J Antimicrob Chemother 2016; **71**: 1174–1177
doi:10.1093/jac/dkv459 Advance Access publication 10 January 2016

Journal of Antimicrobial Chemotherapy

ESBL-producing Enterobacteriaceae in 24 neonatal units and associated networks in the south of England: no clustering of ESBL-producing *Escherichia coli* in units or networks

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Received 31 July 2015; returned 24 November 2015; revised 2 December 2015; accepted 3 December 2015

Objectives: The objectives of this study were to characterize ESBL-producing Enterobacteriaceae present in 24 neonatal units (NNUs) in eight networks participating in a multicentre probiotic study and to test the hypothesis that specific strains would cluster within individual units and networks.

Methods: We performed analysis of stool samples for the presence of ESBL-producing Enterobacteriaceae at 2 weeks post-natal age and 36 weeks post-menstrual age. ESBL-producing Enterobacteriaceae were characterized and typed using molecular methods.

Results: ESBL-producing Enterobacteriaceae ($n=71$) were isolated from 67/1229 (5.5%) infants from whom we received a sample at either sampling time or both sampling times, and from infants in 18 (75%) of the 24 recruiting NNUs. Thirty-three *Escherichia coli*, 23 *Klebsiella* spp. and 6 *Enterobacter* spp. strains were characterized. ESBL-producing *E. coli* were all distinguishable within individual NNUs by antibiotic resistance genotype, serogroup (O25b), phenotype, phylotype or ST. Ten of the 33 were ST131 and 9 of the 10 ST131 isolates were ciprofloxacin resistant. Seven of the 10 ST131 isolates carried genes encoding CTX-M group 1 enzymes. ST131 isolates were isolated from centres within five of the eight NNU networks. There were clusters of indistinguishable ESBL-producing *Klebsiella* and *Enterobacter* isolates associated with specific NNUs.

Conclusions: Strains of *E. coli* ST131 were distributed across neonatal networks in the south of England. There was no evidence of clustering of clonally related ESBL-producing *E. coli* strains, by contrast with *Klebsiella* spp. and *Enterobacter* spp., which did cluster within units. The possibility that ESBL-producing *E. coli* strains are spread by vertical transmission requires further investigation.

Introduction

ESBL-producing Enterobacteriaceae can cause outbreaks in many contexts, including neonatal units (NNUs).[1–3] The objectives of this study were to identify the range of ESBL-producing Enterobacteriaceae prevalent in NNUs participating in a multicentre study in the south of England. There is evidence that virulent clones of ESBL-producing *Escherichia coli* can be transmitted from mothers to infants early in life,[4,5] so a second objective was to explore the relative importance of horizontal and vertical transmission by determining the extent to which specific clones cluster within NNUs or networks.

Methods

The PiPS study was a multicentre probiotic study (https://www.npeu.ox.ac.uk/pips). Ethics approval for the PiPS study was given by the NRES Committee South Central—Oxford A, Reference 09/H0604/30. A secondary objective of the study was to determine rates of carriage of ESBL-producing Enterobacteriaceae in the probiotic and placebo groups. Infants were recruited between July 2010 and July 2013. Stool samples were collected from infants at 2 weeks post-natal age and 36 weeks post-menstrual age, and were transported to the microbiology laboratory at Barts Health NHS Trust using the Thermacor transportation system (Dyecor Ltd, Herefordshire, UK). Specimens were weighed and divided into two equal parts. One half was stored at −80°C to allow additional tests. The other half was diluted 1:10 in a cryopreservative broth (brain heart infusion; Oxoid Ltd, Basingstoke, UK) containing 10% glycerol (w/v), mixed by vortexing for 10 s and then placed in 1 mL aliquots into sterile 1.5 mL Eppendorf tubes before freezing at −80°C.

Culture, identification and enumeration of ESBL-producing Enterobacteriaceae

Vials of frozen samples in cryopreservative were thawed at room temperature and serially diluted in brain heart infusion broth (Oxoid Ltd). Aliquots (100 μL) of the neat sample and 10^{-1}, 10^{-3} and 10^{-5} dilutions were

inoculated on to MacConkey and Brilliance™ ESBL agar (Oxoid Ltd). Inoculated media were incubated at 37°C for 24–36 h in air.

Bacterial colonies were enumerated and identified using standard laboratory methods, including MALDI-TOF MS (Bruker UK Ltd). Gram-negative bacilli that grew on MacConkey or Brilliance ESBL agars were tested using the BSAC disc diffusion method and ESBL production was confirmed using cefotaxime/clavulanate and cefepime/clavulanate combined disc tests (Total ESBL Confirm Kit, Rosco Diagnostica, Taastrup, Denmark). When the same ESBL-producing species was isolated at both sampling times, then only the 2 week post-natal age isolate was characterized.

Identification of resistance genes in ESBL isolates

DNA was released by inoculation of one colony into 100 μL of distilled water and heating to 100°C for 10 min followed by centrifugation for 1 min. Multiplex PCR assays were used to detect the presence of genes encoding major β-lactamase families (TEM, SHV, OXA-1/4/30/48, CTX-M groups 1, 2 and 9, plasmidic AmpCs, VEB, PER, GES, IMP, VIM and KPC-like),[6] aminoglycoside-modifying enzymes (APH, ANT and AAC)[7,8] and the *aac(6′)-Ib-cr* variant associated with fluoroquinolone resistance.[9]

All PCRs were carried out using Thermoprime ReddyMix™ PCR Master Mix (Thermo Scientific, USA). Amplicons were visualized following electrophoresis on 1.5% agarose gels containing ethidium bromide at 100 V for 40 min.

Molecular typing of E. coli

E. coli phylogroup was determined by PCR methods[10] and allele-specific PCR was used to detect the 025b-ST131 subtype.[11] Isolates were assigned to major phylogroups A, B1, B2 or D based on differential amplification of the ChuA, YjaA and TspE4.C2 DNA sequences. An internal control (TrpA 427 bp) was run with each sample for the 025b-ST131 allele PCR.

Sequence typing of E. coli

E. coli isolates belonging to major extra-intestinal pathogenic (ExPEC) STs were identified using PCR and specific primers. The STs and product sizes were: ST131, 310 bp; ST127, 404 bp; ST95, 200 bp; ST73, 490 bp; and ST69, 100 bp.[12]

Typing of Klebsiella spp. and Enterobacter spp.

Klebsiella pneumoniae were typed using variable-number tandem repeat (VNTR) analysis[13] using loci A, E, H, J, K and D, and three additional loci (N1, N2 and N4). *Enterobacter* spp. and *Klebsiella oxytoca* were typed using PFGE of XbaI-digested genomic DNA.[13]

Results

One thousand three hundred and fifteen infants were recruited to the PiPS study from 24 NNUs in eight networks. Five were withdrawn, leaving 1310. Stool samples were received at either time from 1229 infants: from 1186 (94%) of the 1266 infants alive at 2 weeks post-natal age and 1043 (84%) of the 1235 alive at 36 weeks post-menstrual age. Fifteen percent of infants were transferred to another NNU (usually within the same network) on more than one occasion and the largest number of transfers was 12 for a single infant. Transfers outside of networks were either the result of overloaded capacity in a network or a requirement for a surgical or investigational procedure available in a limited number of centres.

ESBL-producing Enterobacteriaceae were isolated from 67/1229 (5.5%) infants from whom samples were received at either sampling time or both sampling times (39 infants from whom 2 week samples were available and 39 infants from whom the 36 week post-menstrual age sample was available; for 11 of the infants samples were available from both sampling times). Two species of ESBL-producing Enterobacteriaceae were isolated from each of four infants at the same sampling time to give a total of 71 isolates. Of these, 65 isolates were available for characterization. These were 33 *E. coli*, 21 *K. pneumoniae*, 4 *K. oxytoca*, 6 *Enterobacter cloacae* and 1 *Enterobacter aerogenes*. Only one colonized infant developed a bacteraemia with an ESBL-producing *K. pneumoniae*.

The site distribution and characteristics of ESBL-producing *E. coli* isolates are shown in Table 1. ESBL-producing *E. coli* were isolated from infants in 18 (75%) of the 24 recruiting units. All were distinguishable within NNUs by antibiotic resistance phenotype, genotype, serogroup (O25b), phylogroup or ST. Ten of 33 (30%) *E. coli* isolates were ST131; of these 10, 9 were ciprofloxacin resistant and 7 produced CTX-M group 1 enzymes. ST131 isolates were isolated from NNUs within five of the eight NNU networks recruiting into the PiPS study. A single ST95 isolate was identified, but the remaining 22 isolates did not belong to any of the three other major ExPEC lineages sought (STs 69, 73, 127).

Klebsiella spp. and Enterobacter spp. typing results

ESBL-producing *Klebsiella* spp. were isolated from infants in eight (33%) of the 24 recruiting centres. *K. pneumoniae* was the most common species isolated, with three clusters of indistinguishable isolates identified by VNTR analysis. The largest cluster, involving isolates from five infants, was associated with one NNU (A5). There was another cluster involving three infants at another NNU (A1) and two infants at each of two NNUs who were found to have strains from the widespread clonal complex ST15.[14] Twelve of the 21 *K. pneumoniae* produced CTX-M group 1 enzymes. Two of the four *K. oxytoca* isolates were indistinguishable by PFGE typing and were associated with two infants in one NNU.

The PFGE typing of ESBL-producing *Enterobacter* spp. showed that five of the six *E. cloacae* isolates were indistinguishable by PFGE. This strain has caused an ongoing outbreak in NNU G1 and two of the PiPS study infants colonized with this strain were recruited at this centre. Both of these infants were re-located to NNU A3 and subsequently a third infant in NNU A3 was found to be colonized with the same strain. The other two infants with this strain had been inpatients in NNU A1, which is in the same network, or NNU D1, which is in the adjacent network to NNU A3.

None of the Enterobacteriaceae recovered was resistant to carbapenems or carried KPC, IMP, VIM or OXA-48-like carbapenemases.

Discussion

The primary and secondary outcomes of the PiPS study have been published elsewhere, including the frequency of carriage of ESBL-producing Enterobacteriaceae in the probiotic and placebo groups.[15] The overall prevalence of ESBL-producing Enterobacteriaceae reported in this study (5.5%) is similar to the figure reported from Germany (5.7%)[16] and lower than that reported in Italy (27.1%).[17] We found no evidence to suggest that there was transmission of ESBL-producing *E. coli* within

Table 1. Characteristics of ESBL-producing *E. coli*

			Antibiogram	Resistance determinants encoded		Molecular typing		
Infant	Network	Unit	CIP/GEN/TOB/CHL	β-lactamases	aminoglycoside-modifying enzymes	phylotype	ST PCR	025b-ST131 allele
1	A	A1	CIP^R GEN^S	SHV, CTX-M-1 Gp	APH(3′)-Ia, AAC(6′)-Ib-cr	B2	ST131	ST131-025b
2	A	A2	CIP^R TOB^R GEN^S	TEM, CTX-1 Gp	ANT(3″)-Ia, AAC(3′)-IIa	NT	ST131	NT
3	A	A1	CIP^R GEN^S	TEM	AAC(6′)-Ib-cr	B2		
4	A	A1	CIP^S GEN^S	CTX-M-1 Gp		B2		
5	A	A2	CIP^S GEN^S TOB^R CHL^S	CTX-M-1 Gp	AAC(3′)-IIa	B2		
6	A	A2	CIP^S GEN^S CHL^R	CTX-M-1 Gp		B2		
7	A	A3	CIP^R TOB^R GEN^S	SHV, CTX-M-1 Gp	ANT(3″)-Ia	B2		
8	A	A4	CIP^R GEN^S	CTX-M-1 Gp		B2	ST131	ST131-025b
9	A	A5	CIP^R GEN^S	CTX-M-2 Gp, CTX-M-9 Gp		A	ST131	NT
10	A	A5	CIP^S TOB^R GEN^S	CTX-M-1 Gp	AAC(3′)-Ia, AAC(6′)-Ib	B2		
11	A	A6	CIP^S GEN^S	TEM		B2	ST95	
12	B	B1	CIP^S GEN^S	TEM, OXA-1/4/30		B2		
13	C	C1	CIP^R TOB^R GEN^R	CTX-M-1 Gp	AAC(3′)-IIa	B2	ST131	ST131-025b
14	C	C1	CIP^R TOB^R GEN^R	TEM, OXA-1/4/30, CTX-M-1 Gp	AAC(3′)-IIa	B2	ST131	ST131-025b
15	C	C1	CIP^S TOB^R GEN^R	TEM	AAC(6′)-Ib-cr, AAC(3′)-IIa	D		
16	C	C1	CIP^R GEN^S	TEM		B2		
17	C	C2	CIP^R GEN^R	TEM	AAC(6′)-Ib-cr, AAC(3′)-IIa	D		
18	D	D1	CIP^R GEN^R	TEM, CTX-M-2 Gp	ANT(3″)-Ia, AAC(3′)-IIa	B2		
19	D	D2	CIP^S GEN^S	TEM	AAC(6′)-Ib-cr	B2		
20	D	D2	CIP^R TOB^R GEN^S	SHV, CTX-M-1 Gp	ANT(3″)-Ia	B2		
21	D	D2	CIP^R GEN^S	SHV, CTX-M-2 Gp, CTX-M-9 Gp		A	ST131	NT
22	D	D1	CIP^S GEN^S	CTX-M-1 Gp	ANT(3″)-Ia	A		
23	D	D1	CIP^S GEN^S		ANT(3″)-Ia, APH(3′)-Ia AAC(6′)-Ib-cr	B2		
24	D	D1	CIP^S TOB^R GEN^R	CTX-M-1 Gp	APH(3′)-Ia, AAC(6′)-Ib-cr, AAC(3′)-IIa	D	ST131	NT
25	E	E1	CIP^S GEN^R	TEM	ANT(3″)-Ia, AAC(3′)-IIa	NT		
26	E	E1	CIP^R GEN^S	SHV, CTX-M-1 Gp		B2		
27	E	E2	CIP^R GEN^S	TEM, OXA-1/4/30, CTX-M-9 Gp	AAC(6′)-Ib-cr	NT	ST131	NT
28	E	E3	CIP^S GEN^S	TEM	ANT(6′)-Ia	B2		
29	F	F1	CIP^S GEN^S	CTX-M-1 Gp		B2		
30	G	G1	CIP^R GEN^S	TEM, SHV, OXA-1/4/30, CTX-M-9 Gp		B2		
31	G	G1	CIP^R GEN^S	CTX-M-1 Gp	AAC(3′)-Ia, AAC(3′)-IIa	B2	ST131	ST131-025b
32	H	H1	CIP^S GEN^S	TEM, CTX-1 Gp		B2		
33	H	H2	CIP^R GEN^S	CTX-M-2 Gp, CTX-M-9 Gp		NT		

CIP, ciprofloxacin; TOB, tobramycin; GEN, gentamicin; CHL, chloramphenicol; ^{R}, resistant; ^{S}, susceptible; Gp, group; NT, not typeable.

units. Ten of the 33 ESBL-producing *E. coli* were ST131. There is evidence of worldwide community spread of ST131.[4] The H30 clade is frequently ESBL producing and ciprofloxacin resistant.[18] ST131 strains have been associated with transmission within households and hospitals, but seem to be transmitted more efficiently amongst household as opposed to hospital contacts.[19] All of the ST131 *E. coli* strains in this study were phylogroup B2, which has a propensity to carry a wide range of virulence genes.[18] Even so, there were no ST131 strains associated with episodes of late-onset infection in the PiPS study.

Carriage of ESBL-producing Enterobacteriaceae by the mother may be an important risk factor for carriage by the infant.[16] The lack of clustering of individual strains of *E. coli* within individual NNUs supports the plausibility of vertical acquisition. By contrast, there was evidence that other ESBL-producing species (*E. cloacae* and *Klebsiella pneumoniae*) were being transmitted within units and networks, and across networks.

Conclusions

Despite being present, ST131 strains were not associated with episodes of late-onset infection in this study. The diversity of ESBL-producing *E. coli* strains observed is not consistent with the view that acquisition is a consequence of horizontal transmission. The role of vertical transmission requires further investigation.[20]

Funding

The PiPS trial was funded by the UK National Institute for Health Research Health Technology Assessment programme (ISRCTN Number: 05511098; EudraCT Number: 2006-003445-17).

Transparency declarations

None to declare.

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