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Extended-spectrum β-lactamases and plasmid-mediated quinolone resistance in enterobacterial clinical isolates in the paediatric hospital of Uruguay

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Objectives: To analyse the prevalence of resistance to β-lactams and plasmid-mediated quinolone resistance in Enterobacteriaceae in the paediatric hospital of Uruguay.

Methods: A total of 368 enterobacterial isolates collected between 1 May and 30 November 2009 were studied for the presence of extended-spectrum β-lactamases (ESBLs), *qnr* alleles and *aac(6′)Ib* by phenotypic and molecular methods. The genomic context and transferability of β-lactamase and *qnr* genes were examined by PCR and conjugation, respectively.

Results: The proportion of inpatients having an infection caused by ESBL-producing enterobacteria was 0.23% (16/7073) in paediatrics wards, 0.64‰ (3/4696) in the neonatology department and 0.03‰ (1/32 557) in the emergency department. ESBL-carrying enterobacteria constituted a total of 21.6% (16/74), 13% (3/23) and 0.37% (1/271) when samples were obtained from paediatrics wards, the neonatology department and the emergency department, respectively. Overall, CTX-M-2 ($n=7$), CTX-M-9 ($n=3$), CTX-M-8 ($n=2$), CTX-M-15 ($n=1$), SHV-5 ($n=5$) and SHV-2 ($n=2$) β-lactamases were detected. Thirteen out of 20 ESBL-producing isolates also carried the *aac(6′)Ib* gene, and the *cr* variant was detected in one of them. *qnr* alleles were detected in four isolates comprising two *qnrA1* genes, a *qnrB8*-like variant and a new *qnrB* gene showing 26 amino acid differences from QnrB1.

Conclusions: The proportion of ESBL-producing enterobacteria in Uruguay's paediatric hospital during the study period was 2.3 per 1000 hospitalized patients. The number of different microorganisms detected, as well as the various EBSLs, suggests the occurrence of sporadic episodes instead of nosocomial outbreaks. Nevertheless, the presence of new resistance genes reinforces the necessity for permanent surveillance programmes.

Keywords: antibiotic resistance, Enterobacteriaceae, integrons

Introduction

Enterobacteriaceae harbouring extended-spectrum β-lactamases (ESBLs) have been associated with an increase in mortality and healthcare-associated costs.[1] Co-resistance to fluoroquinolones due to the dissemination of plasmid-mediated quinolone resistance (PMQR) associated with the classical (mutation-based) resistance mechanisms is frequent.[2]

Although PMQR can be mediated by Qnr proteins (masking of target site), the production of Aac(6′)Ib-cr or QepA and OqxAB efflux pumps,[2] the first two mechanisms are by far the most frequent.

Data on the occurrence of both ESBLs and PMQR in paediatric patients from South America are scarce. So far, the only report on ESBLs from a paediatric population in Uruguay is that on PER-2 in typical enteropathogenic *Escherichia coli* (EPEC) strains isolated during the years 1991–93.[3] Although PMQR has been reported in an adult population[4,5] there are still no data concerning the paediatric population.

Materials and methods

A total of 368 enterobacterial isolates were recovered at the microbiology laboratory of Children's Hospital Pereira Rossell (CHPR) between 1 May and 30 November 2009. Approximately 96% of these isolates were recovered from the following sources: urine culture (82.1%); blood samples (7.9%); faeces (3.8%); or surgical wounds (2.4%). Only one clinically relevant specimen per patient per hospitalization event was included. For re-hospitalized patients, data from different isolates were only recorded if they belonged to different species or to different resistance profiles.

Identification to the species level was performed using the VITEK® 2 Compact system (bioMérieux, Marcy l'Étoile, France).

Antibiotic susceptibility tests were performed by a combination of diffusion tests (following CLSI recommendations[6]) and using the VITEK® 2 Compact system. Additionally, MICs of cefotaxime, amikacin and ciprofloxacin were determined by Etest for those enterobacteria harbouring ESBLs, according to the manufacturer's recommendations. ESBL screening and confirmatory tests were performed by disc diffusion, as suggested by CLSI guidelines,[6] regardless of bacterial genus or species, as previously suggested for areas of high CTX-M enzyme prevalence.[7]

Isolates with positive ESBL screening results were further analysed by PCR for the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{PER-2}}$ and bla_{SHV} genes using specific primers.[4] Positive samples were re-amplified using *Pfu* DNA polymerase (Fermentas Life Sciences) and fully sequenced on both strands.

The genes *qnrA*, *qnrB*, *qnrS* and *aac(6′)Ib* and the *cr* variant were sought in ESBL-producing isolates by PCR and amplicon sequencing as previously described.[4] We then used the deduced amino acid sequence of every available QnrB protein in public domain databases to construct a phylogenetic tree by the neighbour-joining method with the aid of MEGA4 software.[8]

Isolates harbouring *qnr* alleles were also tested for the presence of insertion sequences such as IS*CR1*, IS*26*, IS*903* and IS*Ecp1* according to Eckert *et al.*[9]

All confirmed ESBL-producing isolates were analysed for the presence of class 1 integrons by PCR, using primers I5/I3, 5′CS/3′CS, qacE1F/sul1b and ORFend/F12R.[4]

Conjugation assays were carried out using an *E. coli* J53 Rif^{R} strain as the recipient; transconjugants were selected on MacConkey agar plates supplemented with rifampicin (150 mg/L) and ceftriaxone (1 mg/L).[10]

Incompatibility groups of plasmids carrying ESBL and/or *qnr* or *aac(6′)Ib-cr* genes were determined by PCR replicon typing according to Carattoli *et al.*[11]

Data on patients within the study period were obtained from the hospital's information bureau (Sistema de Información Hospitalaria, El Centro Hospitalario Pereira Rossell en cifras 2009). Data on children in the neonatology service were obtained from the birth register of the CHPR.

Results

Two hundred and seventy-one enterobacteria were recovered from 32 557 children in the emergency department, 23/4696 from the neonatology unit and 74/7073 from inpatients from different services of the CHPR (such as the paediatrics wards, intensive care unit, orthopaedics, haematology/oncology and the surgery department). A total of 4945/7073 inpatients (69.9%) were admitted from the emergency department.

Twenty enterobacterial isolates (20/368) were characterized as ESBL producers (16 from paediatrics wards, 3 from neonatology and 1 from the emergency department). Two different isolates were obtained from the same child in two different hospitalization events, yielding a CTX-M-2-producing *E. coli* strain and a CTX-M-8-producing *Klebsiella pneumoniae* strain.

The proportion of inpatients having at least one infection episode caused by ESBL-producing enterobacteria was 2.26‰ (16/7073) in paediatrics wards, 0.64‰ (3/4696) in the neonatology department and 0.03‰ (1/32 557) in the emergency department. On the other hand the proportion of enterobacteria carrying ESBLs was 21.6% (16/74), 13% (3/23) and 0.37% (1/271) if samples were obtained from paediatrics wards, the neonatology unit and the emergency department, respectively.

ESBL-producing enterobacteria were recovered from 10 urine samples, 7 blood cultures, 1 skin lesion sample, 1 catheter tip sample and 1 synovial fluid sample. ESBL genes are shown in Table 1.

Thirteen out of 20 ESBL-harbouring isolates also carried the gene *aac(6′)Ib* coupled to $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-2}}$ or $bla_{\text{SHV-5}}$. Of these, one isolate carried the *aac(6′)Ib7* variant in a class 1 integron and displayed an MIC of amikacin as low as 3 mg/L, whereas another harboured the *aac(6′)Ib-cr* variant along with $bla_{\text{CTX-M-15}}$.

Four isolates harboured *qnr* variants. Two *Enterobacter cloacae* isolates carried the genes *qnrA1-ampR* linked to IS*CR1*, one *Citrobacter freundii* carried a *qnrB8*-like variant along with $bla_{\text{CTX-M-2}}$, and one *K. pneumoniae* harboured $bla_{\text{CTX-M-8}}$ and a *qnrB* variant linked to IS*Ecp1*. Regarding this isolate, both determinants were simultaneously transferred by conjugation, and transconjugants (TcKp737) showed an approximate 12-fold increase in ciprofloxacin MIC (0.38 mg/L versus 0.032 mg/L for the rifampicin-resistant *E. coli* receptor strain).

The partial nucleotide sequence of the *qnrB* variant (606 bp), obtained with primers qnrBR[4] and tnpAISEcp1,[9] showed 77% similarity with *qnrB17*, whereas the deduced amino acid sequence showed 87% identity with the corresponding protein, displaying 26 differences with QnrB1, 25 of which have not been described in the http://www.lahey.org/qnrStudies web site.

Class 1 integrons were detected in 14/20 strains, displaying eight distinct genetic arrays (Table 1). Such arrays carried 11 different gene cassettes, partially explaining resistance to aminoglycosides [*aadA1*, *aadA2*, *aadA5*, *aadB*, *aac(6′)Ib* and *aac(6′)Ib*$_7$], trimethoprim (*dfrA12*, *dfrA16*, *dfrA17* and *dfrA25*) and β-lactams ($bla_{\text{OXA-2}}$).

The $bla_{\text{OXA-2}}$ gene was always detected in integrons such as InK13, which codes for resistance to amikacin, oxyiminocephalosporins and tazobactam-like β-lactamase inhibitors.[12] Concerning isolates carrying $bla_{\text{CTX-M-2}}$, *E. coli* 954a harboured a complex integron with a different gene array from the one described above [i.e. *aadB-aad2* instead of *aac(6′)Ib-*$bla_{\text{OXA-2}}$*-orfD*]; this particular isolate was susceptible to piperacillin/tazobactam (Table 1).

Conjugation assay and replicon typing results of the 20 ESBL-carrying isolates are shown in Table 1.

Discussion

The proportion of ESBL-producing enterobacteria in the paediatrics wards of the CHPR during the study period was 2.3 per 1000, which is lower than in other reports on the subject.[13] Interestingly, the diversity of the detected microorganisms (and EBSLs), as well as the elapsed time between putatively related isolates (such as 532/593 or 954a/984), suggests the

Table 1. Main features of the ESBL-producing enterobacteria isolated in this study

Number	Service	Isolation date	Sample	Strain	MIC (mg/L)								PMQR	ESBL	5cs-3cs	Inc	Tc
					TZP	CTX	CAZ	AMK	GEN	NAL	CIP	SXT	*aac(6′)Ib*				
836	paediatrics	20/4/09	urine	*K. pneumoniae*	≤4	32	≤1	24	8	4	0.032	≤20	+	—	SHV-2	—	N-FIC-F	–
475	ICU	27/4/09	urine	*K. pneumoniae*	≥128	32	4	8	≥16	≤2	0.023	≤20	+	—	CTX-M-2	*aac(6′)Ib-bla*$_{OXA-2}$-*orfD*	A/C	+
343	neonatology	11/5/09	urine	*C. freundii*	≥128	≥256	4	16	≥16	≥32	1	≤20	+	*qnrB8*-like	CTX-M-2	—	—	–
954a	paediatrics	15/5/09	urine	*E. coli*	≤4	≥256	4	2	4	≥32	1	≤20	–	—	CTX-M-2	*aadB-aadA2*	FIB-F	–
576	ICU	27/5/09	blood	*Serratia marcescens*	≥128	16	16	16	≥16	≥32	0.75	≥320	+	—	SHV-5	*aadA1*	P	–
945	neonatology	2/6/09	urine	*S. marcescens*	≥128	4	16	12	≥16	≥32	0.5	≥320	+	—	SHV-5	*aadA1*	FIC	–
532	ICU	12/6/09	blood	*E. cloacae*	≤4	32	16	1	≤1	8	0.125	≥320	–	*qnrA1*	CTX-M-9	*aadB-aadA2/dfrA16-aadA2*	H11-HI2	+
327	HO	15/6/09	urine	*K. pneumoniae*	≥128	≥256	≥64	12	≤1	4	0.023	≥320	+	—	SHV-5	*dfrA25*	FIC-A/C	+
954b	paediatrics	8/7/09	urine	*K. pneumoniae*	≥128	64	≤1	1.5	≤1	≥32	≥32	≥320	–	—	CTX-M-8	*dfrA12-aadA2/dfrA25*	I1	+
463	orthopaedics	13/7/09	skin lesion	*E. coli*	≥128	≥256	4	16	≥16	≥32	≥32	≤20	+	—	CTX-M-2	*aac(6′)Ib-bla*$_{OXA-2}$-*orfD*	A/C	+
547	paediatrics	1/8/09	urine	*E. coli*	≤4	4	16	1	≤1	≥32	≥32	≥320	–	—	SHV-5	—	FIB	+
314	HO	14/9/09	blood	*S. marcescens*	≥128	≥256	≥64	24	≥16	≤2	0.125	≤20	+	—	CTX-M-2	*aac(6′)Ib-bla*$_{OXA-2}$-*orfD*	A/C	+
004	ICU	21/9/09	synovial fluid	*K. pneumoniae*	≥128	8	≥64	8	≥16	4	0.023	≥320	+	—	CTX-M-9	*aadB-aadA2*	H11-HI2	–
742	neonatology	5/10/09	blood	*K. pneumoniae*	8	32	4	16	≤1	≤2	0.023	≤20	–	—	SHV-2	—	K	+
984	paediatrics	8/10/09	urine	*E. coli*	≤4	≥256	4	1	≤1	4	0.012	≤20	–	—	CTX-M-2	—	FIB-F	–
631	surgery	13/10/09	catheter tip	*S. marcescens*	≤4	4	16	3	≥16	≥32	1	≥320	+	—	SHV-5	*aadA1/aac(6′)Ib7*	P	–
593	ICU	26/10/09	blood	*E. cloacae*	≥128	64	≥64	1.5	4	≥32	0.5	≥320	–	*qnrA1*	CTX-M-9	*aadB-aadA2/dfrA16-aadA2*	H11-HI2	+
025	emergency	29/10/09	urine	*K. pneumoniae*	≥128	≥256	4	16	≥16	4	0.032	≤20	+	—	CTX-M-2	*aac(6′)Ib-bla*$_{OXA-2}$-*orfD*	A/C	+
311	HO	16/11/09	blood	*E. coli*	≥128	4	≤1	16	4	≥32	≥32	≥320	+	*aac(6′)Ib-cr*	CTX-M-15	*dfrA17-aadA5*	FIA-F	–
737	ICU	27/11/09	blood	*K. pneumoniae*	≥128	16	≤1	16	8	≥32	1.5	≤20	+	*qnrBKp737*	CTX-M-8	—	L/M	+

TZP, piperacillin/tazobactam; CTX, cefotaxime; CAZ, ceftazidime; AMK, amikacin; GEN, gentamicin; NAL, nalidixic acid; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; ICU, intensive care unit; HO, haematology/oncology; 5cs-3cs, variable region of class 1 integron (slashes separate different integrons); Inc, plasmid incompatibility group; Tc, transconjugant.

occurrence of sporadic episodes instead of nosocomial outbreaks (Table 1).

Seventy percent of the inpatients admitted to the CHPR came from the emergency department, where the proportion of ESBL-producing enterobacteria is very low. The remaining 30% of inpatients was composed of patients transferred from hospitals throughout our country since the CHPR is the only tertiary referral paediatric hospital in Uruguay. This diversity of geographical zones could, in part, account for the heterogeneity of ESBLs and enterobacterial species. Demonstrating this, strain 954b carrying CTX-M-8 was isolated from a child living in a city bordering Brazil, the only country in South America that has reported the presence of this ESBL.[14]

The implementation of permanent infection control policies may account for the absence of intra-nosocomial outbreaks.

Many of the ESBL-producing isolates were also resistant to aminoglycosides and fluoroquinolones. Thirteen isolates showed MICs of amikacin between ≥8 mg/L and <32 mg/L; *aac(6′)Ib* was detected in 12/13 isolates (see Table 1). The interpretation of these results changes drastically depending on whether CLSI or European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines are used. Thus, whilst according to EUCAST guidelines the 13 isolates would be considered as resistant or intermediate, such microorganisms would be considered as susceptible to amikacin according to CLSI guidelines.

Interpretation of fluoroquinolone susceptibility levels is also troublesome on account of the emergence of PMQR; for example, only one of the *qnr*-carrying isolates would be interpreted as resistant according to CLSI, whereas according to EUCAST the number of resistant isolates would be two.

Thus, EUCAST guidelines appear to be a more powerful tool than CLSI guidelines for the detection of probable resistance mechanisms. Well-designed clinical trials are still required in order to verify whether the existing differences in the breakpoints defined by both guidelines could affect the outcome of a course of treatment with such antibiotics. For example, we detected in this work the occurrence of *qnr* variants conferring MICs of ciprofloxacin as low as 0.125 mg/L; hence, even in cases of MIC values as low as these the treating physician should be alerted about possible treatment failures.

Since the paediatric usage of fluoroquinolones in our country is restricted to life-threatening infections, co-selection of PMQR promoted by the administration of oxyiminocephalosporins or aminoglycosides is a likely explanation for the occurrence of these genes in the CHPR. The diversity of the EBSLs detected in this study is in accordance with previous results[4] that suggest the recent dissemination of CTX-M enzymes. Hence, whilst in Uruguay *K. pneumoniae* isolates carrying *bla*$_{CTX-M-2}$ were detected as early as 1996,[12] no CTX-M-9 and/or CTX-M-15-producing enterobacteria were detected until 2006.[4,5] Nevertheless, CTX-M-2 is still the most frequent CTX-M variant. Its presence in a complex InK13-like integron[12] within a conjugative plasmid (see Table 1), along with the fact that these genetic structures have been circulating in diverse hospitals within our country for the past 15 years, could in part account for this predominance.

Apart from these ESBLs, other resistance genes have appeared in our country, such as *aac(6′)Ib-cr* (associated with *bla*$_{CTX-M-15}$), *qnrA1* (associated with *bla*$_{CTX-M-9}$) and the new *qnrB* variant (*qnrBKp737*) associated with CTX-M-8. Although QnrBKp737 appears to be clustered with the rest of the QnrB

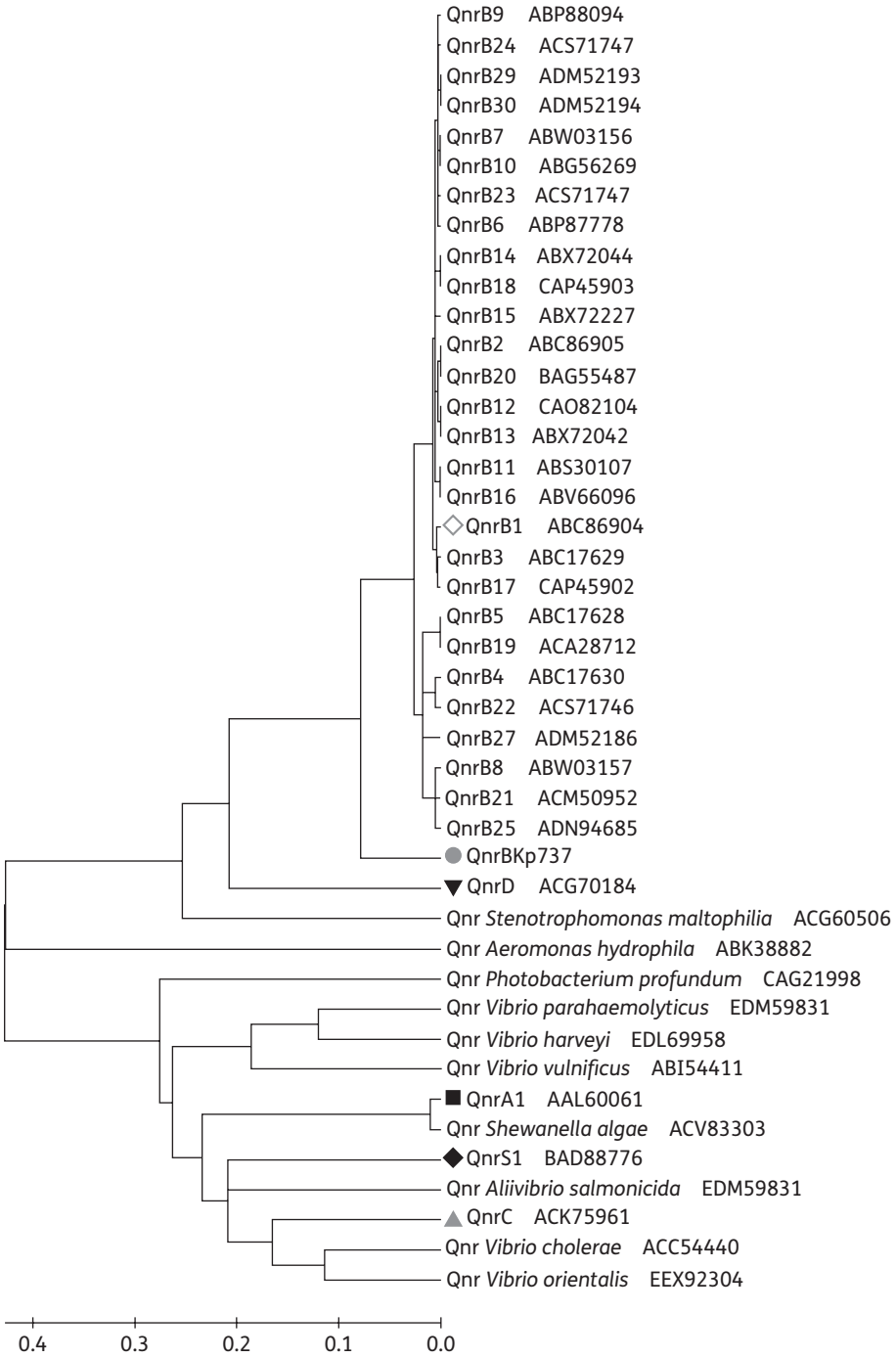

Figure 1. Phylogenetic tree of Qnr proteins. Different Qnr families are indicated by filled geometrical forms. The QnrB variant detected in this work (QnrBKp737) is indicated by a filled circle and QnrB1 is indicated by an open diamond; QnrA1 is indicated by a filled square, QnrD by a filled upside-down triangle, QnrC by a filled triangle and QnrS1 by a filled diamond.

sub-family, the phylogenetic analysis indicates that it is clearly different from the rest of the previously described QnrB proteins (see Figure 1). Additionally, this is the first description of a *qnrB* allele linked to IS*Ecp1*. This insertion sequence has been found next to several antibiotic resistance genes, such as *rmtC* (which confers resistance to aminoglycosides), and to various β-lactamases, mainly CTX-M-15.

Since the occurrence of ESBL-producing enterobacteria in the CHPR apparently is not associated with outbreaks, the clinical details of patients harbouring such microorganisms should be studied to identify any predisposing factor that may account for infections caused by them. Nevertheless, this work represents a starting point for the development of surveillance programmes aimed at the detection of ESBLs and PMQR.

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Transparency declarations

None to declare.

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