Multiple publications have focused on the microbiological and clinical aspects of infections caused by quinolone-resistant \textit{E. coli} in adults (Boyd et al., 2008), but there is little information related to paediatric patients (particularly neonates) as quinolones are not usually recommended for these patients (Van den Oever et al., 1998).

A relationship between antimicrobial, resistance, virulence and phylogenetic group in \textit{E. coli} has been reported (Horcajada et al., 2005). Isolates of the two phylogenetic groups more often isolated from invasive infections (B2 and D) usually contain more virulence factors than those of groups A or B1, which are frequently isolated as commensal isolates (Escobar-Páramo et al., 2004). However, \textit{E. coli} strains resistant to quinolones commonly present less virulence factors than quinolone-susceptible isolates (Horcajada et al., 2005). Again, there is limited information on this relationship when considering neonates.

Quinolones, cotrimoxazole and tetracyclines are not used in the neonatal unit of our hospital (University Hospital Marqués de Valdecilla). Unexpectedly, quinolone-, trimethoprim/ sulfamethoxazole- and tetracycline-resistant clinical isolates of \textit{E. coli} were cultured from some of the admitted neonates. The objectives of this work were to describe the clonal relationship, the phylogenetic group, the mechanisms of resistance to quinolones and the presence of genes coding for virulence factors in these isolates.

A total of 111 \textit{E. coli} isolates were cultured from clinical samples from 1225 neonates admitted to the neonatal unit at our hospital from January 2005 to May 2006. Identification and susceptibility testing, including determination of extended-spectrum $\beta$-lactamase (ESBL) production, were routinely performed with the WalkAway system (Dade Behring). A total of 18 out of the 111 (16.2\%) \textit{E. coli} isolates intermittently sensitive or resistant [as determined by Clinical and Laboratory Standards Institute (CLSI) breakpoints (CLSI, 2010)] to at least three of the following – amoxicillin, amoxicillin/ clavulanate, nalidixic acid, gentamicin and trimethoprim/sulfamethoxazole – were selected for further studies. Clinical data for the 18 corresponding patients (12 males and 6 females) are presented in Table 1. All but 1 were preterm newborns, and 15 of them weighed less than 2000 g. Fourteen (78\%) patients received parenteral nutrition, eleven (61\%) were under mechanical ventilation and seven (39\%) had a central venous catheter. The organisms were cultured from diverse clinical samples, as presented in Table 1. Antimicrobial treatment covering the multiresistant \textit{E. coli} (cefotaxime and/or a carbapenem) was given to six patients. None of the patients died.

MICs of the following antimicrobial agents were determined for the 18 \textit{E. coli} isolates by standardized broth microdilution (CLSI, 2009): amoxicillin (Sigma-Aldrich), amoxicillin/clavulanate (2:1) (GlaxoSmithKline), piperacillin (Sigma-Aldrich)/tazobactam (Wyeth Pharmaceuticals); fixed concentration of 4 $\mu$g tazobactam ml$^{-1}$, amikacin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), tobramycin (Sigma-Aldrich), nalidixic acid (Sigma-Aldrich), ciprofloxacin (Fluka), tetracycline (Sigma-Aldrich), doxycycline (Sigma-Aldrich), and cotrimoxazole [sulfamethoxazole (Sigma-Aldrich) plus trimethoprim (Sigma-Aldrich) (19:1)]. MICs of levofloxacin were determined by Etest (bioMérieux) following the manufacturer’s recommendations.

The clonal relationship of the isolates was determined by Rep-PCR (repetitive extragenic palindromic PCR) (Vila et al., 1996). Two isolates were considered clonally unrelated when two or more different bands were observed (Canó et al., 2009). Determination of phylogenetic groups was performed by multiplex PCR (Clermont et al., 2000). Three Rep-PCR patterns (I, II and III) distributed in different periods, with no overlapping among them, were defined: pattern I contained four isolates (phylogenetic group D), pattern II nine isolates (group B2), and pattern III five isolates (group D). None of the isolates produced an ESBL. All 18 isolates were resistant to amoxicillin, nalidixic acid and gentamicin, and 17 of them were also resistant to cotrimoxazole. MICs of ciprofloxacin for isolates with Rep-PCR patterns II and I ranged from 8 to 64 mg l$^{-1}$, and for isolates of pattern III ranged from 1 to 2 mg l$^{-1}$.

Changes in the quinolone-resistance-determining region of the \textit{gyrA} and \textit{parC} genes were studied by PCR amplification and sequencing (Canó et al., 2009) in 11 isolates representative of the different Rep-PCR and susceptibility patterns. The following mutations in the topoisomerase subunits were observed: S83L plus D87N (GyrA) and S801 (ParC) in Rep-PCR pattern I; S83L plus D87N (GyrA) and S801 plus E84V (ParC) in Rep-PCR pattern II; and S83L (GyrA) and G78C (ParC) in Rep-PCR pattern III. None of the isolates contained plasmid-encoded quinolone-resistance genes \textit{qnrA}, \textit{qnrB}, \textit{qnrS}, \textit{qepA} and \textit{aac(6')-Ib-cr} when these genes were tested by multiplex PCR (Canó et al., 2009). Large plasmids around 100–150 kb were extracted by the Kado and Liu method (Kado & Liu, 1981) and characterized by replicon typing (Carattoli et al., 2005). Isolates of Rep-PCR pattern I carried IncN and IncF (positive for repFIA and repFIB) plasmids, those of pattern II carried IncN and IncF (positive for repFIA, repFIB and repF) plasmids, and those of pattern III carried IncI1, IncN and IncF (positive for repFIB and repF) plasmids. Conjugation experiments were carried out for one isolate of each clonal group using azide-resistant \textit{E. coli} J53 as the recipient strain. Transconjugants were selected with sodium azide (100 mg l$^{-1}$; Sigma) and ampicillin (100 mg l$^{-1}$), gentamicin (8 mg l$^{-1}$) or sulfamethoxazole (1000 mg l$^{-1}$). None of the transconjugants were resistant or presented decreased
Table 1. Clinical and microbiological data from 18 newborns with multiresistant *E. coli*

All isolates were negative for *cnf1, focG, sfaS, sfa/foc operon, bma, gaf, afa/dra, hlyA, kpsMTK1* and *kpsMTIII.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rep-PCR</th>
<th>Group</th>
<th>Virulence factors</th>
<th>Sex</th>
<th>Weight (g)</th>
<th>Admission date</th>
<th>Sample*</th>
<th>Other organisms</th>
<th>Previous treatment (days)†</th>
<th>Invasive procedures‡</th>
<th>LHS (days)§</th>
<th>Treatment†</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>III</td>
<td>D</td>
<td>traT, fyuA, iroN</td>
<td>M</td>
<td>750</td>
<td>24/12/2004</td>
<td>NPL</td>
<td>No</td>
<td>Amp+Tob (10)</td>
<td>MV, NG, PN</td>
<td>60</td>
<td>Cro (2); Cpe + Amk (6); Flu (30)</td>
</tr>
<tr>
<td>92</td>
<td>III</td>
<td>D</td>
<td>traT, fyuA, iroN</td>
<td>F</td>
<td>1070</td>
<td>30/12/2004</td>
<td>Conj. swab</td>
<td>No</td>
<td>Amp+Tob (10)</td>
<td>CVC, MV, NG, PN</td>
<td>57</td>
<td>Ctx (13)</td>
</tr>
<tr>
<td>147</td>
<td>III</td>
<td>D</td>
<td>traT, fyuA, iroN</td>
<td>M</td>
<td>680</td>
<td>11/01/2005</td>
<td>Sputum</td>
<td>No</td>
<td>Amp+Tob (10)</td>
<td>NG</td>
<td>56</td>
<td>Ctx (5)</td>
</tr>
<tr>
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<td>D</td>
<td>traT, fyuA, iroN</td>
<td>M</td>
<td>1130</td>
<td>30/12/2004</td>
<td>NPL</td>
<td>No</td>
<td>Amp+Tob (21)</td>
<td>CVC, LP, MV, NG, PN</td>
<td>61</td>
<td>Ctx + Amk + AFB (25); Tei (6)</td>
</tr>
<tr>
<td>537</td>
<td>III</td>
<td>D</td>
<td>traT, fyuA, iroN</td>
<td>F</td>
<td>2040</td>
<td>28/01/2005</td>
<td>NPL</td>
<td>Enterobacter cloacae</td>
<td>Amp+Tob (21)</td>
<td>MV, PN</td>
<td>107</td>
<td>Amp+Tei + Imp</td>
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<tr>
<td>1885</td>
<td>II</td>
<td>B2</td>
<td>traT, fyuA, PAI, KpsMTII, KpsMTK5</td>
<td>M</td>
<td>590</td>
<td>02/05/2005</td>
<td>Trach. asp.</td>
<td>Enterobacter cloacae</td>
<td>Amp+Tob (21)</td>
<td>MV, PN</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>B2</td>
<td>traT, fyuA, iroN</td>
<td>M</td>
<td>1040</td>
<td>04/06/2005</td>
<td>Conj. swab</td>
<td>No</td>
<td>Amp+Tob (10)</td>
<td>NG, PN</td>
<td>63</td>
<td>Amp + Imp</td>
</tr>
<tr>
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<td>B2</td>
<td>traT, fyuA, iroN</td>
<td>M</td>
<td>1080</td>
<td>23/06/2005</td>
<td>Umb.</td>
<td>No</td>
<td>Amp+Tob (10)</td>
<td>NG, PN</td>
<td>57</td>
<td>None</td>
</tr>
<tr>
<td>2359</td>
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<td>B2</td>
<td>traT, fyuA, iroN</td>
<td>F</td>
<td>1290</td>
<td>11/06/2005</td>
<td>Urine</td>
<td>Klebsiella oxytoca</td>
<td>Amp+Tob (26)</td>
<td>MV, NG, LP, PN</td>
<td>54</td>
<td>AFB</td>
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<tr>
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<td>B2</td>
<td>iroN, PAI, KpsMTII, KpsMTK5</td>
<td>F</td>
<td>2190</td>
<td>14/07/2005</td>
<td>NPL</td>
<td>Enterobacter cloacae</td>
<td>Amp+Tob (21)</td>
<td>MV, CVC, PN, SPP</td>
<td>48</td>
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<td>B2</td>
<td>iroN, PAI, KpsMTII, KpsMTK5</td>
<td>M</td>
<td>1020</td>
<td>20/08/2005</td>
<td>Conj. swab</td>
<td>No</td>
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<td>MV, NG, PN</td>
<td>67</td>
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<tr>
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<td>M</td>
<td>990</td>
<td>20/07/2005</td>
<td>FN</td>
<td>Enterobacter cloacae</td>
<td>Amp+Tob (21)</td>
<td>MV, CVC, NG, PN</td>
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<td>Amx (14)</td>
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<td>iroN, PAI, KpsMTII, KpsMTK5</td>
<td>M</td>
<td>1200</td>
<td>19/09/2005</td>
<td>NPL</td>
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<td>CVC, LP, NG</td>
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<td>iroN, PAI, KpsMTII, KpsMTK5</td>
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<td>2890</td>
<td>10/10/2005</td>
<td>NPL</td>
<td>No</td>
<td>None</td>
<td>None</td>
<td>23</td>
<td>None</td>
</tr>
<tr>
<td>1363</td>
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<td>D</td>
<td>ibeA</td>
<td>M</td>
<td>930</td>
<td>18/04/2006</td>
<td>Conj. swab</td>
<td>No</td>
<td>Amp+Tob (12)</td>
<td>MV, NG, PN, TD</td>
<td>90</td>
<td>Tei + Mer + Ctx + Flu</td>
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<td>1564</td>
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<td>D</td>
<td>ibeA</td>
<td>F</td>
<td>1810</td>
<td>29/04/2006</td>
<td>NPL</td>
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<td>Amp+Tob (10)</td>
<td>MV, NG, PN</td>
<td>23</td>
<td>None</td>
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<tr>
<td>1848</td>
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<td>D</td>
<td>ibeA</td>
<td>F</td>
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<td>22/04/2006</td>
<td>Conj. swab</td>
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<td>None</td>
<td>None</td>
<td>2</td>
<td>None</td>
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<td>1902</td>
<td>I</td>
<td>D</td>
<td>No positive results</td>
<td>M</td>
<td>1450</td>
<td>11/05/2006</td>
<td>Blood</td>
<td>No</td>
<td>Cur + Amk</td>
<td>CVC, LP, PN</td>
<td>36</td>
<td>Tei + Tob</td>
</tr>
</tbody>
</table>

F, Female; M, male.

*Conj. swab, Conjunctival swab; NPL, nasopharyngeal lavage; Trach. asp, tracheal aspirate; Umb., Umbilical swab.*

†AFB, Amphoterericin B; Amk, amikacin; Amp, ampicillin; Amx, amoxicillin; Cpe, cefepime; Cro, ceftriaxone; Ctx, cefotaxime; Cur, cefturoxime; Flu, fluconazole; Imp, imipenem; Mer, meropenem; Tei, teicoplanin; Tob, tobramycin.

‡CVC, Central venous catheter; LP, lumbar puncture; MV, mechanical ventilation; NG, nasogastric tube; PN, parenteral nutrition; SPP, suprapubic puncture; TD, thoracic drainage.

§LHS, Length of hospital stay in the neonatal unit.
susceptibility to quinolones nor contained any of the studied plasmid-encoded quinolone-resistance genes. Transconjugants from the same clonal group presented a similar resistance phenotype and plasmid content: those from Rep-PCR pattern I (selected with ampicillin) were resistant to ampicillin and contained an IncF (FIB replicon) plasmid; those from pattern II (selected with all three antimicrobial agents used) were resistant to ampicillin, gentamicin, sulfamethoxazole and tetracycline, and contained IncF (replicons FIA, FIB, FII) plasmids; and those from pattern III (selected with ampicillin or gentamicin) were resistant to ampicillin and gentamicin, and contained plasmids of IncF (FIB replicon), IncN and IncI1.

Virulence factors encoded by genes hlyA, sfaS, focG, fimH, bma, gaf, afuA, dnaK, iutA, fyuA, traT, sfa/foc operon, ibeA, PAI, kpsMTIII, kpsMTIII, KpsMTK1 and KpsMTK5, were determined by PCR, as described elsewhere (Johnson & Stell, 2000). Detection of iroN was carried out by PCR using primers IroN-F, 5’-GAAAGCTCTGG-TGGAGCGTGTA-3’, and IroN-R, 5’-CAGACA-GAGGATTCGCTGTG-3’. None of the isolates were haemolytic on sheep blood agar nor contained the hlyA gene. All isolates studied contained the genes for virulence determinants iutA and fimH. In two out of three isolates of clonal group I studied the ibeA gene was also detected. All isolates belonging to Rep-PCR pattern II were positive for fyuA, PAI, kpsMTII and kpsMTK5, and two of them also had the traT gene. In all four isolates of clonal group III studied, traT, ibeA and fyuA were detected (Table 1).

In this study we have characterized multiresistant ESBL-negative E. coli from patients admitted to our neonatal unit, where quinolones, trimethoprim/sulfamethoxazole and tetracyclines are not used. Quinolones-resistance rates are usually lower in children than in adults (Boyd et al., 2008).

Quinolone resistance in E. coli is a multifactorial process. Single mutations in gyrA cause resistance to nalidixic acid and a moderate decrease in the activity of fluoroquinolones (Hopkins et al., 2005). Additional mutations in this gene or in parC cause stepwise increases in the level of resistance, as observed in our isolates with Rep-PCR patterns III (with one mutation in gyrA and another one in parC), which were either susceptible (two isolates) or intermittently susceptible (three isolates) to fluoroquinolones. Clinically relevant resistance to fluoroquinolones (as defined by the CLSI) is reached when 3–4 mutations have been selected in gyrA/parC (Calva et al., 1996), just as observed in organisms with Rep-PCR patterns I and II.

Resistance to aminoglycosides has been well documented in enterobacteria isolated from newborns (Van der Zwaet et al., 1999). The genes involved are usually encoded on conjugative plasmids also containing genes responsible for trimethoprim/sulfamethoxazole and tetracycline resistance. In the isolates we studied, resistance to these agents was determined by conjugative plasmids of incompatibility groups IncF, IncN and/or IncI1, usually related to antimicrobial resistance in enterobacteria (Carattoli, 2009).

Most reports indicate that quinolone-resistant E. coli are usually of phylogenetic group D or A (Johnson & Stell, 2000). This agrees with our isolates of clonal groups I and III, both included in group D. In addition, the isolates of pattern II corresponded to group B2. Isolates of clonal group II/phylogenetic group B2 contained a higher number of virulence determinant genes than those of the other two clonal groups of phylogenetic group D, a situation also in agreement with previous studies.

Clonal analysis showed that the 18 isolates were obtained in 3 separated periods. This multiclonality suggests that the introduction of the resistant organisms in the unit occurred independently on every occasion. Two clusters persisted for just 1 or 2 months, but another one lasted for 7 months. Other authors have also demonstrated, using molecular methods, the existence of several clusters (with a small to large number of patients affected) of multiresistant organisms within the same neonatal unit (De Oliveira Garcia et al., 2008).

Quinolones are not used in our unit and, therefore, they cannot be blamed as the selecting agents of the multiresistant isolates. We may speculate that the indicated chromosomal mutations have been selected elsewhere and that the newborns have acquired the resistant organisms either directly or indirectly from an external source: their mother or other relatives who may have been in contact with them, health workers or other previously colonized or infected newborns. Unfortunately, the retrospective nature of this study precludes a more precise definition of these possibilities. As the isolates were also resistant to ampicillin and resistant or of intermediate sensitivity to tobramycin, and the newborns were empirically treated with a combination of these agents, it is also possible that these compounds may have favoured the colonization and persistence of these resistant organisms.

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B. Ruiz del Castillo,1 C. García de la Fuente,1 J. Agüero,1,2 J. Oteo,3 J. Gómez-Ullate,4 V. Bautista,5 and L. Martínez-Martínez2,6

1Microbiology Service, University Hospital Marqués de Valdecilla – IFIMAV (Instituto de Formación e Investigación Marqués de Valdecilla), Santander, Spain
2Department of Molecular Biology, University of Cantabria, Santander, Spain
3Antibiotic Laboratory, Bacteriology Service, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain
4Service of Paediatrics, University Hospital Marqués de Valdecilla – IFIMAV (Instituto de Formación e Investigación Marqués de Valdecilla), Santander, Spain

Correspondence: L. Martínez-Martínez (lmartinez@humv.es)


