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Differential Distribution of Plasmid-Mediated Quinolone Resistance Genes in Clinical Enterobacteria with Unusual Phenotypes of Quinolone Susceptibility from Argentina

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We studied a collection of 105 clinical enterobacteria with unusual phenotypes of quinolone susceptibility to analyze the occurrence of plasmid-mediated quinolone resistance (PMQR) and *oqx* genes and their implications for quinolone susceptibility. The *oqxA* and *oqxB* genes were found in 31/34 (91%) *Klebsiella pneumoniae* and 1/3 *Klebsiella oxytoca* isolates. However, the *oqxA*- and *oqxB*-harboring isolates lacking other known quinolone resistance determinants showed wide ranges of susceptibility to nalidixic acid and ciprofloxacin. Sixty of the 105 isolates (57%) harbored at least one PMQR gene [*qnrB19*, *qnrB10*, *qnrB2*, *qnrB1*, *qnrS1*, or *aac*(6′)-*Ib-cr*)], belong to 8 enterobacterial species, and were disseminated throughout the country, and most of them were categorized as susceptible by the current clinical quinolone susceptibility breakpoints. We developed a disk diffusion-based method to improve the phenotypic detection of *aac*(6′)-*Ib-cr*. The most common PMQR genes in our collection [*qnrB19*, *qnrB10*, and *aac*(6′)-*Ib-cr*] were differentially distributed among enterobacterial species, and two different epidemiological settings were evident. First, the species associated with community-acquired infections (*Salmonella* spp. and *Escherichia coli*) mainly harbored *qnrB19* (a unique PMQR gene) located in small ColE1-type plasmids that might constitute its natural reservoirs. *qnrB19* was not associated with an extended-spectrum β-lactamase phenotype. Second, the species associated with hospital-acquired infections (*Enterobacter* spp., *Klebsiella* spp., and *Serratia marcescens*) mainly harbored *qnrB10* in IS*CR1*-containing class 1 integrons that may also have *aac*(6′)-*Ib-cr* as a cassette within the variable region. These two PMQR genes were strongly associated with an extended-spectrum β-lactamase phenotype. Therefore, this differential distribution of PMQR genes is strongly influenced by their linkage or lack of linkage to integrons.

Functions of plasmid-mediated quinolone resistance (PMQR) genes include (i) protection of the DNA gyrase mediated by *qnr* genes, five classes of which are known to date (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*, some of which present several alleles); (ii) acetylation of ciprofloxacin and norfloxacin mediated by *aac*(6′)-*Ib-cr*, an allele of the aminoglycoside acetyltransferase-encoding gene *aac*(6′)-*Ib*; and (iii) a quinolone-specific efflux pump belonging to the MFS family and encoded by the *qepA* gene (1). The last mechanism displays variable activity according to the hydrophilicity of the quinolone (from maximal activity for the hydrophilic ciprofloxacin or norfloxacin to no activity for the hydrophobic nalidixic acid [NAL]) (2). In addition, the OqxAB multidrug efflux pump, which belongs to the RND family and confers low-level resistance to quinolones, was found in a plasmid of an *Escherichia coli* isolate from pigs in 2004 (3). However, besides quinolones, this mechanism also confers resistance to other antibiotics, antiseptics, disinfectants, and detergents (4). Recently, OqxAB was also found in clinical isolates of *E. coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*, and, mainly, *Klebsiella pneumoniae*, where OqxAB is mostly encoded on the chromosome. However, the presence of the OqxAB-encoding genes, *oqxA* and *oqxB*, did not correlate with the ciprofloxacin MICs, probably due to differences in their expression levels (5, 6, 7). Given these differences in substrate profiles, plasmid/chromosome locations, and gene expression between the previously described PMQR genes and *oqxA* and *oqxB*, we will consider them separately in this work.

The first PMQR determinant described was *qnrA1*, found in a *K. pneumoniae* strain isolated in 1994. The *qnrB8*-like and *qnrB9*-like alleles were later found in earlier enterobacterial isolates from 1988 (8), and now, the *qnrB* alleles and *aac*(6′)-*Ib-cr* seem to be the most prevalent PMQR genes by far (9). The extensive use of quinolones, together with the mobility at the molecular and cellular level of most PMQR determinants, has contributed to the spread of these genes among almost all clinical enterobacteria across the world (1), and their incidence seems to have increased in recent years (9).

Although the PMQR genes *per se* only confer low-level resistance similar to that produced by other mechanisms, they facilitate and complement the selection of additional resistance mechanisms, enabling bacteria to become fully resistant. As a consequence, despite the efforts to find them, specific phenotypic traits that allow the easy detection of PMQR genes at the clinical laboratory level have not yet been reported (1). Moreover, such a

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diversity of resistance mechanisms has led to the recent emergence of several phenotypic profiles of quinolone susceptibility. For example, data from a set of 1,064 nonselected (consecutive) enterobacteria collected from 66 hospitals in Argentina during a period of 5 days in 2007 (10) showed that 87% of the isolates could be included in two major phenotypic profiles defined by disk diffusion susceptibility (11): (i) full quinolone resistance (no inhibition zone for NAL plus resistance to ciprofloxacin and levofloxacin; 17% of the isolates) and (ii) full quinolone susceptibility (with ciprofloxacin inhibition zones of ≥31 mm; 70% of the isolates). The remaining 13% were distributed in seven minor or unusual (≤5% each) phenotypes: six of them with resistance or in the intermediate category to NAL and in variable susceptibility categories for ciprofloxacin and levofloxacin and one with decreased ciprofloxacin susceptibility (the same as group ii above but with ciprofloxacin inhibition zones in the range of 21 to 30 mm) (see Table S1 in the supplemental material). These unusual phenotypes constitute the "gray zone" of quinolone susceptibility, since most of them represent a challenge at the clinical laboratory level to categorize quinolone susceptibility under the current breakpoints (11, 12, 13).

So far, two *qnr* determinants have been reported in Argentina, *qnrB9*-like (8) and *qnrB10*, which is sometimes associated with *aac(6′)-Ib-cr* in complex class 1 integrons (14). However, these findings were obtained from a collection of strains isolated between 1981 and 1991 (8) or a small number of more recent multiresistant clinical isolates (14). Here, we studied a collection of 105 recently isolated clinical enterobacteria with unusual quinolone susceptibility phenotypes to analyze the occurrence of PMQR and *oqx* genes and their implications for quinolone susceptibility.

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MATERIALS AND METHODS

Bacterial collection and susceptibility tests. We used as the source of the collection of bacterial isolates with unusual quinolone susceptibility phenotypes all the enterobacteria (one per patient; $n = 120$) that had been sent by clinical laboratories to the National Reference Laboratory (the Antimicrobial Agents Division) in the period 2005 to 2008 to further investigate their quinolone susceptibility profiles. These profiles suggested the presence of low-level quinolone resistance mechanisms, and, then, the susceptibility testing results were difficult to interpret at the clinical centers. Therefore, isolates fully resistant to quinolones (with no inhibition zone for NAL plus resistance to ciprofloxacin and levofloxacin) were not sent. Since a disk diffusion breakpoint to decreased ciprofloxacin susceptibility from the Clinical and Laboratory Standards Institute (CLSI) was not available for all enterobacteria (11), we selected the isolates from the primary bacterial set that showed ciprofloxacin disk diffusion inhibition zones of ≤30 mm (9 isolates with inhibition zones of 31 to 35 mm were discarded). This cutoff was defined using a population criterion by analyzing the distribution of the ciprofloxacin disk diffusion inhibition zones of the collection of nonselected enterobacteria from Argentina mentioned above (10) and was the same as that inferred for *E. coli* and *Salmonella enterica* (15) or the recently revised CLSI ciprofloxacin breakpoint for *Salmonella* (13). In addition, when several isolates of the same bacterial species showing similar antibiotic susceptibility profiles were collected in the same hospital during a short period, only one representative isolate was selected to avoid the inclusion of possible outbreaks (6 isolates were discarded). The resulting collection of 105 isolates consisted of 34 *K. pneumoniae*, 3 *K. oxytoca*, 25 *E. coli*, 20 *Salmonella* sp., 8 *E. cloacae*, 2 *Enterobacter aerogenes*, 8 *S. marcescens*, 2 *Shigella flexneri*, 2 *Proteus mirabilis*, and 1 *Morganella morganii* isolates. These isolates were collected from January 2005 to December 2008 in 31 hospitals from 11 provinces of Argentina and Buenos Aires City (BAC). The distribution of unusual phenotypes of quinolone susceptibility is shown in Table S1 in the supplemental material. Hospital-acquired infections (HAI) were defined as infections acquired at least 48 h after hospital admission. Community-acquired infections (CAI) were defined as infections that occurred among nonhospitalized ambulatory patients or within 48 h after hospitalization (16, 17).

Antimicrobial susceptibility tests were performed by the disk diffusion (NAL, ciprofloxacin, norfloxacin, levofloxacin, kanamycin, tobramycin, amikacin, and gentamicin) and agar dilution (NAL, ciprofloxacin, levofloxacin, kanamycin, tobramycin, and amikacin) methods according to the CLSI guidelines (11). Phenotypic detection of extended-spectrum β-lactamases (ESBLs) was done by assaying the synergy between cefotaxime/ceftazidime and clavulanic acid by disk diffusion (11).

General methods. All the PCR primers used in this work are listed in Table S2 in the supplemental material. Standard PCRs were performed as described previously (18). In the case of amplification of the *qepA* gene (72% G+C) the PCR× Enhancer Solution (Invitrogen, Carlsbad, CA) was added to the reaction mixture at a 1× final concentration, since it optimized the amplification of GC-rich templates.

Sequencing reactions were performed using the BigDye terminator methodology with an ABI 3130xl Genetic Analyzer (Applied Biosystems/PerkinElmer, Foster City, CA). Nucleotide sequence editing and analyses were performed using ClustalX2 v2.1 (19; http://www.clustal.org/clustal2/), BioEdit v7.0.9 (20; http://www.mbio.ncsu.edu/bioedit/bioedit.html), and the Basic Local Alignment Search tool (BLAST) (21; http://www.ncbi.nlm.nih.gov/BLAST/).

Transference of PMQR-containing plasmids was assayed by biparental conjugation with *E. coli* J53 azide resistant (AzR) as described previously (14). Transconjugants were selected with 100 μg/ml of sodium azide and 100 μg/ml of ampicillin for the isolates harboring *qnrB10*, *qnrB2*, or *qnrS1* or 0.12 μg/ml of ciprofloxacin for the isolates harboring *qnrB19*. The identities of *qnrS1* and the *qnrB* alleles were checked back in the transconjugants by DNA sequencing.

Analysis of the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC*. The QRDRs of *gyrA* from the 82 isolates that showed resistance or an intermediate category to NAL were amplified by PCR and sequenced. The QRDRs of *parC* were additionally analyzed in those isolates where at least one mutation associated with quinolone resistance (MAQR) (22) was detected in the QRDR of *gyrA* (the primers used are listed in Table S2 in the supplemental material). The *gyrA* QRDR sequences were compared with reference sequences of *E. coli*, *E. aerogenes*, *E. cloacae*, *K. oxytoca*, and *K. pneumoniae* (GenBank accession numbers AF052254 to AF052258, respectively) (23) or with the sequences of previously characterized quinolone-susceptible isolates: *P. mirabilis* ATCC 29906 (24), *S. flexneri* 2457T (ATCC 700930) (25), *S. marcescens* ATCC 14756 (26), and several serovars of *S. enterica* subsp. *enterica* (27) (GenBank accession numbers GG668576, AE014073, U56906, and AE006468, CP001113, CP001120, CP001138, and CP001144, respectively). The *parC* QRDR sequences were compared with reference sequences of *E. coli* and *K. pneumoniae* (GenBank accession numbers NC000913 and AF303641, respectively) (28) or with the sequences of the same quinolone-susceptible isolates of *P. mirabilis*, *S. flexneri*, *S. marcescens*, and *S. enterica* subsp. *enterica* used for the *gyrA* QRDR comparisons (GenBank accession numbers GG668579, AE014073, AF227958, and AE006468, CP001113, CP001120, CP001138, and CP001144, respectively).

The QRDR sequences of *M. morganii* were compared with available sequences in GenBank (strain KT, accession numbers ALJX01000007 for *gyrA* and ALJX01000008 for *parC*).

Detection of *oqxA*, *oqxB*, and PMQR genes. The presence of *oqxA*, *oqxB* (in *oqxA*-positive isolates), *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS* was tested by PCR. Differentiation among the 51 *qnrB* alleles described to

date (http://www.lahey.org/qnrStudies; last accessed 20 July 2012) was performed using a PCR-restriction fragment length polymorphism (RFLP)-based system. The 523-bp-long amplicons generated using the *qnrB* PCR primers (see Table S2 in the supplemental material) were digested with restriction enzymes, and the fragments were analyzed using 2% agarose gel electrophoresis (see Fig. S3 in the supplemental material). At least one *qnrB* allele corresponding to each PCR-RFLP profile was sequenced (complete gene).

The presence of *aac*(6′)-*Ib-cr* and/or *aac*(6′)-*Ib*, which differ in 2 amino acids (29), was determined with separate allele-specific PCRs, using two primers sets directed against each variant (see Table S2 in the supplemental material) (30, 31). The results were validated by DNA sequencing.

Genetic environments of *qnrB* and *qnrS* genes. Previously described genetic environments for *qnrS1* (32), *qnrB1* (33), *qnrB2* (34), and *qnrB10* (14) were identified by PCR cartography.

Small plasmids harboring *qnrB19* were detected by PCR using the divergent primers indicated in Table S2 in the supplemental material (35). In order to determine the plasmid variants, the obtained amplicons were analyzed by PCR-RFLP using two different restriction enzymes, ApoI and RsaI (New England BioLabs, Ipswich, MA). Fragments were visualized on 1.8% agarose gel electrophoresis. At least one plasmid corresponding to each PCR-RFLP profile was completely sequenced.

The unknown genetic environments of *qnrB19* (*E. coli* M9820 and M11059) were analyzed by reverse PCR. Briefly, the total DNA (PureLink Genomic DNA Minikit; Invitrogen) from each isolate was digested with PstI and ligated with T4 DNA Ligase (New England BioLabs). The ligation products were used as the templates for reverse PCR using the divergent primers for *qnrB19* (see Table S2 in the supplemental material). The amplicons were cloned using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's recommendations, and the inserts were sequenced using the M13 universal primers and sequence-based-designed primers (by DNA walking). To discard possible *in vitro* DNA rearrangements, the genetic structure was confirmed by PCR cartography in the original clinical isolates.

Nucleotide sequence accession numbers. The sequences of the QRDRs of *gyrA* and *parC* from the *M. morganii* isolate have been deposited in the GenBank database under the accession numbers KC503934 and KC503935, respectively, and the sequences of the *qnrB19* genetic environments from *E. coli* M9820 and M11059 under the accession numbers JX298079 and JX298080, respectively.

RESULTS AND DISCUSSION

Analysis of the QRDRs of *gyrA* and *parC*. MAQRs in *gyrA* (22) were detected in all 13 isolates (6 *Salmonella* sp., 4 *E. coli*, 2 *S. marcescens*, and 1 *M. morganii* isolates) with high resistance to NAL (disk diffusion inhibition zones of 6 mm; MICs 128 to >512 μg/ml). In addition, we also found MAQRs in *gyrA* in all the NAL-resistant isolates of *S. flexneri* (both isolates) and *P. mirabilis* (1 isolate) (disk diffusion inhibition zones of 9 or 13 mm and MICs of 64 or 16 μg/ml, respectively). These 16 isolates showed only single *gyrA* MAQRs as follows (number of isolates): *Salmonella* spp., S83F (4), S83Y (1), and D87N (1); *E. coli* S83L (3) and S83A (1); *S. marcescens* S83R (2); *S. flexneri* D87G (1) and D87N (1); *P. mirabilis* S83R (1); and *M. Morganii* S83I (1). The last isolate was unique in harboring a MAQR in *parC* (22): the S80R substitution (ParC numbering of *E. coli*). To the best of our knowledge, this is the first report of *gyrA* and *parC* MAQRs in *M. morganii*. This strain showed high resistance to NAL and ciprofloxacin (disk diffusion inhibition zones of 6 mm and MICs of >512 μg/ml and 16 μg/ml, respectively) but susceptibility to levofloxacin (disk diffusion inhibition zone of 20 mm and MIC of 1 μg/ml). The additional presence of *aac*(6′)-*Ib-cr* may explain this unusual phenotype. Other PMQR genes were found in another 2 of the 16 isolates with MAQRs: *qnrB19* (*E. coli*) and *qnrB10* plus *aac*(6′)-*Ib-cr* (*S. marcescens*).

TABLE 1 Distribution of NAL and ciprofloxacin susceptibilities of the 32 *oqxA*- and *oqxB*-harboring *K. pneumoniae* and *K. oxytoca* isolates according to the PMQR genes detected

No. of isolates	PMQR gene(s) detected[a]		MIC_{50} (range) (μg/ml)		Disk diffusion inhibition zone [median (range)] (mm)	
	qnr	*aac*(6′)-*Ib-cr*	NAL	CIP	NAL	CIP
14[b]	−	−	32 (4–128)	0.12 (0.015–1)	16 (9–25)	25 (15–29)
3	−	+	4 (4–32)	0.12 (0.06–1)	20 (8–24)	24 (15–26)
5	+	−	32 (16–64)	1 (1–2)	16 (9–18)	17 (13–20)
10	+	+	32 (16–256)	4 (1–32)	15 (9–16)	13 (6–17)

[a] −, not detected; +, detected. The *qnr* genes screened were *qnrA*, *-B*, *-C*, *-D*, and *-S*.
[b] This group includes a *K. oxytoca* isolate.

Analysis of *oqxA* and *oqxB*. The *oqxA* gene was detected only in the *Klebsiella* isolates: 31 of 34 (91%) *K. pneumoniae* and 1 of 3 *K. oxytoca*. The *oqxB* gene was found in all the *oqxA*-positive isolates. The *oqxA* and *oqxB* genes had not been previously reported in Argentina, and the high proportion observed in *K. pneumoniae* was in agreement with the high prevalence rates (74 to 100%) previously found for the species (5, 6, 7). It was reported that OqxAB confers low-level resistance to NAL, fumequine, ciprofloxacin, and norfloxacin (4). Therefore, the NAL and ciprofloxacin susceptibility data for the 32 *oqxA*- or *oqxB*-harboring isolates of our bacterial collection were analyzed according to the PMQR genes detected (Table 1). Of note, the 14 *oqxA*- and *oqxB*-harboring isolates without any known PMQR gene showed a wide range of MICs and disk diffusion inhibition zones for both NAL (4 to 128 μg/ml and 9 to 25 mm) and ciprofloxacin (0.015 to 1 μg/ml and 15 to 29 mm). This result was in agreement with a range of ciprofloxacin MICs from 0.008 to 0.25 μg/ml previously observed for *K. pneumoniae* isolates that harbored *oqxA* and *oqxB* as the unique determinant of quinolone resistance (7). It has been proposed that this lack of correlation between the presence of the *oqxA* and *oqxB* genes and the level of quinolone resistance could be explained by different expression levels of the *oqxAB* operon, due to yet-undefined regulatory elements (5, 6, 7). Therefore, the analyses of PMQR genes described below were carried out regardless of the presence of *oqxA* and *oqxB*.

PMQR genes: distribution, detection, and impacts on quinolone susceptibility. Sixty (57%) out of the 105 isolates harbored at least one PMQR gene: 20 *K. pneumoniae*, 2 *K. oxytoca*, 14 *E. coli*, 14 *Salmonella* sp., 5 *E. cloacae*, 2 *E. aerogenes*, 2 *S. marcescens*, and 1 *M. morganii* isolates. These PMQR-harboring isolates belonged to 8 of the 10 enterobacterial species comprised in the collection (only the *P. mirabilis* and *S. flexneri* isolates did not harbor PMQR genes) and were found in 24 out of 31 hospitals (9 from BAC and 15 from 10 provinces). These results show a wide distribution of PMQR genes, both among enterobacterial species and throughout Argentina.

Thirty-six and 5 isolates harbored a *qnr* gene or *aac*(6′)-*Ib-cr*, respectively, whereas 19 isolates contained both genes. The most common PMQR gene was *qnrB*, which was detected in 50 (83%) out of the 60 PMQR-containing isolates. We found 4 *qnrB* alleles: *qnrB19* (26 isolates), *qnrB10* (20 isolates), *qnrB2* (3 isolates), and

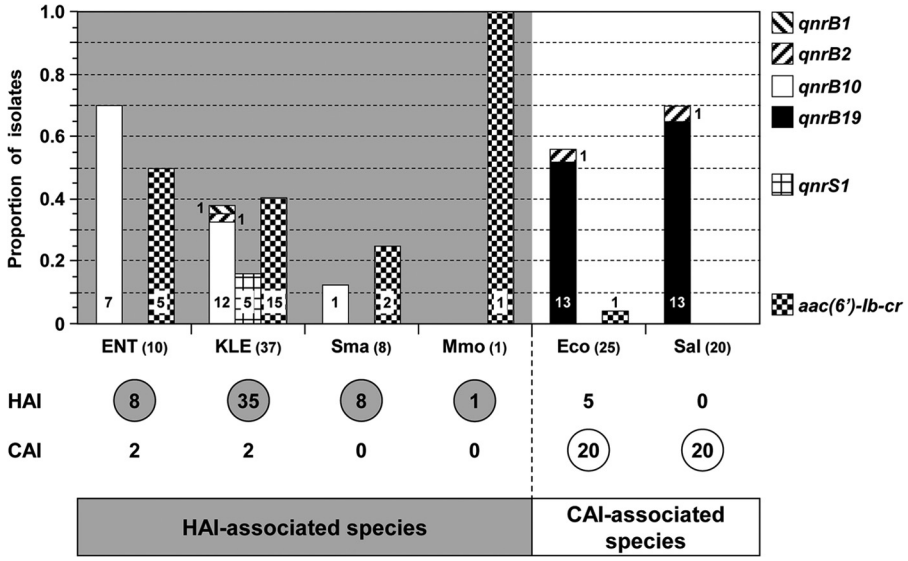

FIG 1 Differential distribution of the PMQR genes among enterobacterial species. For each enterobacterial species, the proportion of isolates harboring each indicated PMQR gene is shown, regardless of whether they had other PMQR genes or not. For simplicity, *E. aerogenes* and *E. cloacae* were considered a unique group (ENT), as well as *K. oxytoca* and *K. pneumoniae* (KLE). The other enterobacterial species indicated are as follows: Sma, *S. marcescens*; Mmo, *M. morganii*; Eco, *E. coli*; and Sal, *Salmonella* spp. (*P. mirabilis* and *S. flexneri* isolates did not harbor PMQR genes.) The numbers of isolates harboring each PMQR gene are indicated inside the bars, and the total number of isolates in each group is shown in parentheses. The numbers of HAI and CAI are shown below each group, and the groups of species associated with HAI are highlighted with a gray background (the 2 *P. mirabilis* isolates were from HAI, while the 2 *S. flexneri* isolates were from CAI).

qnrB1 (1 isolate). The *qnrB19* gene was found only in *E. coli* and *Salmonella* spp. These species were mostly associated with CAI (89% [40/45]) (Fig. 1). On the other hand, *qnrB10* was present only in *Enterobacter* spp., *Klebsiella* spp., and *S. marcescens*, which were mostly associated with HAI (93% [51/55]) (Fig. 1). Recently, a high prevalence of *qnrB* alleles was found in the genus *Citrobacter*, which has been proposed as their source (36). The lack of *Citrobacter* sp. isolates in our collection may be due to the very low proportion of isolates of the genus among clinical enterobacteria in Argentina. Indeed, among the 1,064 nonselected enterobacteria collected in 66 hospitals in our country in 2007 (10), there were only 15 (1.4%) *Citrobacter* sp. isolates, and just 2 of them (0.2%) showed unusual phenotypes of quinolone susceptibility (unpublished data). The *qnrS1* gene was found in 5 *Klebsiella* spp. (8% of PMQR-positive isolates), while *qnrA*, *qnrC*, *qnrD*, and *qepA* were not detected in our bacterial collection. Some of these genes were found in other Latin American countries. The *qnrA* gene was detected in enterobacterial isolates from Brazil and Uruguay (1), and *qepA* was recently found in 4 out of 58 CTX-M-producing *E. coli* isolates from Bolivia (37). However, comparisons among these findings should be avoided because the criteria for bacterial selection used in each study were different.

The *aac*(6′)-*Ib* gene confers resistance to several aminoglycosides (e.g., kanamycin, tobramycin, and amikacin) (38), but its recently isolated variant *aac*(6′)-*Ib-cr* can also acetylate norfloxacin and ciprofloxacin (29). Therefore, the screening of both variants, based on allele-specific PCR, was only done in the subset of 73 isolates that showed nonsusceptibility to kanamycin (disk diffusion inhibition zones of ≤17 mm; 16 isolates with inhibition zones of ≥18 mm, used as a control group, were negative). Forty-one of the 73 isolates gave positive PCR results: the *aac*(6′)-*Ib* and the *aac*(6′)-*Ib-cr* alleles were detected in 17 and 9 isolates, respectively, while 15 isolates contained both. Nineteen out of the 24 isolates with *aac*(6′)-*Ib-cr* also harbored a *qnr* gene (*qnrB10*, 16 isolates; *qnrB1*, *qnrB2*, and *qnrS1*, 1 isolate each), whereas in 5 isolates (3 *Klebsiella* sp., 1 *S. marcescens*, and 1 *M. morganii* isolates), *aac*(6′)-*Ib-cr* was found as the unique PMQR gene. Similar to *qnrB10*, *aac*(6′)-*Ib-cr* was mainly found in *Enterobacter* spp., *Klebsiella* spp., and *S. marcescens*, whereas it was almost absent in *E. coli* and *Salmonella* spp. (Fig. 1). Moreover, *qnrB10* and *aac*(6′)-*Ib-cr* were significantly associated due to coexpression [*aac*(6′)-*Ib-cr* was present in 80% of *qnrB10*-harboring isolates but in only 9% of those without *qnrB10*; $P < 0.0001$; Fisher's test].

The nucleotide sequence of the *aac*(6′)-*Ib-cr* variant previously found in Argentina (14) differs from that originally reported by Robicsek et al. (29) in a silent change located in one of the two mutations that confer catalytic activity on ciprofloxacin and norfloxacin (AGG instead of CGG). In our bacterial collection, 18 of the 24 *aac*(6′)-*Ib-cr*-containing isolates harbored the AGG codon and only 6 of them showed the CGG codon. This fact must be taken into account if the detection of *aac*(6′)-*Ib-cr* is done using molecular methods based on the detection of single-nucleotide polymorphisms, such as allele-specific PCR or RFLP (39). The allele-specific PCR described here allowed us to detect not only *aac*(6′)-*Ib* and *aac*(6′)-*Ib-cr*, but also both variants of the latter.

The relevant antibiotic susceptibility profiles of the bacterial collection, excluding the 16 isolates with MAQRs, were grouped according to the PMQR genes detected (Table 2). The MIC_{50}s of NAL, ciprofloxacin, and levofloxacin in the group of isolates that harbored any PMQR gene were 4 times higher than those in the group without a known PMQR. Accordingly, the disk diffusion inhibition zones of these three quinolones were smaller in the former group. However, Table 2 also shows that there was partial overlap between the distributions of the MICs or disk diffusion

TABLE 2 Relevant MICs and disk diffusion inhibition zones of the bacterial collection[a]

Gene(s) harbored (no. of isolates)	MIC_{50} (range) (μg/ml)				Disk diffusion inhibition zone [median (range)] (mm)			
	NAL	CIP	LVX	AMK	NAL	CIP	LVX	AMK
No PMQR (32)[b]	8 (1–128)	0.12 (0.015–1)	0.25 (0.03–2)	2 (0.5–64)	18 (9–28)	26 (15–30)	27 (16–32)	21 (10–25)
Any PMQR (57)	32 (2–256)	0.5 (0.06–32)	1 (0.06–8)	2 (0.5–32)	15 (8–26)	21 (6–27)	23 (13–33)	21 (11–25)
Any *qnr* gene (35)[c]	32 (8–64)	0.5 (0.12–2)	1 (0.25–4)	2 (0.5–16)	13 (9–20)	23 (13–27)	24 (16–29)	23 (11–25)
aac(6′)-Ib-cr (4)	4 (2–32)	0.25 (0.06–1)	0.06 (0.06–2)	8 (4–32)	22 (8–26)	23 (15–26)	28 (22–33)	17 (16–20)
qnr + *aac(6′)-Ib-cr* (18)[d]	32 (16–256)	2 (1–32)	1 (0.25–8)	8 (2–32)	15 (9–19)	14 (6–21)	22 (13–28)	17 (12–22)

[a] The 16 isolates with MAQRs were excluded.
[b] PMQR screening was for *qnrA*, *-B*, *-C*, *-D*, and *-S*; *qepA*; and *aac(6′)-Ib-cr*.
[c] Thirty-one *qnrB* (alleles *B2*, *B10*, and *B19*) and 4 *qnrS1*.
[d] Seventeen *qnrB* (alleles *B1*, *B2*, and *B10*) and 1 *qnrS1*.

inhibition zones of these groups for ciprofloxacin or levofloxacin. Of note, the ciprofloxacin-selective activity of AAC(6′)-Ib-cr was clearly observed when the group of PMQR-harboring isolates was further subdivided according to the kind of PMQR gene detected [any *qnr*, *aac(6′)-Ib-cr*, or both combined]. In the groups of isolates harboring *aac(6′)-Ib-cr*, either alone or combined with any *qnr* gene, the medians of the inhibition zones of ciprofloxacin were 5 and 8 mm smaller, respectively, than those of levofloxacin (Table 2). However, only subtle differences were observed when the MIC_{50}s of ciprofloxacin and levofloxacin were compared, either in the group with *aac(6′)-Ib-cr* alone or in that with *aac(6′)-Ib-cr* plus any *qnr* gene (2 and 1 dilutions, respectively) (Table 2), suggesting that disk diffusion has higher sensitivity than agar dilution to detect *aac(6′)-Ib-cr*. Therefore, we used these findings to develop a phenotypic approach for the screening of *aac(6′)-Ib-cr* by disk diffusion, comparing the susceptibilities to ciprofloxacin and levofloxacin. As can be seen in Fig. 2, a difference of ≥5 mm between the inhibition zones of levofloxacin and ciprofloxacin

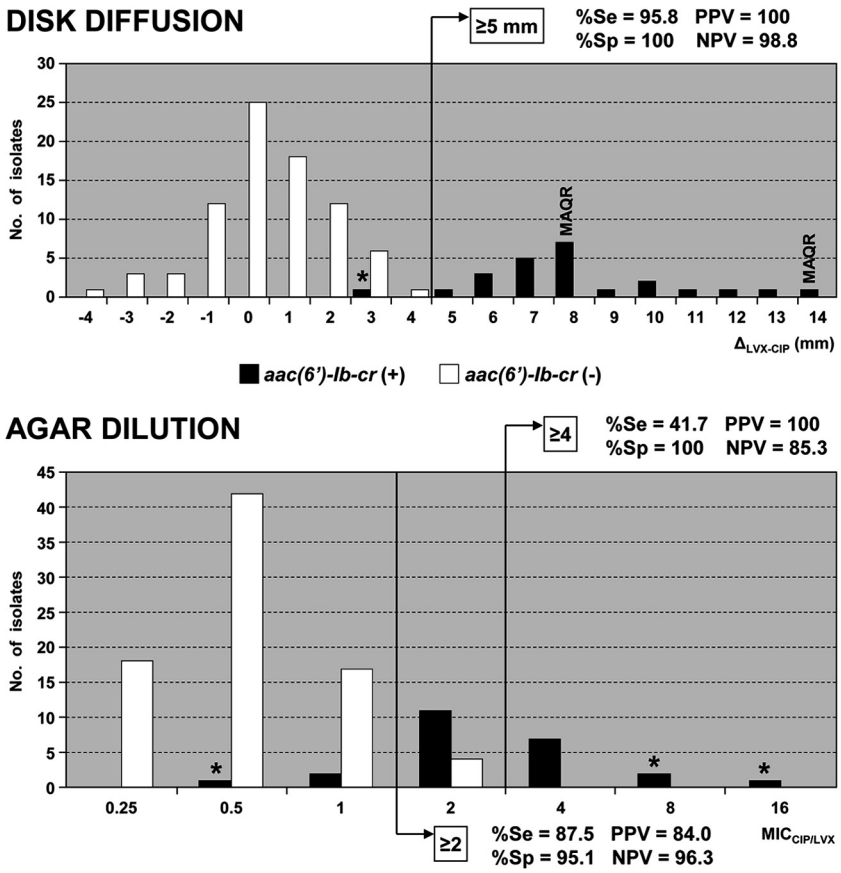

FIG 2 Phenotypic approaches to detect *aac(6′)-Ib-cr*. The isolates with (n = 24) or without (n = 81) *aac(6′)-Ib-cr* in the complete bacterial collection were stratified according to the diameter of the levofloxacin inhibition zone minus that of ciprofloxacin ($\Delta_{LVX-CIP}$) for the disk diffusion approach (5-μg disks were used for both fluoroquinolones) or the ratio of the MIC of ciprofloxacin to that of levofloxacin ($MIC_{CIP/LVX}$) for the agar dilution approach. The $\Delta_{LVX-CIP}$ values for the 2 isolates with MAQRs in *gyrA* and *parC* are indicated. The vertical thin lines represent the cutoffs used for the calculation of the percentages of sensitivity (%Se) and specificity (%Sp) and the positive and negative predictive values (PPV and NPV, respectively). In those cases where outlier values were obtained (asterisks), the susceptibility assays were repeated at least three times (median values were used for the calculations).

TABLE 3 Quinolone susceptibilities of PMQR-containing isolates by CLSI and EUCAST clinical breakpoints

Guideline	MIC susceptibility breakpoint (μg/ml)			% Susceptible isolates (*n*)[a]			Disk diffusion susceptibility breakpoint (mm)			% Susceptible isolates (*n*)[a]		
	NAL	CIP	LVX	NAL	CIP	LVX	NAL	CIP	LVX	NAL	CIP	LVX
CLSI 2012[b]	≤16	≤1	≤2	23 (14)	77 (46)	93 (56)	≥19	≥21	≥17	13 (8)	57 (34)	95 (57)
EUCAST 2012	NA[c]	≤0.5	≤1	NA	50 (30)	87 (52)	NA	≥22	≥22	NA	45 (27)	72 (43)

[a] Percentages and numbers of PMQR-containing isolates that were considered susceptible under the susceptibility breakpoints of each guideline (total number of PMQR-containing isolates, 60).
[b] Ciprofloxacin MICs and disk diffusion susceptibility breakpoints for *Salmonella enterica* serovar Typhi and extraintestinal *Salmonella* spp. were revised and are ≤0.06 μg/ml and ≥31 mm, respectively.
[c] NA, not applicable.

($\Delta_{LVX-CIP}$) was a strong predictor of the presence of *aac*(6′)-*Ib-cr*. As a comparison, the increase in the MIC of ciprofloxacin with respect to that of levofloxacin was a worse predictor (Fig. 2). The disk diffusion method was able to detect the presence of *aac*(6′)-*Ib-cr* even in the 2 isolates with MAQRs in *gyrA* and *parC* (Fig. 2). Of course, this method cannot be applied to those isolates without disk diffusion inhibition zones for ciprofloxacin and halos of ≤10 mm for levofloxacin (40). However, the detection of *aac*(6′)-*Ib-cr* becomes clinically irrelevant in these cases, since they are already highly fluoroquinolone resistant.

Besides the impact on quinolone susceptibility pointed out above, a relevant number of PMQR-containing strains were not detected by the quinolone susceptibility breakpoints defined by the CLSI (11) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (12) (Table 3). Thirteen percent (disk diffusion) and 23% (MIC) of PMQR-containing isolates showed NAL susceptibility according to CLSI. These susceptibility percentages were several times higher for ciprofloxacin and highest for levofloxacin (87% [EUCAST] to 93% [CLSI] under the MIC breakpoints or 72% [EUCAST] to 95% [CLSI] for disk diffusion [Table 3]). Of note, only 5% and 8% of the PMQR-harboring isolates were considered wild type (i.e., without resistance mechanisms) using the epidemiological cutoffs (ECOFFs) defined by the EUCAST from the MIC distributions of ciprofloxacin and levofloxacin, respectively (41).

Genetic environments of *qnrB* and *qnrS*. As can be seen in Fig. 3A to C, the *qnrB2* and *qnrB10* alleles were associated with IS*CR1* but *qnrB1* and *qnrB19* were not. A *pspF*-like gene, the transcriptional activator of the stress-inducible *psp* operon, was found immediately upstream of all the *qnrB* alleles (14, 33, 35, 36). Interestingly, *qnrB19* was located in 3 different genetic environments, which suggests that several mobile genetic elements were involved in its mobilization (Fig. 3C). In 24 out of the 26 *qnrB19*-harboring isolates, this gene was located in 4 small highly related ColE1-type plasmids, pPAB19-1, pPAB19-2, pPAB19-3, and pPAB19-4, that were fully characterized in a previous work, where a model for plasmid evolution was proposed (35). The distribution of these plasmids in our bacterial set was as follows: pPAB19-1 (8 *Salmonella* sp. isolates and 8 *E. coli* isolates), pPAB19-2 (1 *Salmonella* sp. isolate and 2 *E. coli* isolates), pPAB19-3 (1 *E. coli* isolate), and pPAB19-4 (4 *Salmonella* sp. isolates). In the remaining 2 *qnrB19*-harboring isolates, *E. coli* M9820 and *E. coli* M11059 (different hospitals in BAC), the gene was not located in such plasmids, since PCRs with the *qnrB19* divergent primers and those directed against the *rep* region (see Table S2 in the supplemental material) were negative (Fig. 3C). In both isolates, the 433-bp sequences located upstream of *qnrB19* were identical to that found in plasmid p61/9, previously described in a clinical isolate of *S. enterica* from Italy (42). However, the downstream regions presented some differences from that in p61/9. In strain M9820, the comparison to the p61/9 sequence showed that the region was interrupted by an internal fragment of the *tnpA* gene of Tn*1721*, which in turn was truncated by a Tn*3* insertion. In M11059, we found the same *qnrB19* downstream region as in M9820 but lacking the Tn*3* insertion (Fig. 3C).

The *qnrS1* gene was not associated with IS*CR1*, and its genetic environment was identical to others previously described (32) (Fig. 3D).

Horizontal gene transfer. We selected 3 *E. coli* isolates with *qnrB19* (M9996, M9820, and M11059, one for each genetic environment) (Fig. 3C), 2 *K. pneumoniae* isolates and 1 *E. cloacae* isolate with *qnrB10* plus *aac*(6′)-*Ib-cr*, 1 *E. coli* isolate with *qnrB2* plus *aac*(6′)-*Ib-cr*, and 1 *K. pneumoniae* isolate with *qnrS1* for mating experiments. All of these PMQR genes were transferred to the recipient strain, *E. coli* J53-AzR, which underscores their potential for horizontal dissemination. Interestingly, the 4 *qnrB19*-harboring ColE1-type plasmids found contain identical *oriT* regions, but they lack the *mob* and *tra* genes (35). Therefore, the transfer of pPAB19-2 from isolate M9996 to *E. coli* J53-AzR strongly suggests that all these highly related plasmids may be mobilized, even when they are not self-transferable (43).

Association with other relevant resistance mechanisms. Forty (38%) out of the 105 studied isolates had an ESBL phenotype. The proportion of isolates with an ESBL phenotype among the PMQR-containing isolates was not significantly different from that among the isolates without PMQR (45% [27/60] and 29% [13/45], respectively; $P = 0.107$; Fisher's test). However, when we analyzed the subset of species that were mostly associated with HAI (*Enterobacter* spp., *Klebsiella* spp., *S. marcescens*, *P. mirabilis*, and *M. morganii*; $n = 58$) (Fig. 1), the presence of any PMQR gene was significantly associated with an ESBL phenotype (78% [25/32] of PMQR-containing isolates and 46% [12/26] of the isolates without PMQR had an ESBL phenotype; $P = 0.015$; Fisher's test). Interestingly, 2 isolates of this subset produced the carbapenemases KPC-2 (*K. pneumonie* M9171) (44) and IMP-1 (*E. cloacae* M9921) (45), in addition to *aac*(6′)-*Ib-cr* and *qnrB10* plus *aac*(6′)-*Ib-cr*, respectively.

In the remaining species of our collection, *E. coli*, *Salmonella* spp., and *Shigella* spp. ($n = 47$) (Fig. 1), which were mainly associated with CAI, only 3 isolates showed an ESBL phenotype, and it was not associated with the presence of PMQR genes (7% [2/28]

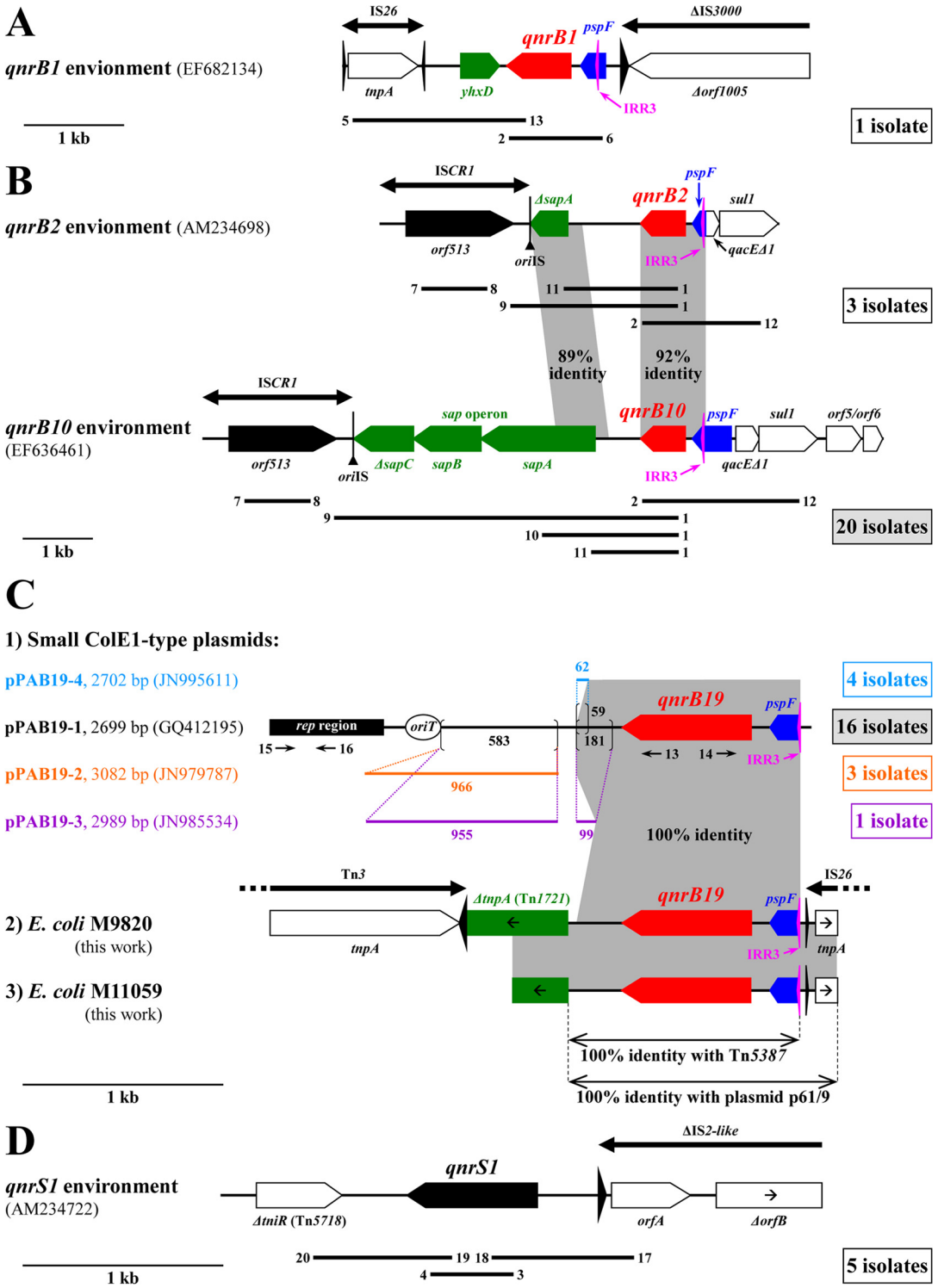

FIG 3 Genetic environments of the *qnrB* alleles and *qnrS1* gene. The genetic environments of the *qnr* genes detected in this work (the numbers of isolates harboring each structure are shown on the right) were compared to previously reported genetic platforms (for the sake of clarity, only the relevant regions are shown). Genes/open reading frames (ORFs) are represented by arrow-shaped boxes (arrows inside boxes indicate the transcriptional orientation for 3′-truncated genes/ORFs). The thick lines with single or double arrowheads indicate the insertion elements (ISs) and transposons found (black triangles represent their inverted repeats [IRs]). The thick horizontal lines in panels A, B, and D depict the amplicons obtained by PCR cartography, and the numbers indicate the corresponding primers in Table S2 in the supplemental material (for each structure, the amplicons from at least one isolate were sequenced). (A, B, and C) For clarity, the *qnrB* alleles are in red and their flanking 3′ and 5′ genes are in green and blue, respectively. The gray-shaded areas highlight the nucleotide identity among the conserved *qnrB* immediate vicinities. The purple triangles indicate the locations of IRR3, a 14-bp sequence that has partial identity with the IRR of IS*Ecp1C* and might be recognized as such by the IS*Ecp1* transposase (see reference 35 for details). The three *qnrB19* genetic environments found are shown in panel C. Features for the three variants of pPAB19-1 are color coded. Only the fragments that differentiate each variant are shown, and the corresponding (replaced) fragments in pPAB19-1 are indicated between square brackets (the numbers are fragment lengths in bp). The short black arrows below pPAB19-1 show primer locations, and the numbers indicate the corresponding ones in Table S2 in the supplemental material. Thin lines with double arrowheads indicate regions identical to those in other *qnrB19*-containing genetic platforms.

of PMQR-containing isolates and 5% [1/19] of the isolates without a PMQR had an ESBL phenotype; $P = 1$; Fisher's test).

We previously reported the coexpression of *rmtD2* and *qnrB10* in a clinical *Citrobacter freundii* isolate from Argentina (10). In the current study, none of the isolates showed the typical absence of inhibition zones for both gentamicin and amikacin (10, 46), which strongly suggests the absence of RmtD2 activity or any other known methyltransferase that methylates 16S rRNA at position G1405.

Differential distribution of PMQR genes among enterobacterial species. The most common PMQR genes in our bacterial collection were *qnrB10*, *qnrB19*, and *aac*(6′)-*Ib-cr*. The results of the PMQR distribution among enterobacterial species and genetic environments and the association with other resistance mechanisms are consistent with two different epidemiological settings. In one, the species associated with CAI (e.g., *Salmonella* spp. and *E. coli*), which usually do not have ESBLs, mainly harbored the *qnrB19* gene in small ColE1-type plasmids, which might constitute natural reservoirs of the allele. The high prevalence of *qnrB19* located in small ColE1-type plasmids in commensal enterobacteria from Peru and Bolivia observed by Pallecchi et al. (47, 48) supports this notion. In the other, the species associated with HAI (e.g., *Enterobacter* spp., *Klebsiella* spp., and *S. marcescens*) mainly harbored *qnrB10* in complex class 1 integrons (i.e., IS*CR1*-containing integrons) that may also have *aac*(6′)-*Ib-cr* as a cassette within the variable region, as we described previously (14). Multiresistance, including ESBLs, is a common feature of the hospital setting that enables bacteria to survive under strong antibiotic pressures, and complex class 1 integrons are genetic vehicles for several resistance genes. Therefore, the genetic linkage of *qnrB10* and *aac*(6′)-*Ib-cr* to class 1 integrons may contribute to their dissemination among multiresistant bacteria.

Concluding remarks. The PMQR genes are frequently found (57%) in clinical enterobacteria with unusual phenotypes of quinolone susceptibility, and their differential distribution among enterobacterial species is strongly influenced by their linkage or lack of linkage to integrons. Our bacterial collection constitutes a 4-year picture of unusual phenotypes of quinolone susceptibility for which the interpretation of the susceptibility testing was problematic, and most of the PMQR-containing isolates were categorized as susceptible by the current CLSI and EUCAST (clinical) susceptibility breakpoints. However, the PMQR-containing isolates were properly differentiated by the ECOFFs for ciprofloxacin and levofloxacin MIC distributions. In this context, the $\Delta_{\text{LVX-CIP}}$ disk diffusion method that we presented here may constitute a very useful tool to improve the phenotypic detection of *aac*(6′)-*Ib-cr*.

Although the sampling design used allowed us to gain some insights into the presence of PMQR and *oqxA* and *oqxB* genes in Argentina, it is noteworthy that our bacterial collection was biased toward resistance or decreased susceptibility to quinolones. Therefore, it was not possible to estimate PMQR prevalence rates or to draw conclusions fully applicable to the whole clinical setting. A nationwide study using a collection of nonselected, consecutive enterobacterial isolates (10) is ongoing to address this issue.

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