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Complex Class 1 Integrons with Diverse Variable Regions, Including *aac(6′)-Ib-cr*, and a Novel Allele, *qnrB10*, Associated with IS*CR1* in Clinical Enterobacterial Isolates from Argentina▽

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Transferable quinolone resistance has not previously been reported in Argentina. Here we describe three complex class 1 integrons harboring the novel allele *qnrB10* in a unique region downstream of *orf513*, one of them also containing *aac(6′)-Ib-cr* within the variable region of integrons. The three arrays differed from *bla*$_{\mathbf{CTX\text{-}M\text{-}2}}$-bearing integrons, which are broadly distributed in Argentina.

Three types of transferable (i.e., plasmid-encoded) quinolone resistance mechanisms have been reported so far, i.e., (i) a quinolone-protective mechanism encoded by the *qnr* genes (15), (ii) a double class antibiotic-modifying enzyme encoded by a two-point mutation allele of *aac(6′)-Ib* [named *aac(6′)-Ib-cr*] which acetylates ciprofloxacin and norfloxacin (14), and (iii) an efflux pump encoded by the *qepA* gene (12, 19). Different transferable *qnr* determinants have been described so far, i.e., *qnrA*, *qnrB*, and *qnrS* (15). The *qnrB* determinants were associated with either the *orf1005* gene, which encodes a putative transposase (7), or the *orf513* recombinase gene (5), the latter included in a region recently named IS*CR1* (16). The origin of *qnrB* is unknown, although QnrB-like proteins were recently found in members of the *Vibrionaceae* family (13). Here we describe a novel quinolone resistance determinant, *qnrB10*, downstream of *orf513* and embedded within different complex class 1 integrons.

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To investigate the occurrence of *qnr* genes in Argentina, we focused on clinical isolates resistant to ciprofloxacin and to at least two different families of antibiotics. Eighteen enterobacterial isolates from three hospitals from Buenos Aires were analyzed for the presence of the *qnrA*, *qnrB*, and *qnrS* genes by PCR (Table 1). None of them contained *qnrA* or *qnrS*, but eight isolates were *qnrB* positive (Table 2). Sequence analysis (NCBI BLAST V2.0) confirmed the identification of a novel *qnrB* allele, designated *qnrB10* (accession number DQ631414), which showed maximal nucleotide identity (97%) with *qnrB5* (6). The deduced QnrB10 protein (226 amino acids) has 98% and 95% identity with QnrB5 and QnrB1, respectively. The *qnrB10* gene was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) by following the manufacturer's recommendations. The expression of this gene, under induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), increased the ciprofloxacin MIC 32-fold compared to that for the host strain, *Escherichia coli* DH10B (Table 3).

Two *qnrB10*-bearing isolates showing different resistance profiles, *Enterobacter cloacae* E705 and *Klebsiella pneumoniae* M7943, were selected for further studies. Relevant susceptibility data (4) are shown in Table 3. To gain insights into the possible mechanisms of dissemination of *qnrB10*, we characterized its genetic environments in both isolates. For this, we used standard PCR long amplification conditions (fragments > 4 kb) implemented with the Elongase kit (Invitrogen) in accordance with the manufacturer's recommendations, followed by DNA sequencing (Table 1; Fig. 1A). Each isolate harbored a complex class 1 integron with a different variable region 1 (vr-1) composed of the 5′ conserved sequence (5′-CS) and the 3′-CS but exhibiting another unique variable region, vr-2, located between IS*CR1* and the second copy of the 3′-CS (Table 2; Fig. 1A). The E705 integron, named In131::IS*CR1*::*qnrB10* (see below for a discussion of the nomenclature), showed a new vr-1 comprising the *aac(6′)-IId* and *aadA1a* cassettes, while the In37::IS*CR1*::*qnrB10* integron harbored *aac(6′)-Ib-cr*, *bla*$_{OXA\text{-}30}$, *catB3*, and *arr-3*. This last array has been previously found in In37 from an *E. coli* isolate from China, which has *qnrA1* in vr-2 (accession number AY259086) (18), and in the integron from a *K. pneumoniae* strain isolated in France which harbors *qnrB4* in vr-2 [plasmid pRBDHA, accession number

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TABLE 1. Primers used in PCR and DNA sequencing

Target and primer[a]	No.[b]	Sequence (5′–3′)	Reference
qnr genes			
qnrA or *-C* (screening)			
qnr-F		TCAGCAAGAGGATTTCTCA	This study
qnr-R		GGCAGCACTATTACTCCCA	
qnrS (screening)			
qnrS-F		ACGACATTCGTCAACTGCAA	6
qnrS-R		TAAATTGGCACCCTGTAGGC	
qnrB (screening)			
qnrB-F	1	CCGACCTGAGCGGCACTGA	This study
qnrB-R	2	CGCTCCATGAGCAACGATGCCT	
qnrB10 (complete gene)[c]			
qnrB-Met-F		ATGTTGTCATTACTGTATA	This study
qnrB-STOP-R		CTAACCAATCACAGCGATG	
Complex class 1 integrons			
intI1			
Inti1-R	3	TTCGAATGTCGTAACCGC	11
Inti1-F		CGAGGCATAGACTGTAC	
Sulpro3 (F)	4	GCCTGACGATGCGTGGA	8
vr-1			
5′CS (F)	5	GGCATCCAAGCAGCAAG	8
3′CS (R)	6	AAGCAGACTTGACCTGA	
orf513 (3′-CS junction), orf513-R5′	7	CGTAACCGTTTGTTTGAGTG	This study
orf513 (screening)			
orf513-F		ATGGTTTCATGCGGGTT	2
orf513-R		CTGAGGGTGTGAGCGAG	11
vr-2			
orf513-F3′	8	GTTCGATCCATCACAGAG	This study
sul1-R	9	TTTGAAGGTTCGACAGC	1
orf5, orf5 lower (R)		TGTCGACGTGGGTGAAT	This study
orf6, In21-R	10	CGGTCGGACTGCAAGTGA	This study
Resistance cassettes and other genes of the integrons characterized in this study			
aadA1			
aadA1-F		TTGCTGGCCGTACATTTG	This study
aadA1-R		TCATTGCGCTGCCATTC	
aac(6′)*-Ib* (any variant) and *aac*(6′)*-IId*			
aacF7334	11	CATCACAAAGTACAGCATCGTGACCAACAG	This study
aacR7907	12	TTAGGCAACACTGCGTGTTCGCTCGAATGC	
aac6IB (R)		TGTGACGGAATCGTTGC	8
aacA4R (F)		AAACACGCCAGGCATTC	1
$bla_{\mathrm{OXA\text{-}30}}$			
oxaIII-F		CATTATTTGAAGGAACTGAAG	This study
oxaIII-R		CACCAGTTTTCCCATACAGT	
catB3			
catout3′-1 (F)	13	TTTCTGCTCTATCGGGAG	This study
arr3out5′-1 (R)		TTGTTAGACGGCAAACTC	
arr-3, *sapC-sapB*	14	GACTTGCTAACCACAGGG	This study
arr-3, arr3-F			
sapC1-R		GAATGTGATTCAGTATCG	This study
sapB4-F		AACCCGTCGGCACAGGCCCG	
arr-3, *sapA*			
sapA-F	15	CAGTGGGTTCGTCTATTGC	This study
sapA-R		CGTTACTGTCCGTCGCC	
arr-3, *sapA-qnrB10* Junction			
sapA3-R		GCAGCAGCCTGACCACTC	This study
qnrB-F3′		CTTGGCATCGCTGTGATTG	

[a] Only relevant primers (F, forward; R, reverse) are shown. Other sequence-based primers used in primer walking were excluded for brevity.
[b] Numbers correspond to primer locations in the PCR chart shown in Fig. 1A.
[c] Primers used for cloning *qnrB10* in the pCR2.1 vector.

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TABLE 2. Relevant characteristics of clinical isolates and transconjugants used in this study

Isolate or strain	Yr of isolation	Hospital	Relevant resistance phenotype[a]	*qnrB10*[b]	vr-1 of *qnrB10*-bearing integrons
C. freundii CF701	2005	H2	AMP-AMC-CAZ-FEP-TZP-AMK-GEN-CHL-TET-SXT-NAL-CIP	+	*aac(6′)-IId-aadA1a*
C. freundii CF702	2005	H3	AMP-AMC-CAZ-FEP-TZP-MEM-CHL-SXT-NAL-CIP	+	*aac(6′)-Ib-aadA1a*
E. aerogenes E704	2005	H3	AMP-AMC-CAZ-TZP-GEN-CHL-SXT-NAL-CIP	+	*aac(6′)-IId-aadA1a*
E. cloacae E701	2005	H3	AMP-AMC-CAZ-TZP-SFI-NAL-CIP	+	*aac(6′)-Ib-aadA1a*
E. cloacae E702	2005	H2	AMP-AMC-CAZ-FEP-TZP-AMK-GEN-CHL-TET-SXT-NAL-CIP	+	*aac(6′)-IId-aadA1a*
E. cloacae E703	2005	H2	AMP-AMC-CAZ-FEP-TZP-GEN-CHL-SXT-NAL-CIP	+	*aac(6′)-IId-aadA1a*
E. cloacae E705	2005	H2	AMP-AMC-CAZ-TZP-GEN-CHL-TET-SXT-NAL-CIP	+	*aac(6′)-IId-aadA1a*
K. pneumoniae M7943	2006	H1	AMP-AMC-CTX-CAZ-FEP-TZP-AMK-GEN-CHL-TET-SXT-NAL-CIP	+	*aac(6′)-Ib-cr-bla*$_{OXA-30}$-*catB3-arr-3*
E. coli M7943-TC2			AMP-AMC-FEP-TZP-AMK-CHL-SFI-NAL-CIP	+	*aac(6′)-Ib-cr-bla*$_{OXA-30}$-*catB3-arr-3*

[a] Antimicrobial resistance and reduced susceptibility were tested by the disk diffusion method (4). Abbreviations: AMC, amoxicillin-clavulanic acid; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; GEN, gentamicin; MEM, meropenem; NAL, nalidixic acid; SFI, sulfisoxazole; SXT; trimethoprim-sulfamethoxazole; TET, tetracycline; TZP, piperacillin-tazobactam.
[b] The presence of *qnrB10* was tested by PCR and sequence analysis (Table 1).

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AJ971343; *aac(6′)-Ib-cr* had not been recognized at the time it was published] (17). However, as shown in Fig. 1B, vr-1 of the In37::IS*CR1*::*qnrB10* integron differed from the other two since it lacks a 101-bp duplication of the 3′-CS located at the 5′ end of the *aac(6′)-Ib-cr* cassette. Also, the W→R mutation in this gene presented a silent change (CGG→AGG) with respect to the previously reported variant (14).

The genetic environments of *qnrB10* in the two integrons described here, Δ(3′)*sapC-sapB-sapA* and Δ(5′)*pspF*, mirrored a conserved architecture found in all of the *qnrB* contexts previously reported (accession numbers AM234698 and AJ971343) that includes the location of *qnrB* alleles in an orientation opposite to that of *orf513* (Fig. 1A) (3, 5, 17). The *sap* operon encodes a putative peptide transport system in gram-negative bacteria, while the *pspF* gene is a transcriptional activator of the stress-inducible *psp* operon. However, a deeper analysis of the *qnrB2*, *qnrB4*, and *qnrB10* environments showed relevant differences. The *sap* and *psp* operons were truncated at different outer points, and the intergenic regions between *sapA* and the *qnrB* alleles were different (Fig. 1A). Moreover, analysis of the inner boundary of the second 3′-CS showed deletions of different lengths in this element when comparing the complex class 1 integrons described so far (Fig. 1C). These data suggest that the *qnrB* alleles are located in similar genetic contexts from probably different genomic sources, as has been described previously for *bla*$_{CTX-M-2}$ alleles (1, 16).

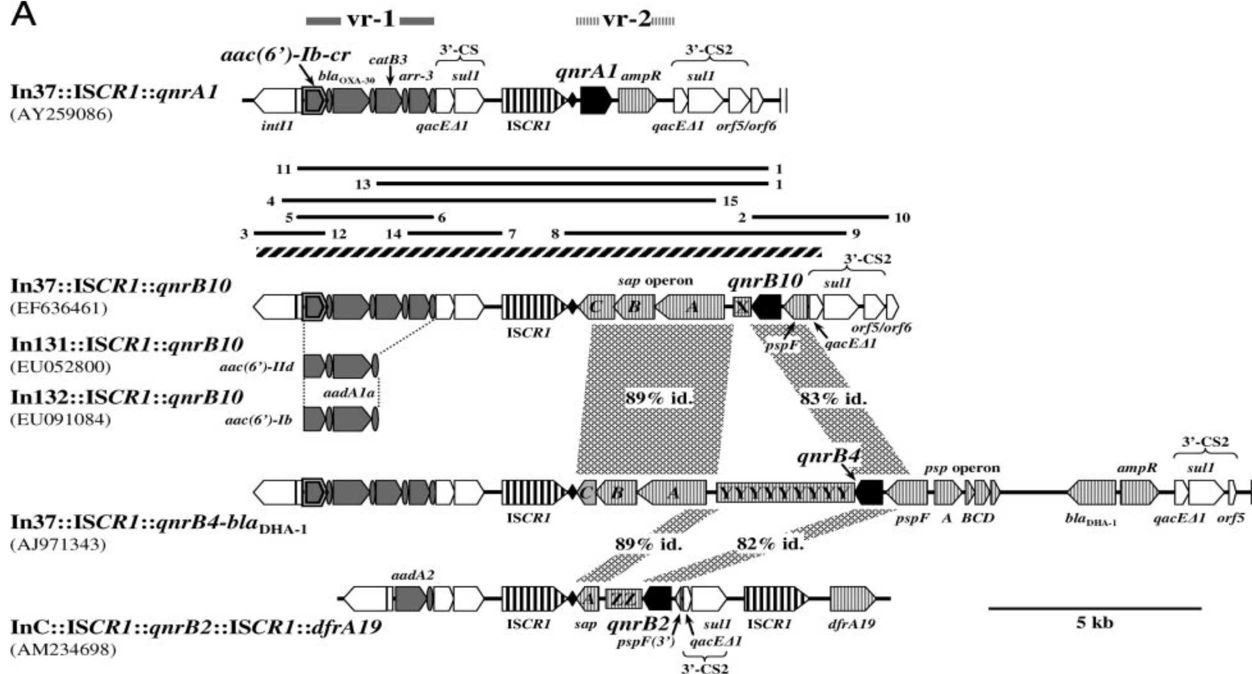
To our knowledge, there is no real consensus on the naming of complex class 1 integrons. Therefore, we tried to follow the simplest criterion. We show here three class 1 integrons with essentially the same vr-1 [*aac(6′)-Ib-cr*, *bla*$_{OXA-30}$, *catB3*, and *arr-3*] but different vr-2s. Since these three harbored the same array of cassettes with alleles that are supposed to confer the same antimicrobial resistance profile, we named the integron from *K. pneumoniae* M7943 In37, focusing on vr-1. In order to specify the genetic platform of the complex class 1 integrons, we added the genetic specification of vr-2, resulting In37::IS*CR1*::*qnrA1* and In37::IS*CR1*::*qnrB4-bla*$_{DHA-1}$ for the integrons described by Wang et al. (18) and Verdet et al. (17), respectively, and In37::IS*CR1*::*qnrB10* for our finding in *K. pneumoniae* M7943.

Transfer of In131::IS*CR1*::*qnrB10* and In37::IS*CR1*::*qnrB10* was assayed by biparental conjugation as described before (10), with *E. coli* J53-AzR (azide resistant) as the recipient strain. Transconjugants were selected on Mueller-Hinton agar supplemented with sodium azide (100 μg/ml) and ampicillin (100 μg/ml), sulfamethoxazole (100 μg/ml), or kanamycin (50 μg/ml). Only In37::IS*CR1*::*qnrB10* was detected in a transconjugant, named M7943-TC2, that had the quinolone and amino-

TABLE 3. Susceptibility profiles of relevant clinical isolates, bacterial recipient for conjugation experiments, transconjugants, clones, and strains used in this study

Strain	MIC (μg/ml)[a]									
	NAL	CIP	NOR	LVX	OFX	GAT	MXF	KAN	TOB	AMK
E. cloacae E705	≥256	≥64	≥64	≥64	≥64	32	≥64	≥256	32	8
K. pneumoniae M7943	128	32	32	4	8	4	8	128	16	16
E. coli M7943-TC2	16	2	2	0.5	0.5	0.5	1	64	16	4
E. coli J53-AzR	4	0.015	0.03	0.03	0.06	0.03	0.06	2	0.25	0.5
E. coli DH10B/pCRQB10[b]	8	0.125	0.125	0.06	0.06	0.06	0.125	≥256	0.25	2
E. coli DH10B/pCR2.1	2	0.004	0.008	0.004	0.004	0.004	0.004	≥256	0.25	2
E. coli DH10B	2	0.004	0.008	0.004	0.004	0.004	0.004	1	0.25	1

[a] MICs (4) were analyzed under induction by 1 mM IPTG. The antibiotics used for MIC determination were as follows: NAL, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; LVX, levofloxacin; OFX, ofloxacin; GAT, gatifloxacin; MXF, moxifloxacin; KAN, kanamycin; TOB, tobramycin; AMK, amikacin.
[b] The complete *qnrB10* gene was cloned into pCR2.1 in the sense orientation with respect to the *lac* promoter.



B

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In37::ISCR1::qnrA1     1 TTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTTTTATTATTTTTAAGCGTGCATAAT
In37::ISCR1::qnrB10      --------------------------------------------------------------------------------

In37::ISCR1::qnrA1    81 AAGCCCTACACAAATTGGGAGTTAGACATCATGAGCAACGCAAAAACAAAGTTAGGCATCACAAAGTACAGCATCGTGAC
                                                       M  S  N  A  K  T  K  L  G  I  T  K  Y  S  I  V  T
In37::ISCR1::qnrB10    1 ---------------------TTAGGCATTAG----AACTTCACGGCGGATTTAAAGA--AAAGGAGAAGACATCGTGAC

In37::ISCR1::qnrA1   161 CAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG....CGG....GCCTAACCCTTC....TGTCAAACG 770
                           N  S  N  D  S  V  T  L  R  L  M  T  E  ..  R  ..  A  *
In37::ISCR1::qnrB10   54 CAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAG....AGG....GCCTAACCCTTC....TGTCAAACG 663
                                                        M  T  E  ..  R  ..  A  *
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C

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In0                         1 TTTAAGCGTG CATAATAAGC CCTACACAAA TTGGGAGATA TATCATGAAA GGCTGGCTTT TTCTTGTTAT
In131::ISCR1::qnrB10          GGCAAGCGTG CATAATAAGC CCTACACAAA TTGGGAGATA TATCATGAAA GGCTGGCTTT TTCTTGTTAT
In37::ISCR1::qnrB10           GGCAAGCGTG CATAATAAGC CCTACACAAA TTGGGAGATA TATCATGAAA GGCTGGCTTT TTCTTGTTAT
In35::ISCR1::bla_CTX-M-2      ATACGCCGTG CATAATAAGC CCTACACAAA TTGGGAGATA TATCATGAAA GGCTGGCTTT TTCTTGTTAT
In7::ISCR1::dfrA10            ATACGTGATT ACTAATAAGC CCTACACAAA TTGGGAGATA TATCATGAAA GGCTGGCTTT TTCTTGTTAT
In37::ISCR1::qnrA1            CAGAAATGCA GTGCACTCTC CTCAACATCC GCCAGTGCGC CGACTTCCAG CCAGACATCC GTTACCCGCG
In6::ISCR1::cat2              TGCTTTTTTA TTTGTCCGCC GGGCGCGGAT AATGGATCAG ATTATGCAGT GTCACAATGG CCTTACCGGG

In0                        71 CGCAATAGTT GGCGAAGTAA TCGCAACATC CGCATTAAAA TCTAGCGAGG GCTTTACTAA GCTTGCCCCT
In131::ISCR1::qnrB10          CGCAATAGTT GGCGAAGTAA TCGCAACATC CGCATTAAAA TCTAGCGAGG GCTTTACTAA GCTTGCCCCT
In37::ISCR1::qnrB10           CGCAATAGTT GGCGAAGTAA TCGCAACATC CGCATTAAAA TCTAGCGAGG GCTTTACTAA GCTTGCCCCT
In35::ISCR1::bla_CTX-M-2      CGCAATAGTT GGCGAAGTAA TCGCAACATC CGCATTAAAA TCTAGCGAGG GCTTTACTAA GCTTGCCCCT
In7::ISCR1::dfrA10            CGCAATAGTT GGCGAAGTAA TCGCAACATC CGCATTAAAA TCTAGCGAGG GCTTTACTAA GCTTGCCCCT
In37::ISCR1::qnrA1            CAATGCCGTG CTGTTCAGCC TGTTCGCGGA TAATGTCCGC CGCACCGAGG GCTTTACTAA GCTTGCCCCT
In6::ISCR1::cat2              ATTGGCGTAA GCGTGCGGGA TATCCGCATG GAAGCGCAGG GATTCCCCGG CAGAAACGGT GTGCCACTCA

In0                       141 TCCGCCGTTG TCATAATCGG TTATGGCATC GCATTTTATT TTCTTTCTCT
In131::ISCR1::qnrB10          TCCGCCGTTG TCATAATCGG TTATGGCATC GCATTTTATT TTCTTTCTCT
In37::ISCR1::qnrB10           TCCGCCGTTG TCATAATCGG TTATGGCATC GCATTTTATT TTCTTTCTCT
In35::ISCR1::bla_CTX-M-2      TCCGCCGTTG TCATAATCGG TTATGGCATC GCATTTTATT TTCTTTCTCT
In7::ISCR1::dfrA10            TCCGCCGTTG TCATAATCGG TTATGGCATC GCATTTTATT TTCTTTCTCT
In37::ISCR1::qnrA1            TCCGCCGTTG TCATAATCGG TTATGGCATC GCATTTTATT TTCTTTCTCT
In6::ISCR1::cat2              TCCCCCAGCC GCAGTTGTAA TGCGCCTTCC AGTACAATGA CATGTTCTCT
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FIG. 1. Genetic organization of *qnrB10*-containing complex class 1 integrons. (A) Genes are represented by arrowed boxes (*qnr* genes are in black), *attC* is represented by vertical ovals, and the putative origin of replication of IS*CR1* (16) is represented by a black diamond. The 3′-CS and the second copy of this element (3′-CS2) are shown by white arrowed boxes. The hatched horizontal bar indicates the sequenced regions (13,281 bp in In37::IS*CR1*::*qnrB10* and 10,762 bp in In131::IS*CR1*::*qnrB10*). Thick horizontal lines indicate the principal amplicons obtained by PCR cartography of the *qnrB10*-bearing integrons (numbers indicate the corresponding primers in Table 1). The integrons In37::IS*CR1*::*qnrA1* (18) and In37::IS*CR1*::*qnrB4-bla*$_{\text{DHA-1}}$ (17), having essentially the same vr-1 as In37::IS*CR1*::*qnrB10*, were included for comparison (sequences were truncated at the two vertical thin lines). Shaded areas with percentages depict identities (id.) between vr-2s. The X, Y, and Z boxes indicate regions of no homology with GenBank sequences. (B) Comparison of the *aac(6′)-Ib-cr* cassettes of In37::IS*CR1*::*qnrA1* and In37::IS*CR1*::*qnrB10* (nucleotides 1492 to 2261 and 1040 to 1702 in the sequences with accession numbers AY259086 and EF636461, respectively). Sequences are numbered from the first nucleotide following the junction with the 5′-CS, and dashes represent gaps introduced to maximize alignment (ClustalX software, available at ftp://ftp-igbmc.u-strasbg.fr/pub/). Identical nucleotides in the two sequences are in bold. The 101-bp duplication of the 3′-CS is underlined. The start (boxed) and stop (*) codons of each *aac(6′)Ib-cr* gene are indicated, and the amino acid sequence of the deduced protein is shown below each nucleotide sequence (14). For brevity, other regions showing 100% nucleotide (or amino acid) identity are represented by dots. The silent CGG-to-AGG change (see the text for details) is also shown. (C) Deletions at the inner boundary of 3′-CS2. Nucleotide sequences correspond to In0 (normal 3′-CS, M73819), In131::IS*CR1*::*qnrB10* (EU052800), In37::IS*CR1*::*qnrB10* (EF636461), In35::IS*CR1*::*bla*$_{\text{CTX-M-2}}$ (AY079169), In7::IS*CR1*::*dfrA10* (L06418), In37::IS*CR1*::*qnrA1* (AY259086), and In6::IS*CR1*::*cat2* (U04277). The deletion endpoint in 3′-CS2 is defined by the first base appearing in bold. Base 1 of In0 corresponds to the nucleotide at position 65 from the normal 3′-CS.

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glycoside resistance profiles for *aac(6′)-Ib-cr* and *qnrB10* genes (Table 2). The presence of In37::IS*CR1*::*qnrB10* was also confirmed by PCR cartography.

The other six *qnrB10*-producing isolates studied here showed the same vr-2 rearrangement and either vr-1 of In131::IS*CR1*::*qnrB10* or a different one composed of the *aac(6′)-Ib* and *aadA1a* cassettes (Table 2; Fig. 1A), which was named In132. None of the *qnrB10*-bearing integrons studied here showed the In35 rearrangement [*aac(6′)-Ib-bla*$_{OXA-2}$-orfD], which is the most widespread complex class 1 integron involved in the dispersion of *bla*$_{CTX-M-2}$ in the bacterial population from Argentina (2).

Recently, Toleman et al. proposed that IS*CR1* is involved in the spread of class 1 integrons through a mechanism that mobilizes adjacent DNA sequences (16). This is in agreement with the finding that cassettes are more often transferred as part of the entire integron structure than as individual gene cassettes (9). The finding of In37 derivatives from different geographical regions with diverse vr-2s is consistent with the speculations of Toleman et al. On the basis of this hypothesis, we would also expect to find In35 associated with *qnrB10* in our nosocomial isolates.

In conclusion, the particular epidemiology of complex class 1 integrons found in isolates harboring a novel *qnrB* allele, as well as the emergence of *aac(6′)-Ib-cr* cassettes in our bacterial population, evidences the need for national surveillance in order to estimate the prevalence of these mechanisms.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this work have been submitted to the GenBank database and assigned accession no. DQ631414, EF636461, EU052800, and EU091084.

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