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Short communication

Detection of class 1 and 2 integrons, extended-spectrum β-lactamases and *qnr* alleles in enterobacterial isolates from the digestive tract of Intensive Care Unit inpatients☆

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ABSTRACT

In this study, we searched for extended-spectrum β-lactamases (ESBLs), class 1 and 2 integrons, and *qnrA*, *qnrB* and *qnrS* genes in 56 oxyimino-cephalosporin and/or ciprofloxacin-resistant enterobacterial isolates obtained from the gastrointestinal tract of patients admitted in an Intensive Care Unit in Uruguay. ESBLs were detected in 11 isolates (6 CTX-M-2, 3 CTX-M-9, 1 CTX-M-15 and 1 PER-2). *qnr* genes and integrons were detected in 5 and 24 isolates, respectively. Eight different antibiotic resistance gene cassettes were found within six different genetic arrangements. Two types of complex class 1 integrons carrying insertion sequence IS*CR1* were found, one showing *bla*$_{CTX-M-2}$–orf3 and the other *qnrA1–ampR*. Ten of the thirteen isolates carrying class 2 integrons presented the element IS*5* inserted between *intI2* and *dfrA1*, whereas another class 2 integron lacked the internal stop codon usually present in *intI2*. This is the first report of the occurrence of *qnrA*, *qnrB* and *bla*$_{CTX-M-9}$ in Uruguay. Dissemination of the different groups of CTX-M enzymes (i.e. groups 1, 2 and 9) appears to be a recent phenomenon in South America.

1. Introduction

The increase in antibiotic resistance is a growing problem worldwide, both in the hospital and the community setting [1,2], owing to the appearance of new antibiotic resistance genes and/or the optimisation of mechanisms responsible for their mobilisation. Amongst the new resistance genes that have gained notoriety in recent years, those coding for transferable quinolone resistance [3] as well as the pandemic expansion of extended-spectrum β-lactamases (ESBLs) of the CTX-M family [1] may be different faces of multiresistance evolution.

The occurrence in South America of *qnr* genes and of the enzyme AAC(6′)-Ib-cr is a recent phenomenon [3]. Moreover, ESBLs from the CTX-M group appear to follow two distinct geographical distribution patterns; whilst in Argentina only CTX-M-2 group enzymes have been reported [4], the occurrence of various CTX-M groups in other countries appears to be the rule [2]. In Uruguay, the presence of CTX-M-2 has already been reported [5,6].

In addition, new forms of gene acquisition by mobile genetic elements have also emerged, such as class 1 integrons linked to the insertion sequence IS*CR1* (complex integrons) [1] and class 2 integrons with a functional integrase capable of inserting new gene cassettes into their variable region [7].

Recently, we reported for the first time in Uruguay the presence of two *Escherichia coli* isolates carrying *bla*$_{CTX-M-15}$ and *aac(6′)-Ib-cr* along with a class 1 integron carrying *dfr17–aadA5* as gene cassettes [8].

The objectives of this study were to detect and characterise class 1 and 2 integrons, ESBLs and *qnr* genes in enterobacterial isolates obtained from Intensive Care Unit (ICU) inpatients in Uruguay.

2. Material and methods

2.1. Patients

In total, 104 patients requiring mechanical respiratory assistance were included in this study. The patients were admitted to the

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ICU of the University Hospital of Montevideo, Uruguay, between 1 March and 31 October 2006.

Patients had rectal swabs obtained 1, 4, 7, 10, 13 and 16 days after admission. Samples were streaked on two MacConkey agar plates supplemented with 4 mg/L ceftazidime or 2 mg/L ciprofloxacin, respectively. Putative enterobacterial colonies were identified as previously described [9]. Only the first antibiotic-resistant isolate per patient was included in the study.

2.2. Bacterial strains

Fifty-six fluoroquinolone- and/or oxyimino-cephalosporin-resistant enterobacterial isolates were studied. Strains *Klebsiella pneumoniae* 120, *E. coli* 137 and *K. pneumoniae* 2296 (provided by J. Martinez and J. Calvo Montes) were used as positive controls for *qnrA*, *qnrB* and *qnrS*, respectively.

2.3. Antibiotic susceptibility testing and extended-spectrum β-lactamase screening

Antibiotic susceptibility was determined by disk diffusion on Mueller–Hinton agar and was interpreted according to Clinical and Laboratory Standards Institute (CLSI) recommendations [10]. Tested antibiotics included ampicillin, amoxicillin/clavulanic acid, cefalothin, cefotaxime, ceftazidime, meropenem, cefoxitin, ciprofloxacin, cefuroxime, gentamicin, amikacin, cefepime and imipenem (Oxoid Ltd., Basingstoke, UK).

All isolates underwent ESBL detection by comparative testing using ceftazidime/clavulanic acid and cefotaxime/clavulanic acid disks (30/10 μg) (Oxoid Ltd.) with plain ceftazidime and cefotaxime disks according to CLSI recommendations [10].

The ciprofloxacin minimum inhibitory concentration was determined by Etest following CLSI guidelines [10].

2.4. Extended-spectrum β-lactamase detection

Positive ESBL screening results were further analysed by polymerase chain reaction (PCR) for the presence of bla_{CTX-M} using generic [11] and specific primers for the groups $bla_{CTX-M-1}$ [8], $bla_{CTX-M-2}$ [6] and $bla_{CTX-M-9}$ (CTX-M-9F, 5′ATGGTGACAAAGAGARTGCAA; and CTX-M-9R, 5′TTACAGCCCTTCGGCGATGAT). bla_{TEM}, bla_{PER-2} and bla_{SHV} genes were also sought using specific primers [9,11]. PCR products were sequenced using the same primers.

2.5. qnr gene screening

The genes *qnrA*, *qnrB* and *qnrS* were sought by means of multiplex PCR and sequencing using primers previously described by Robicsek et al. [12].

2.6. Characterisation of class 1 integrons

All 56 isolates were analysed for the presence of class 1 integrons by PCR using primers I5/I3 [6]. Positive isolates were further studied looking for the variable region, the 3′-conserved region and IS*CR1* using primers 5′CS/3′CS, qacE1F/sul1b and ORFend/F12R, respectively [6].

The region located between IS*CR1* and the second *qacEΔ1–sul1* was amplified with primers orf513InK13/qacE1b and the PCR product was sequenced using a gene walking strategy as reported previously [6]. The region between *qnrA* and the second 3′CS structure was elucidated with the primer qnrAFend (5′AGTAATAGTGCTTGCCGGATTA), as shown in Fig. 1A.

2.7. Detection and mapping of class 2 integrons

Class 2 integrase was amplified by PCR and was sequenced employing primers IntI2.F/IntI2.R as described by Marquez et al. [7] in order to include the stop codon usually present in this gene. The genetic arrangement was determined by PCR mapping and sequencing of PCR products using specific primers for the genes *aadA1* (aadA1F, 5′GGCAGCCAATGACATTCTT; aadA1R, 5′CCAAGCGATCTTCTTGTCCAA), *dfr1* [13], *sat1* (sat1F, 5′CTATCTACCAGAAGTGTGAGC; sat1R, 5′GTTTCGTTCGAGACTTGAGG) and orfX (orfXA, 5′GCGATCTGTTGAAGGTGGTT; orfXB, 5′GATACATGATCTTCAGGCC). Primers for *sat1* and orfX were kindly pro-

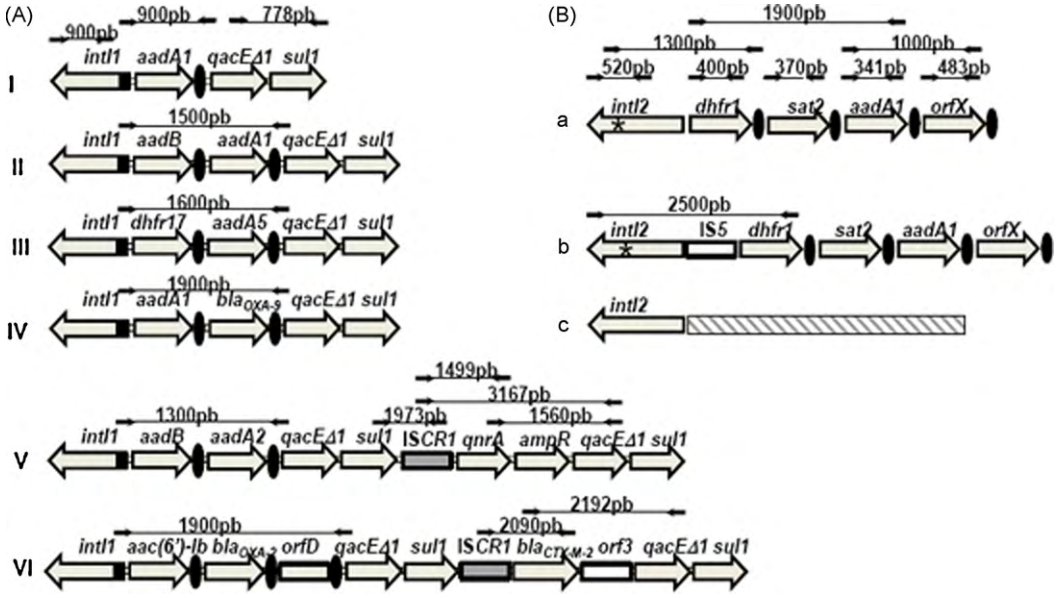

Fig. 1. Diagram depicting the different (A) class 1 and (B) class 2 integrons detected in this work. Roman numbers in (A) and lower case letters in (B) indicate the type of genetic arrangement. Small black arrows represent the different primers used to map the integrons; their location is relative to the DNA sequence to which they hybridise. Filled ovals represent the 59-bp element. Black boxes indicate the *intI1* promoter region and the *attI* site. Asterisks in (B) depict the internal stop codon usually present in these integrons. As shown in genetic arrangement c, the internal stop codon has disappeared, whereas the rest of this integron could not be characterised (symbolised by a striped box).

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vided by Dr Pablo Power (Facultad de Farmacia y Bioquímica, Buenos Aires, Argentina). The strategy used for gene walking is shown in Fig. 1B.

2.8. Pulsed-field gel electrophoresis (PFGE)

Resistant microorganisms acquired after admission to the ICU harbouring any of the genes under study and suspected of belonging to an episode of clonal dissemination were analysed by PFGE as previously reported [9].

2.9. Replicon typing

Strains carrying ESBLs and/or *qnr* genes underwent PCR replicon typing according to Carattoli et al. [14] to determine the incompatibility groups of plasmids carrying such genes.

3. Results

In total, 56 isolates showed resistance to ceftazidime and/or ciprofloxacin (24 *Enterobacter* spp., 16 *E. coli*, 12 *K. pneumoniae* and 4 *Citrobacter freundii*), of which 29 carried at least one of the genes under study (Table 1). Eleven isolates (six *K. pneumoniae*, one *E. coli*, three *Enterobacter* spp. and one *C. freundii*) yielded positive results in presumptive and confirmatory ESBL detection tests.

Five isolates (three *C. freundii* and two *Enterobacter cloacae*) harboured *qnr* alleles. Twenty-four strains presented either class 1 or 2 integrons. Table 1 depicts the main features of the 29 strains.

Of the 56 colonised patients, 29 already carried resistant microorganisms upon admission into the ICU, whereas the remaining 27 acquired resistant microorganisms after admission.

Furthermore, 16 (28.6%) of the 56 patients had the same drug-resistant microorganisms (at the species level and susceptibility profile) isolated either from the gut and/or pharynx as well as from diverse clinical samples (e.g. peritoneal fluid, surgical wound, tracheal aspirate, blood culture, urine culture, etc.) (data not shown). Ten of the sixteen microorganisms isolated from the gut or pharynx of such patients carried at least one of the genes under study (Table 1).

Unfortunately, most of the information regarding clinical samples was obtained retrospectively; hence, microorganisms isolated from such samples were no longer available for molecular typing assays.

3.1. Extended-spectrum β-lactamase identification

CTX-M-derived enzymes were detected in 10 of 11 ESBL-producing isolates; six (*K. pneumoniae*) corresponded to CTX-M-2, one (*E. coli*) to CTX-M-15 and three (two *E. cloacae* and one *Enterobacter aerogenes*) to CTX-M-9. In addition, PER-2 was detected in one *C. freundii* isolate. No TEM- or SHV-derived ESBL were detected in these strains.

3.2. Screening and determination of qnr allelic variants

Two *E. cloacae* isolates (4R-HC112 and 2R-HC158) and three *C. freundii* isolates (1R-HC157, 1R-HC164 and 1R-HC175) harboured *qnr* variants. These isolates were obtained from different ICU inpatients; interestingly, all microorganisms carrying *qnrB* alleles were isolated upon admission to the unit. Both *E. cloacae* isolates harboured *qnrA1*, whereas the *C. freundii* isolates presented three different *qnrB* variants, namely *qnrB4* (1R-HC175), *qnrB13* (1R-HC157) and *qnrB17* (1R-HC164). All of these isolates remained susceptible to ciprofloxacin (Table 1).

Surprisingly, whereas *qnrA1*-carrying isolates displayed a multiresistant profile [including resistance to trimethoprim/sulfamethoxazole (SXT), gentamicin and oxyimino-cephalosporins], those carrying *qnrB13* and *qnrB17* alleles were only resistant to oxyimino-cephalosporins.

3.3. Characterisation of class 1 integrons

Class 1 integrons were detected in 21 of the 56 studied strains (Table 1), clustered in six different genetic arrays. Four such arrays were simple integrons and the other two were complex integrons carrying the IS*CR1* element.

Apart from *sul1*, responsible for resistance to sulfonamides, eight different antibiotic resistance gene cassettes were found within the six arrays, namely resistance to aminoglycosides [*aadA1*, *aadA2*, *aadA5*, *aadB* and *aac(6′)-Ib*], β-lactams (bla_{OXA-2} and bla_{OXA-9}) and trimethoprim (*dfrA17*).

The complex class 1 integrons showed the following genes linked to IS*CR1*: $bla_{CTX-M-2}$ (resistance to oxyimino-cephalosporins); and *qnrA1* (diminished susceptibility to fluoroquinolones) and the regulator of class C β-lactamases, *ampR* (genetic arrangements VI and V, respectively; see Fig. 1A).

$bla_{CTX-M-2}$ was always found embedded in a complex class 1 integron, with a variable region formed by the cassettes *aac(6′)-Ib*–bla_{OXA-2}–orfD. This particular complex class 1 integron confers resistance to aminoglycosides (amikacin) and oxyimino-cephalosporins, two major antibiotic groups used within ICUs. The arrangement *aac(6′)-Ib*–bla_{OXA-2}–orfD was detected in six isolates, with this arrangement being second in frequency to variable regions composed of a single gene cassette, namely *aadA1*, present in nine strains (Table 1).

Another relevant integron is that identified as arrangement V (Fig. 1A), whose variable region is constituted by the genes *aadB*–*aadA2*, whereas the genes associated with IS*CR1* are *qnrA1*–*ampR*. This integron confers resistance to gentamicin (mediated by *aadB*) as well as diminished susceptibility to fluoroquinolones on account of *qnrA1*; this integron was found to be encoded in a conjugative plasmid >50 kb that also carries the gene $bla_{CTX-M-9}$ (data not shown).

3.4. Characterisation of class 2 integrons

Class 2 integrons were detected in 13 isolates. Interestingly, most of these isolates (10/13) also carried class 1 integrons.

The genetic arrangement *dfrA1*–*sat2*–*aadA1*–orfX was found in 12 class 2 integrons. Although this particular arrangement does not confer resistance to any antibiotic of clinical use, the simultaneous presence of class 1 and 2 integrons in a bacterial strain confers resistance to SXT. Ten such class 2 integrons had the IS*5* element located between the genes *intI2* and *dfrA1* (Fig. 1B). To our understanding, this is the first report of an IS*5* element inserted in a class 2 integron. This insertion sequence has been implicated in loss of porins and the emergence of resistance to carbapenems [15]. Considering that the gene cassette promoter in class 2 integrons is not clearly defined, the presence of this insertion sequence upstream of such cassettes could contribute not only to their horizontal transfer but also their expression.

Finally, one of the class 2 integrons (isolate 1R-HC081) lacked the stop codon (amino acid position 174) usually present in *intI2* genes. In this particular case, we were unable to determine the variable region, regardless of the various combinations of primers used. More studies are necessary to elucidate the genes encoded in this integron.

3.5. Pulsed-field gel electrophoresis patterns

Six *K. pneumoniae* and eight *E. cloacae* isolates were analysed by PFGE. Five of the *K. pneumoniae* isolates were grouped in

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Table 1
Main features of isolates carrying extended-spectrum β-lactamases (ESBLs), *qnr* alleles, and class 1 and 2 integrons.

Sample no.[a]	LoS (days)[b]	Microorganism	Class 1 integron		Class 2 integron		ESBL	*qnr*	PFGE pulsotype	Inc. group[c]	CIP MIC (μg/mL)	Resistance profile
			int1	Genetic arrangement	*int2*	Genetic arrangement						
2R-HC080[d]	25	*Citrobacter freundii*	–	–	–	–	PER-2	–		P,F	1	TGC, FQ, GEN, SXT
1R-HC081	10	*Escherichia coli*	–	–	+	c	–	–			6	FQ, SXT
1R-HC093	6	*E. coli*	+	III	–	–	–	–			8	TGC, FQ, GEN, SXT
1R-HC094[d]	27	*Enterobacter cloacae*	+	I	+	a	–	–			>32	TGC, FQ, SXT
2F-HC096	22	*Klebsiella pneumoniae*	+	VI	–	–	CTX-M-2	–	B1	F,A/C	0.064	TGC, GEN, SXT
2F-HC109[d]	47	*E. cloacae*	+	I	–	–	–	–	3c		>32	TGC, FQ, AMK, GEN, SXT
2R-HC110	13	*K. pneumoniae*	+	II	+	a	–	–	C		>32	TGC, FQ, AMK, SXT
4R-HC112	13	*E. cloacae*	+	V	–	–	CTX-M-9	*qnrA*1	1	HI1, HI2	0.38	TGC, GEN, SXT
1F-HC113[d]	9	*Enterobacter aerogenes*	–	–	–	–	CTX-M-9	–		K	0.023	TGC, GEN, SXT
1R-HC114	7	*E. coli*	+	IV	+	a	–	–			>32	TGC, FQ, AMK, GEN, SXT
1R-HC124	9	*E. cloacae*	+	II	+	b	–	–			>32	TGC, FQ, AMK, GEN, SXT
3R/F-HC125	8	*K. pneumoniae*	+	VI	–	–	CTX-M-2	–	B2	F, A/C	0.023	TGC, GEN, SXT
3R-HC136[d]	60	*E. cloacae*	+	I	+	a	–	–	3d		>32	TGC, FQ, SXT
3R/F-HC146[d]	16	*E. cloacae*	+	I	+	a	–	–	2b		>32	TGC, FQ, AMK, GEN, SXT
5R-HC149[d]	19	*E. cloacae*	+	I	+	a	–	–	2a		>32	TGC, FQ, AMK, GEN, SXT
1R/F-HC154[d]	2	*E. cloacae*	–	–	+	b	–	–			>32	TGC, FQ, SXT
1R-HC152	42	*K. pneumoniae*	+	VI	–	–	CTX-M-2	–		A/C	>32	TGC, FQ, AMK, GEN, SXT
1R-HC151[d]	66	*E. cloacae*	+	I	+	a	–	–			>32	TGC, FQ, AMK, GEN, SXT
1R-HC157	41	*C. freundii*	–	–	–	–	–	*qnrB13*		B/O,K	0.125	TGC
2R-HC158	11	*E. cloacae*	+	V	–	–	CTX-M-9	*qnrA*1	1	HI1, HI2	0.25	TGC, GEN, SXT
2R-HC159	14	*E. coli*	–	–	–	–	CTX-M-15	–		F	>32	TGC, FQ, GEN, SXT
6R-HC160	17	*K. pneumoniae*	+	VI	–	–	CTX-M-2	–	A	A/C	>32	TGC, FQ, GEN, SXT
2R-HC161	8	*E. cloacae*	+	I	+	a	–	–	3a		>32	TGC, FQ, AMK, GEN, SXT
1R-HC164	6	*C. freundii*	–	–	–	–	–	*qnrB17*		K	0.125	TGC
5R-HC167	17	*K. pneumoniae*	+	VI	–	–	CTX-M-2	–	A	A/C	>32	TGC, FQ, AMK, GEN
6R-HC168[d]	29	*K. pneumoniae*	+	VI	–	–	CTX-M-2	–	A	A/C	>32	TGC, FQ, AMK, GEN
4R-HC170	17	*E. cloacae*	+	I	+	a	–	–	3b		>32	TGC, FQ, AMK, GEN, SXT
1R-HC174	12	*Enterobacter intermedium*	+	I	–	–	–	–			>32	TGC, FQ, AMK, GEN, SXT
1R-HC175	1	*C. freundii*	–	–	+	a	–	*qnrB4*		K	0.25	TGC, GEN, SXT

PFGE, pulsed-field gel electrophoresis; CIP, ciprofloxacin; MIC, minimum inhibitory concentration; TGC, third-generation cephalosporins; FQ, fluoroquinolones; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; AMK, amikacin.

[a] Numerals indicate the number of the sample that yielded the first antibiotic-resistant isolate; R/F indicates whether the sample was of rectal or pharyngeal origin.

[b] Length of Intensive Care Unit stay.

[c] Plasmid incompatibility group determined by replicon typing.

[d] Patients in which the same microorganism (species level and susceptibility profile) was obtained from clinical samples and rectal/pharyngeal swabbing.

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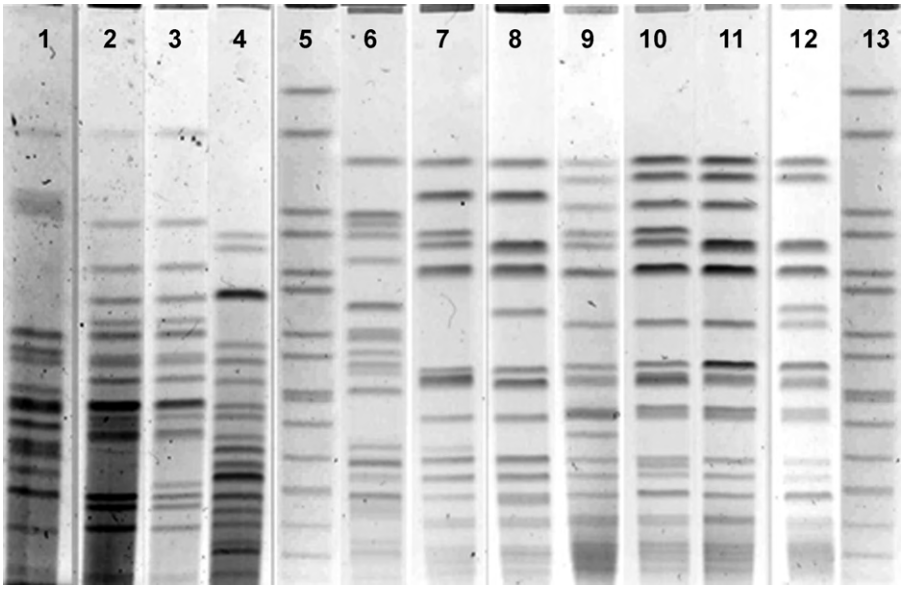

Fig. 2. Pulsed-field gel electrophoresis (PFGE) analysis of nosocomial isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae*. Lanes 1–4, pulsotypes of *K. pneumoniae* (lane 1, pulsotype A; lane 2, pulsotype B1; lane 3, pulsotype B2; and lane 4, pulsotype C); lanes 5 and 13, *Salmonella* serotype Braenderup; lanes 6–12, pulsotypes of *E. cloacae* (lane 6, pulsotype 1; lane 7, pulsotype 2a; lane 8, pulsotype 2b; lane 9, pulsotype 3a; lane 10, pulsotype 3b; lane 11, pulsotype 3c; and lane 12, pulsotype 3d).

two clusters: pulsotype A (6R-HC160, 5R-HC167 and 6R-HC168), and subtypes B1 (2F-HC096) and B2 (3R/F-HC125) (Table 1; Fig. 2). Whilst isolates belonging to pulsotype A were resistant to ciprofloxacin, those belonging to pulsotype B were susceptible to this antibiotic.

On the other hand, *E. cloacae* isolates 2R-HC158 and 4R-HC112 (carrying $bla_{\text{CTX-M-9}}$ and *qnrA1*) shared the same pulsotype (i.e. pulsotype 1). Interestingly, they showed no resemblance to pulsotypes 2 or 3, which did not harbour ESBLs.

3.6. Replicon typing

Isolates carrying ESBL genes were linked to various incompatibility groups. In this regard, those bearing $bla_{\text{CTX-M-2}}$ were associated with groups A/C, those carrying $bla_{\text{CTX-M-9}}$ belonged to groups HI and K, and the isolate carrying $bla_{\text{CTX-M-15}}$ belonged to group F. On the other hand, plasmids bearing *qnrA* belonged to group HI, whereas those harbouring the allelic variants of *qnrB* belonged to group K (Table 1).

4. Discussion

This is the first report regarding the situation of CTX-M ESBLs within an ICU in Uruguay. Despite the small number of samples, the results show notable differences with those obtained in Argentina [4] and with a previous report from Uruguay in which the detected enzymes corresponded only to the CTX-M-2 group [5,6].

Although CTX-M-2 is the most frequently found enzyme in this report (6/11), enzymes CTX-M-9 and CTX-M-15 constitute an important fraction (4/11) of ESBL-producing strains.

The variants detected in Uruguay belong to the same group of ESBLs described recently by Pallecchi et al. in Peru and Bolivia [2]. The current results could be compatible with the observations made by these authors regarding the dissemination and expansion of this group of β-lactamases in Latin America.

The ESBL diversity detected in this work probably constitutes a fraction of the actual number of enzymes circulating within the ICU under study. Considering the relatively high concentration of ceftazidime (4 mg/L) used in this work to select resistant microorganisms, strains harbouring ESBLs with weak activity against this drug (such as some variants of the CTX-M enzymes) may have been missed.

In this work, we also report for the first time the detection of *qnr* genes in Uruguay. *qnrA* alleles were found to be inserted in a complex class 1 integron with a genetic arrangement undescribed so far: *intI1–aadB–aadA2–qacEΔ 1–sul1*::IS*CR1*::*qnrA1*::*ampR*. To our understanding, this constitutes the first description of the genomic context of *qnrA1* in Latin America.

Regarding the occurrence of class 1 and 2 integrons, an important presence of genes conferring resistance to broad-spectrum antibiotics such as amikacin [*aac(6′)-Ib*], gentamicin (*aadB*), oxyimino-cephalosporins ($bla_{\text{CTX-M-2}}$) and quinolones (*qnrA1*) was found. This differs from a recent report by Marquez et al. [5] regarding *E. coli* isolates causing urinary tract infections in outpatients. Their report describes most gene cassettes as variants of *aadA* and *dfrA*, which confer resistance to streptomycin and trimethoprim, respectively. The extensive presence of *aadA* in both settings could be due to gratuitous selection on account of its widespread presence in class 1 integrons.

Irrespective of this, the difference in the amount of antibiotic resistance genes in integrons in isolates from hospital and community settings could be explained by the selective pressure exerted by the different antibiotics present in such environments.

On the other hand, the integron carrying $bla_{\text{CTX-M-2}}$ is of particular interest since it has been stably present in our region for over a decade [6].

Finally, the presence in an ICU of a class 2 integron with a presumptively functional integrase could set the stage for recombination events and acquisition of new gene cassettes by this genetic structure. So far there have been few reports describing such class 2 integrases, but only strains of human origin were linked to antibiotic resistance genes (*dfrA14*) [7].

The presence of microorganisms with a functional *intI2* gene within an ICU where most class 1 integrons either code for *aac(6′)-Ib* or *aadB* could imply that it is only a matter of time before we find class 2 integrons carrying those gene cassettes, thus further contributing to their horizontal dissemination.

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