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Extended Spectrum ß-Lactamases and Plasmid Mediated Quinolone Resistance in Enterobacterial Clinical Isolates in the Paediatric Hospital of Uruguay.

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Keywords:	Antibiotic resistance, Enterobacteriaceae, Integrons

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1 Extended Spectrum β–Lactamases and Plasmid Mediated Quinolone Resistance in
2 Enterobacterial Clinical Isolates in the Paediatric Hospital of Uruguay.

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18 Short running title:

19 ESBL and PMQR in Hospitalized Children in Uruguay

20 Keywords: Antibiotic resistance, Enterobacteriaceae, Integrons

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21 Synopsis

22 Objectives: To analyze the prevalence of resistance to β-lactamics, and plasmid-
23 mediated quinolone resistance in Enterobacteriaceae in the paediatric hospital of
24 Uruguay.

25 Methods: A total of 368 enterobacterial isolates collected between May 1st and
26 November 30th (2009) were studied for the presence of Extended-Spectrum
27 β–lactamases (ESBLs), *qnr* alleles and *aac(6')-Ib* by phenotypic and molecular
28 methods. β-lactamase and *qnr* genes, their genomic context and transferability were
29 examined by PCR and conjugation, respectively.

30 Results: The proportion of inpatients having an infection caused by ESBL-producing
31 enterobacteria was 0.23% (16/7073) in paediatric wards, 0.64‰ (3/4696) in the
32 neonatology department and 0.03‰ (1/32,557) in the emergency department. ESBL-
33 carrying enterobacteria constituted a total of 21.6% (16/74), 13% (3/23) or 0.37%
34 (1/271) whether samples were obtained from paediatric wards, the neonatology
35 department or the emergency service, respectively. Overall, CTX-M-2 (n=7), CTX-M-9
36 (n=3), CTX-M-8 (n=2), CTX-M-15 (n=1), SHV-5 (n=5) and SHV-2 (n=2) β-lactamases
37 were detected. Thirteen out of 20 ESBL-producing isolates also carried the gene
38 *aac(6')-Ib*, and the *cr* variant was detected in one of them. *qnr* alleles were detected in
39 four isolates comprising two *qnrA1* genes, a *qnrB8*-like variant and a new *qnrB* gene
40 showing 26 aminoacidic differences regarding QnrB1.

41 Conclusions: The proportion of ESBL-producing enterobacteria in Uruguay's Paediatric
42 Hospital during the study period was 2.3 per 1000 hospitalised patients. The amount of
43 different microorganisms detected as well as the various EBSLs suggests the occurrence
44 of sporadic episodes instead of nosocomial outbreaks. Nevertheless, the presence of
45 new resistance genes reinforces the necessity for permanent surveillance programs.

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54 **Introduction:**

55 Enterobacteriaceae harbouring ESBLs have been associated with an increase in
56 mortality and health-care associated costs. [1] Co-resistance to fluoroquinolones due to
57 the dissemination of plasmid mediated quinolone resistance (PMQR) associated to the
58 classical (mutation based) resistance mechanisms is frequent. [2]

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

62 Data on the occurrence of both ESBLs and PMQR in paediatric patients from South
63 America are scarce. So far, the only report on ESBLs from a paediatric population in
64 Uruguay is that on PER-2 in typical enteropathogenic *E. coli* (EPEC) strains isolated
65 during the years 1991-93. [3] Although PMQR has been reported in an adult population [4,
66 5] there are still no data concerning the paediatric population.

67

68 **Material and methods:**

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

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76 Identification to the species level was performed using VITEK® 2 Compact system
77 (bioMérieux, Marcy l'Étoile, France).
78
79 Antibiotic susceptibility tests were performed by a combination of diffusion tests
80 (following CLSI recommendations [6]) and using the VITEK® 2 Compact system.
81 Additionally, MICs to cefotaxime, amikacin, and ciprofloxacin were determined by E-
82 test for those enterobacteria harbouring ESBLs, according to the manufacturer's
83 recommendations. ESBL screening and confirmatory tests were performed by disk
84 diffusion as suggested by CLSI guidelines [6] regardless of bacterial genus or species, as
85 previously suggested for areas of high CTX-M enzymes prevalence. [7]
86
87 Isolates with positive ESBL-screening results were further analysed by polymerase
88 chain reaction (PCR) for the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{PER-2}}$ and bla_{SHV} genes
89 using specific primers. [4] Positive samples were re-amplified using *Pfu* DNA
90 polymerase (Fermentas, Life Sciences) and fully sequenced on both strands.
91
92 The genes *qnrA*, *qnrB*, *qnrS*, *aac(6')Ib* and the *cr* variant were sought in ESBL-
93 producing isolates by PCR and amplicon sequencing as previously described. [4] We then
94 used the deduced aminoacidic sequence of every available QnrB protein in public
95 domain databases to construct a phylogenetic tree by the Neighbour-Joining method
96 with the aid of MEGA4 software. [8]
97
98 Isolates harbouring *qnr* alleles were also tested for the presence of insertion sequences
99 such as IS*CR1*, IS*26*, IS*903* and IS*Ecp1* according to Eckert *et al*. [9]

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

102 Conjugation assays were carried out using an *E. coli* J53 Rif^{R} strain as recipient;
103 transconjugants were selected on MacConkey agar plates supplemented with rifampin
104 (150 mg/L) and ceftriaxone (1 mg/L). [10]

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

107

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

112

113 **Results:**

114 Two hundred and seventy-one enterobacteria were recovered from 32,557 children
115 (271/32,557) at the emergency department, 23/4696 from the neonatology unit and
116 74/7073 from inpatients from different services of the CHPR (such as the paediatrics
117 ward, intensive care unit, orthopaedics, haematology/oncology, and the surgery
118 department). A total of 4945/7073 inpatients (69.9%) were admitted from the
119 emergency department.

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121 Twenty enterobacterial isolates (20/368) were characterized as ESBL-producers (16
122 from paediatric wards, 3 from neonatology, and one from the emergency department).
123 Two different isolates were obtained from the same child in two different
124 hospitalization events, rendering a CTX-M-2-producing *E. coli* strain and a CTX-M-8-
125 producing *K. pneumoniae* strain.

126

127 The proportion of inpatients having at least one infection episode by ESBL-producing
128 enterobacteria was 2.26‰ (16/7073) in paediatric wards, 0.64‰ (3/4696) in
129 neonatology department and 0,03‰ (1/32,557) in the emergency department. On the
130 other hand the proportion of enterobacteria carrying ESBLs was 21.6% (16/74), 13%
131 (3/23) and 0.37% (1/271) if samples were obtained from paediatric wards, the
132 neonatology unit or the emergency department, respectively.

133

134 ESBL-producing enterobacteria were recovered from urine samples (10), blood cultures
135 (7) and one skin lesion, catheter tip and synovial fluid samples. ESBL genes are
136 displayed in table 1.

137 Thirteen out of 20 ESBL-harbouring isolates also carried the gene *aac(6´)Ib* coupled
138 either to $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{SHV\text{-}2}$ or $bla_{SHV\text{-}5}$. Of these, one isolate
139 carried the *aac(6')Ib7* variant in a class-1 integron and displayed a MIC to amikacin as
140 low as 3 mg/L, whereas another harboured the *aac(6')Ib-cr* variant along with $bla_{CTX\text{-}M\text{-}}$
141 $_{15}$.

142 Four additional isolates harboured *qnr* variants. Two *E. cloacae* isolates carried the
143 genes *qnrA1-ampR* linked to IS*CR1*, one *C. freundii* carried a *qnrB8*-like variant along

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144 with *bla*$_{CTX-M-2}$, and one *K. pneumoniae* harbouring *bla*$_{CTX-M-8}$ and a *qnrB* variant linked
145 to IS*Ecp1*. Regarding this isolate, both determinants were simultaneously transferred by
146 conjugation, and transconjugants (TcKp737) showed an approximate 12-fold increase in
147 ciprofloxacin MIC (0.38 mg/L vs. 0.032 mg/L of the *E. coli* Rif^{r} receptor strain).

148 The partial nucleotide sequence of the *qnrB* variant (606bp), obtained with primers
149 qnrBR[4] and tnpAIS*Ecp1*[9] showed a 77% similarity with *qnrB17*, whereas the deduced
150 amino acid sequence showed an 87% identity with the corresponding protein,
151 displaying 26 differences with QnrB1, 25 of which have not been described in the
152 http://www.lahey.org/qnrStudies website.

153 Class-1 integrons were detected in 14/20 strains, displaying eight distinct genetic arrays
154 (table 1). Such arrays carried 11 different gene cassettes explaining partially, resistance
155 to aminoglycosides (*aadA1, aadA2, aadA5, aadB, aac(6')-Ib and aac(6')Ib*$_{7}$),
156 trimethoprim (*dfrA12, dfrA16, dfrA17, dfrA25*) and ß-lactams (*bla*$_{OXA-2}$).

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

163 Conjugation assays and replicon typing results of the 20 ESBL-carrying isolates are
164 displayed in table 1.

165

166 **Discussion:**

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167 The proportion of ESBL-producing enterobacteria in the paediatric wards of the CHPR
168 during the study period was 2.3 per 1000 which is lower than other reports on the
169 subject[13]. Interestingly, the diversity of the detected microorganisms (and EBSLs) as
170 well as the elapsed time between putatively related isolates (such as 532/593, or
171 954a/984), suggests the occurrence of sporadic episodes instead of nosocomial
172 outbreaks (see table 1).

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

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

183

184 Many of the ESBL-producing isolates were also resistant to aminoglycosides and
185 fluoroquinolones. Thirteen isolates showed MICs to amikacin between ≥8 mg/L and
186 less than 32 mg/L; *aac(6')Ib* was detected in 12/13 isolates (see table 1). The
187 interpretation of these results changes drastically depending on whether CLSI or
188 EUCAST guidelines are used. Thus, whilst according to EUCAST guidelines the 13
189 isolates would be considered as resistant or intermediate, such microorganisms would
190 be considered as susceptible to amikacin according to CLSI guidelines.

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191 Interpretation of fluoroquinolone susceptibility levels is also troublesome on account of
192 the emergence of PMQR; in this sense, only one of the *qnr*-carrying isolates would be
193 interpreted as resistant according to CLSI, whereas according to EUCAST the number
194 of resistant isolates would be two.

195 Thus, EUCAST guidelines appear to be a more powerful tool than CLSI guidelines for
196 the detection of probable resistance mechanisms. Well-designed clinical trials are still
197 required in order to verify whether the existing differences in the breakpoints defined by
198 both guidelines could affect the outcome of a course of treatment with such antibiotics.

199 In this sense, we detected in this work the occurrence of *qnr* variants conferring MICs
200 to ciprofloxacin as low as 0.125mg/L; hence, even in cases of MIC values as low as
201 these, the treating physician should be alerted about possible treatment failures.

202

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

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215 Apart from these ESBLs, other resistance genes have appeared in our country, such as
216 *aac(6')Ib-cr* (associated with *bla*$_{CTX-M-15}$), *qnrA1* (associated with *bla*$_{CTX-M-9}$) and the
217 new *qnrB* variant (*qnrBKp737)* associated with CTX-M-8. Although QnrBKp737
218 appears to be clustered with the rest of the QnrB sub-family, the phylogenetic analysis
219 indicates that it is clearly different from the rest of the previously described QnrB
220 proteins (see fig. 1). Additionally, this is the first description of a *qnrB* allele linked to
221 IS*Ecp1*. This insertion sequence has been found next to several antibiotic resistance
222 genes such as *rmtC* (which confers resistance to aminoglycosides), and to various β-
223 lactamases, mainly CTX-M-15.

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

229

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237

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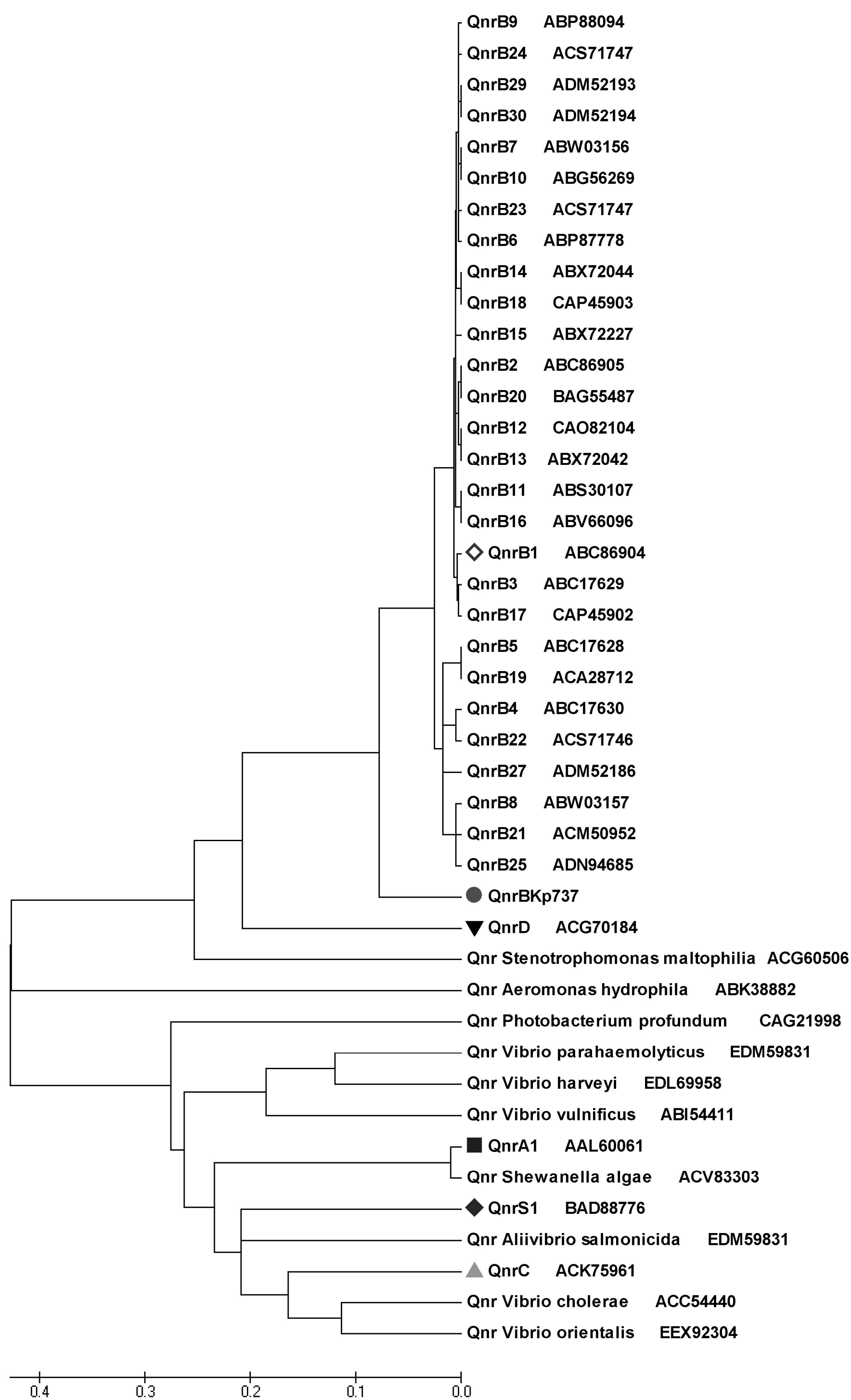
284 Fig.1



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289 Fig. 1) Phylogenetic tree of Qnr proteins. Different Qnr families are indicated by filled
290 geometrical forms. The QnrB variant detected in this work (QnrBKp737) is indicated by
291 a filled circle and QnrB1 is indicated by a diamond.

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

293 PTZ: piperacillin-tazobactam; CTX: cefotaxime; CAZ: ceftazidime; AMK: amikacin;
294 GEN: gentamicin; NAL: nalidixic acid; CIP: ciprofloxacin; SXT: trimethoprim-
295 sulfamethoxazole. MIC values are expressed in mg/L.
296 [a] HO: Haematology/Oncology; ICU: Intensive Care Unit.
297 [b] PMQR: Plasmid-Mediated Quinolone Resistance.
298 [c] ESBL: Extended-Spectrum β-lactamase.
299 [d] 5cs-3cs: Variable region of class-I integrons.
300 [e] Inc: Plasmid incompatibility groups.
301 [f] TC: Transconjugants.
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Number	Service [a]	Isolation Date (dd.mm.yy)	Sample	Strain	PTZ	CTX	CAZ	AMK	GEN	NAL	CIP	SXT	*aac(6')Ib*	PMQR [b]	ESBL [c]	5cs-3cs [d]	Inc [e]	Tc [f]
836	Paediatrics	20.4.09	uroculture	*K.pneumoniae*	≤ 4	32	≤ 1	24	8	4	0.032	≤ 20	+	-	SHV-2	-	N-FIC-F	-
475	ICU	27.4.09	uroculture	*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	uroculture	*C. freundii*	≥ 128	≥ 256	4	16	≥ 16	≥ 32	1	≤ 20	+	*qnrB8-like*	CTX-M-2	-	-	-
954a	Paediatrics	15.5.09	uroculture	*E.coli*	≤ 4	≥ 256	4	2	4	≥ 32	1	≤ 20	-	-	CTX-M-2	*aadB-aadA2*	FIB-F	-
576	ICU	27.5.09	bloodculture	*S.marcescens*	≥ 128	16	16	16	≥ 16	≥ 32	0.75	≥ 320	+	-	SHV-5	*aadA1*	P	-
945	Neonatology	2.6.09	uroculture	*S.marcescens*	≥ 128	4	16	12	≥ 16	≥ 32	0.5	≥ 320	+	-	SHV-5	*aadA1*	FIC	-
532	ICU	12.6.09	bloodculture	*E.cloacae*	≤ 4	32	16	1	≤ 1	8	0.125	≥ 320	-	*qnrA1*	CTX-M-9	*aadB-aadA2* *dfrA16-aadA2*	HI1-HI2	+
327	HO	15.6.09	uroculture	*K.pneumoniae*	≥ 128	≥ 256	≥ 64	12	≤ 1	4	0.023	≥ 320	+	-	SHV-5	*dfrA25*	FIC-A/C	+
954b	Paediatrics	8.7.09	uroculture	*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	uroculture	*E.coli*	≤ 4	4	16	1	≤ 1	≥ 32	≥ 32	≥ 320	-	-	SHV-5	-	FIB	+
314	HO	14.9.09	bloodculture	*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*	HI1-HI2	-
742	Neonatology	5.10.09	bloodculture	*K.pneumoniae*	8	32	4	16	≤ 1	≤ 2	0.023	≤ 20	-	-	SHV-2	-	K	+
984	Paediatrics	8.10.09	uroculture	*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	bloodculture	*E.cloacae*	≥ 128	64	≥ 64	1.5	4	≥ 32	0.5	≥ 320	-	*qnrA1*	CTX-M-9	*aadB-aadA2* *dfrA16-aadA2*	HI1-HI2	+
025	Emergency	29.10.09	uroculture	*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	bloodculture	*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	bloodculture	*K.pneumoniae*	≥ 128	16	≤ 1	16	8	≥ 32	1.5	≤ 20	+	*qnrBKp737*	CTX-M-8	-	L/M	+