii* (Fig. 2B), which is consistent with the notion that this species is likely the source of *qnrB* alleles.⁷ This concept could also account for the high PMQR prevalence found here for *C. freundii*, since all the PMQR-harboring isolates of this species contained *qnrB* alleles (Fig. 2B). In other surveys based on phenotypically unselected enterobacteria, it was also found that the *qnr* genes in *C. freundii* were almost exclusively *qnrB* alleles and that this species had the highest *qnr* prevalence (25%–71%).^{16,19,21,22}

Coexistence of QRDR mutations and PMQR genes

In this study, 45 of the 55 PMQR-containing isolates showed high resistance to NAL, suggesting the presence of QRDR mutations in the topoisomerase II-encoding genes.^{3,23} The presence of QRDR mutations in *gyrA* and *parC* was investigated in all the PMQR-containing isolates and in the isolates

that were nonsusceptible to NAL (DD inhibition zones ≤ 18 mm) of those without PMQR genes. QRDR mutations were only found in two groups (Table 2). First, all the isolates that were highly resistant to NAL (DD inhibition zones ≤ 8 mm, MIC ≥ 128 $\mu\text{g/ml}$) harbored QRDR mutations in the codon 83 of *gyrA*. The 30% of this group had a second QRDR mutation in *gyrA* (codon 87), and 83% showed at least one QRDR mutation in *parC*. Second, only the isolates of *E. coli* and *Shigella* sp. with NAL inhibition zones of 9–14 mm (MIC of 32–64 $\mu\text{g/ml}$) harbored the single QRDR mutations in *gyrA*, S83A, or D87G/Y, which were reported to confer low-level NAL resistance.^{37–39} These specific QRDR genotypes were observed in 6 of the 7 NAL^{I-LR} *E. coli* (S83A, D87Y, and D87G, 1, 2, and 3 isolates, respectively) and in the unique NAL-resistant *Shigella* sp. (D87G) (Table 2), giving a prevalence of 0.9% in *E. coli* and of 1.5% in *Shigella* spp.. The limited distribution of these QRDR genotypes can be related, at least in part, with the low-level quinolone resistance that they confer³⁸ and is in agreement with previous reports. Indeed, single substitutions in codon 87 were the unique QRDR mutations found in NAL-resistant *S. flexneri* from Argentina,²³ whereas S83A was also

TABLE 2. DISTRIBUTION OF THE QRDR MUTATIONS FOUND IN *GYRA* AND *PARC*

Gene, codon (c)	QRDR mutation	% of QRDR mutation found in: ^a										
		<i>Kpn</i>	<i>Kox</i>	<i>Ecl</i>	<i>Eae</i>	<i>Cfr</i>	<i>Sma</i>	<i>Eco</i>	SHI	<i>Pmi</i> ^b	<i>Mmo</i> ^b	<i>Pst</i> ^b
Group 1 (137 isolates): DD ≤ 8 mm for NAL (MIC ≥ 128 $\mu\text{g/ml}$)												
<i>gyrA</i> , c83	None (WT)	—	—	—	—	—	—	—	—	—	—	—
	S83I ^c	75	100	—	100	100	—	—	—	19	60	50
	S83R	—	—	—	—	—	100	—	—	81	40	50
	S83Y	6	—	91	—	—	—	—	—	—	—	—
	S83F	19	—	9	—	—	—	—	—	—	—	—
	S83 L	—	—	—	—	—	—	—	100	—	—	—
<i>gyrA</i> , c87	None (WT)	88	100	18	100	100	100	33	—	95	100	100
	D87G	6	—	64	—	—	—	—	5	—	—	—
	D87 N	—	—	9	—	—	—	—	61	—	—	—
	D87A	6	—	9	—	—	—	—	—	—	—	—
	D87Y	—	—	—	—	—	—	—	6	—	—	—
<i>parC</i> , c80	None (WT)	13	—	27	100	—	100	22	—	—	—	—
	S80I	75	—	73	—	100	—	72	—	95	100	100
	S80R	—	100	—	—	—	—	6	—	5	—	—
	S80T	13	—	—	—	—	—	—	—	—	—	—
<i>parC</i> , c84	None (WT)	100	100	100	100	100	100	89	—	100	100	100
	E84V	—	—	—	—	—	—	11	—	—	—	—
Group 2 (seven isolates): DD of 9–14 mm for NAL (MIC 32–64 $\mu\text{g/ml}$) ^d												
<i>gyrA</i> , c83	None (WT)	—	—	—	—	—	—	83	100	—	—	—
	S83A	—	—	—	—	—	—	17	—	—	—	—
<i>gyrA</i> , c87	None (WT)	—	—	—	—	—	—	17	—	—	—	—
	D87G	—	—	—	—	—	—	50	100	—	—	—
	D87Y	—	—	—	—	—	—	33	—	—	—	—

Only the isolates with at least one QRDR mutation were considered ($n=144$).

^a*Kpn*, *Klebsiella pneumoniae*; *Kox*, *Klebsiella oxytoca*; *Ecl*, *E. cloacae*; *Eae*, *E. aerogenes*; *Cfr*, *C. freundii*; *Sma*, *Serratia marcescens*; *Eco*, *E. coli*; *SHI*, *Shigella* spp.; *Pmi*, *P. mirabilis*; *Mmo*, *M. morgani*; *Pst*, *P. stuartii*. The unique isolate of *P. vulgaris* that contained QRDR mutations (*gyrA*, S83I and *parC*, S80I)⁶ is not included in the table for simplicity. The percentages of the individual amino acid variants found in each indicated codon of *gyrA* or *parC* were calculated over the total number of isolates of each enterobacterial species in Group 1 or Group 2 (values for the prevalent amino acid variants are indicated in bold; “—”: variant not found). The gray background indicates that there were no isolates with a particular phenotype (SHI in Group 1 and all the species, except *Kpn*, in Group 2) or the isolates with a particular phenotype did not harbor QRDR mutations (*Kpn* in Group 2).

^bData from our preliminary work.⁶

^cT83I in *Kox*, *Eae*, and *Cfr*.

^dThis group includes six of the seven *E. coli* isolates that showed intermediate or low resistance to NAL (NAL^{I-LR}). The remaining NAL^{I-LR} isolate showed a DD of 17 mm for NAL and did not harbor QRDR mutations. In this group, the ciprofloxacin MICs were 0.015–0.12 $\mu\text{g/ml}$ and the levofloxacin MICs were 0.015–0.5 $\mu\text{g/ml}$ (DD of 28–36 mm and 25–35 mm, respectively).

NAL, nalidixic acid; WT, wild type.

found occasionally among clinical enterobacteria from other regions.^{28,40} It is important to highlight that the seven isolates with these uncommon QRDR genotypes were susceptible to ciprofloxacin and levofloxacin according to the MIC and DD breakpoints²⁵ (Table 1). Moreover, one of the D87Y-harboring *E. coli* and the D87G-containing *Shigella* sp. resulted in being susceptible when they were tested with the pefloxacin 5- μ g disk⁴¹ that was proposed for the screening of ciprofloxacin nonsusceptibility in *Salmonella*.²⁵

In *Salmonella* Typhi, the single *gyrB* substitution S464F has been associated with an unusual quinolone resistance phenotype.^{4,5} This phenotype was very similar to that of the unique quinolone-resistant *Salmonella* of our collection (Table 1), which showed wild-type *gyrA* and *parC* QRDRs, but the analysis of the *gyrB* QRDR of this isolate did not reveal any QRDR mutation. Here, we are stating two conditions for reaching high-level ciprofloxacin resistance in *Proteaceae*: (1) single QRDR mutations in both *gyrA* and *parC* were not sufficient, and (2) it is necessary the presence of additional substitutions S464F/Y.⁶ To know whether these mutations could also be involved in the high-level ciprofloxacin resistance observed in both *K. pneumoniae* and *E. cloacae*, the *gyrB* QRDRs of four isolates of each species were analyzed but no substitution was found in codon 464.

The occurrence of QRDR mutations was significantly associated with the presence of PMQR genes: At least one QRDR mutation was present in 82% (45/55) of the PMQR-harboring isolates but in only 23% (99/430) of those without PMQR genes ($p < 0.0001$, Fisher's Test) (Tables 1 and 3). Such a high proportion of QRDR mutations in PMQR-harboring isolates was also observed in other surveys based on phenotypically unselected enterobacteria,^{10,11,13,14} but the occurrence of QRDR mutations in isolates without PMQR genes was not analyzed in any of them, hampering the statistical association provided here.

The high level of coexistence of QRDR mutations and PMQR genes could be due, at least in part, to the possible

presence of several clonally related PMQR-harboring isolates. However, in the analysis of susceptibility to 19 antibiotics other than quinolones, we observed that the isolates of the same species that harbored the same PMQR gene, or combination of PMQR genes, had different susceptibility profiles (Table 4). Moreover, these isolates were mostly collected in different hospitals (Table 4). These facts suggest extensive genetic heterogeneity among the PMQR-harboring isolates.

It is well known that the PMQR genes facilitate the selection of higher levels of quinolone resistance.⁷ *In vitro* studies on *E. coli* showed that these genes promoted the selection of mutations that conferred higher levels of quinolone resistance, which were located preferentially in several targets that were different from the type II topoisomerase-encoding genes.⁴²⁻⁴⁵ However, a different outcome was observed *in vivo*. First, several observational studies showed that QRDR mutations in *gyrA* were commonly found in clinical PMQR-harboring isolates of *E. coli* and other enterobacteria.^{10,11,13,14,30,46} Second, the inpatient selection of QRDR mutations in *gyrA* and *parC* over a *qnr*-positive background was observed during fluoroquinolone treatment of infections caused by *E. coli*⁴⁷ or *Salmonella* Typhimurium.⁴⁸ Moreover, two longitudinal surveys provided additional support for a role of the PMQR genes in promoting the emergence of QRDR mutations in *gyrA* and *parC* in clinical settings.^{17,20} Although our results do not allow us to figure out which of these mechanisms occurs first in the development of high-level resistance, the finding of PMQR-harboring isolates without QRDR mutations indicates that the PMQR genes, including several *qnrB* alleles and *aac(6')-Ib-cr* (Table 1), might be acquired before the selection of these mutations.

Genetic environments of the prevalent PMQR genes

The genetic environments of *qnrB2*, *qnrB10*, *qnrB19*, and *aac(6')-Ib-cr* were analyzed. In all cases, *qnrB2* and *qnrB10* were located in the same genetic structures of complex class

TABLE 3. COEXISTENCE OF PMQR GENES AND QRDR MUTATIONS FOUND IN *GYRA* AND *PARC*

Species (total number of analyzed isolates)	Isolates with PMQR genes (n=55)		Isolates without PMQR genes (n=430)	
	Without QRDR mutations	With QRDR mutations (%) ^a	Without QRDR mutations	With QRDR mutations (%) ^a
<i>Klebsiella</i> spp. (143)	6	26 (81)	96	15 (14)
<i>E. coli</i> (100) ^b	0	10 (100)	53	37 ^c (41)
<i>Enterobacter</i> spp. (52)	0	5 (100)	36	11 (23)
<i>Proteus</i> spp. (65) ^d	0	2 (100)	43	20 (32)
<i>Citrobacter</i> spp. (15)	3	2 (40)	10	0 (0)
<i>Salmonella</i> spp. (9)	1	0 (0)	8	0 (0)
<i>Shigella</i> spp. (66)	0	0 (0)	65	1 (2)
<i>Serratia</i> spp. (19)	0	0 (0)	13	6 (32)
<i>M. morgani</i> (12) ^d	0	0 (0)	7	5 (42)
<i>P. stuartii</i> (4) ^d	0	0 (0)	0	4 (100)
Total	10	45 (82)	331	99 (23)

For simplicity, species of the same genus are grouped together.

^aPercentages were calculated over the total number of isolates with or without PMQR genes into each species.

^b*E. coli* sample of 100 isolates described in the section Analysis of Quinolone Susceptibility.

^cThis subgroup includes the six isolates with intermediate or low resistance to nalidixic acid (NAL^{1-LR}) that harbored S83A or D87G/Y in *gyrA* as the unique QRDR mutation found.

^dData from our preliminary work.⁶

TABLE 4. DIVERSITY OF ANTIMICROBIAL SUSCEPTIBILITY PROFILES AND HOSPITALS WHERE THE PMQR-HARBORING ISOLATES WERE COLLECTED

Species ^a (total no. of isolates)	PMQR gene ^b	No. of isolates	No. of susceptibility profiles ^c	No. of hospitals
With QRDR mutations <i>Klebsiella</i> spp. (26)	<i>qnr</i>	9	9	9
	<i>aac(6′)-Ib-cr</i>	13	13	7
	<i>qnr + aac(6′)-Ib-cr</i>	4	4	4
	<i>qnr</i>	3	3	2 ^d
	<i>aac(6′)-Ib-cr</i>	6	6	6
<i>E. coli</i> (10)	<i>oqxA and oqxB</i>	1	1	1
	<i>qnr</i>	2	2	2
	<i>aac(6′)-Ib-cr</i>	2	2	2
<i>E. cloacae</i> (5)	<i>qnr + aac(6′)-Ib-cr</i>	1	1	1
	<i>qnr</i>	1	1	1
	<i>qnr + aac(6′)-Ib-cr</i>	1	1	1
Without QRDR mutations <i>Klebsiella</i> spp. (6)	<i>qnr</i>	3	3	3
	<i>aac(6′)-Ib-cr</i>	1	1	1
	<i>qnr + aac(6′)-Ib-cr</i>	2	2	2
<i>C. freundii</i> (3)	<i>qnr</i>	3	3	3

The isolates are grouped according to: (i) whether they had or had not at least one QRDR mutation in *gyrA*; (ii) the enterobacterial species; and (iii) the PMQR genes found in each isolate.

^aThe species that only contained 1 PMQR-harboring isolate were excluded (*P. mirabilis*, *P. vulgaris*, and *Salmonella* spp.).

^bFor simplicity, the different *qnr* genes found (the *qnrB* alleles and *qnrS1*) were not shown. The combinations of *qnr* genes with *aac(6′)-Ib-cr* are indicated by a plus sign.

^cNumber of different profiles of susceptibility to 19 antibiotics other than quinolones observed in each group.

^dThe two *E. coli* isolates collected in the same hospital contained different *qnr* genes (*qnrB19* and *qnrS1*).

1 integrons (variable region 2) that were previously found in Argentina, which have proved to be contained in conjugative plasmids.²³

In 5 out of 7 *qnrB19*-containing isolates, this gene was found in the ColE1-type plasmid pPAB19-1 that was proposed as a natural reservoir.²³ This plasmid and its close genetic variants were almost exclusively found in *E. coli* and *Salmonella* spp. and could be transferred by conjugation to *E. coli*, even when they are not self-transferable.^{23,49} Interestingly, besides *E. coli* and *Salmonella* spp., here, we found pPAB19-1 in 2 *K. pneumoniae* isolates, which suggests that the mobilization of this plasmid also occurred *in vivo*. In the remaining 2 *qnrB19*-containing isolates (*K. pneumoniae*), this gene was located in the genetic context of the ISEcp1C-based Tn2012.²⁹ This is the first report of such a *qnrB19* genetic environment in Argentina, which, along with the three previously described,²³ highlights the abundance of genetic elements involved in its mobilization.

In 23 out of 30 (77%) *aac(6′)-Ib-cr*-harboring isolates, this gene was found as a cassette of class 1 integrons. These included six out of seven isolates in which *aac(6′)-Ib-cr* was combined with *qnrB2* or *qnrB10*. Therefore, the significant association observed between these genes and *aac(6′)-Ib-cr* (see section *Occurrence and Prevalence of PMQR Genes*) might be explained by genetic linkage in complex class 1 integrons. In the other 4 (13%) isolates, *aac(6′)-Ib-cr* was located in Tn6238, which was only described in *K. pneumoniae* from Argentina.³¹ Interestingly, besides 1 *K. pneumoniae*, we found Tn6238 in 2 *E. cloacae* and 1 *E. coli*, suggesting dissemination wider than expected among clinical enterobacteria. In the remaining three isolates, *aac(6′)-*

Ib-cr was found to be bracketed by IS26 (upstream) and *bla*_{OXA-1} (downstream), as described.³⁰

Concluding Remarks

To the best of our knowledge, this is the first report on the prevalence of PMQR genes in phenotypically unselected clinical enterobacteria where all the genes currently known have been screened. We observed an overall prevalence of 8.1% and a wide distribution of these genes throughout Argentina. One main strength of this work is that it constitutes a large national point prevalence study on PMQR genes. This was based on the enterobacterial collection method used that allowed us to obtain a large number of unselected isolates collected at the same time point from many hospitals all along the country. Although this approach also provided a diverse spectrum of isolates, it had the limitation that some species, such as *Salmonella* spp., *C. freundii*, *C. koseri*, *P. vulgaris*, and *P. stuartii* (9, 8, 7, 7, and 4 isolates, respectively), could be underrepresented. Since the isolates were consecutively collected during the hospital routine, the low numbers of isolates of these species in the enterobacterial collection are probably a consequence of their abundance in our clinical settings. Given the high number of isolates tested, we could not perform a molecular subtyping analysis (MLST, repPCR, or PFGE) to address their genetic relatedness, which constitutes another limitation of our study.

In *E. coli* and *Shigella* spp., we found that the QRDR genotypes based on single *gyrA* mutations (S83A or D87G/Y) confer low NAL resistance. Although these genotypes

have low prevalence, the fact that they cannot be detected by the current susceptibility breakpoints of ciprofloxacin and levofloxacin for enterobacteria, nor by the pefloxacin-disk test recommended for *Salmonella*, represents a threat for fluoroquinolone treatment. In this context, it should be stressed that the NAL susceptibility (DD or MIC determination) was very suitable to detect these QRDR genotypes.

Finally, our data provide additional evidence for the co-existence of PMQR genes and QRDR mutations in clinical enterobacteria. Indeed, the fact that 82% of the PMQR-harboring isolates also contained QRDR mutations involves an advantage for the clinical laboratories, since most of the PMQR-harboring enterobacteria can be easily detected and only a few of them (those without QRDR mutations) could represent a challenge to categorize quinolone susceptibility.

Acknowledgments

This work was supported by a grant from ANPCYT (PICT 2007-01804), Buenos Aires, Argentina, to A.P. E.A was supported by fellowships from ANPCYT and CONICET.

WHONET-Argentina participants include the following: Daniela Ballester, Hosp. Piñero; Ana Di Martino, Sanatorio de la Trinidad Mitre; Laura Errecalde, Hosp. Fernández; Angela Famiglietti, Hosp. de Clínicas; Analía Fernández, Hosp. Universitario Fundación Favalaro; Nora Gómez, Hosp. Argerich; Horacio Lopardo, Hosp. de Pediatría Garrahan; Nora Orellana, FLENI; Miryam Vazquez, Hosp. de Niños Gutiérrez; Miriam Mortarini, Hosp. Muñiz (all in Buenos Aires City); Adriana Di Bella, Hosp. Posadas, El Palomar; Marcelo Zotta, Instituto Nacional de Epidemiología Juan Jara, Mar del Plata; Mónica Machain, Hosp. Piñeyro, Junín; Andrea Pacha, Hosp. San Juan de Dios, La Plata; Marisa Almuzara, Hosp. Eva Perón, San Martín; Ana Togneri, Hosp. Evita, Lanús; María Luz Benvenuti, Hosp. Penna, Bahía Blanca, and Cecilia Vescina, Hosp. de Niños Sor Maria Ludovica, La Plata (all in the province of Buenos Aires); Viviana David, Hosp. Interzonal San Juan Bautista, and Patricia Valdez, Hosp. de Niños Eva Perón, Catamarca (province of Catamarca); Norma Cech, Hosp. 4 de Junio-Ramón Carrillo, Pte. Roque Saenz Peña, and Isabel Marques, Hosp. Perrando, Resistencia (province of Chaco); Omar Daher, Hosp. Zonal de Esquel (province of Chubut); Claudia Aimaretto, Hosp. Regional de Villa María; Marina Bottiglieri, Clínica Reina Fabiola; Catalina Culasso, Hosp. de Niños de la Santísima Trinidad; Liliana González, Hosp. Infantil Municipal; Ana Littvik, Hosp. Rawson, and Lidia Wolff, Clínica Privada Vélez Sarsfield, city of Córdoba (all in the province of Córdoba); Ana María Pato, Hosp. Llano, and Viviana García Saitó, Hosp. Pediátrico Juan Pablo II, Corrientes (province of Corrientes); María Moulins, Hosp. Masvernat, Concordia, and Francisco Salamone, Hosp. San Martín, Paraná (province of Entre Ríos); Nancy Pereira, Hosp. Central, and María Vivaldo, Hosp. de la Madre y el Niño, Formosa (province of Formosa); Marcelo Toffoli, Hosp. de Niños Quintana, and María Weibel, Hosp. Pablo Soria, Jujuy (province of Jujuy); Gladys Almada, Hosp. Molas, Santa Rosa, and Adriana Pereyra, Hosp. Centeno, Gral. Pico (province of La Pampa); Sonia Flores, Hosp. Vera Barros, La Rioja (province of La Rioja); Lorena Contreras, Hosp. Central, and Beatriz García,

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Disclosure Statement

No competing financial interests exist.

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