Multi-Locus Sequence Typing of *Escherichia coli* Isolates with acquired *ampC*

genes and *ampC* Promoter Mutations

Jonathan A. Lewis (1)

Philippa C. L. Moore (1)

Dawn L. Arnold (2)

Lynne M. Lawrance (2)

Affiliations

(1)

Department of Microbiology

Gloucestershire Royal Hospital

Great Western Road

Gloucester

GL1 3NN

(2)

Faculty of Health and Applied Sciences

University of the West of England

Coldharbour Lane

Bristol

BS16 1QY

Corresponding Author

Jonathan A. Lewis

Email: jonathan.a.lewis@nhs.net

Tel: 0300 422 5050

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Abstract

Multi-locus sequence typing was used to reveal a high degree of diversity amongst the *E. coli* isolates

with AmpC plasmid genes, and a high prevalence of the -32 mutation present.

Cephalosporins are broad-spectrum antibiotics used to treat a wide range of clinical infections. Some serious infections, such as sepsis, can be harder to treat and result in higher mortality rates if strains are cephalosporin-resistant (de Kraker et al. 2011). The most common cause of cephalosporin resistance in *Escherichia coli* is production of the CTX-M beta-lactamase enzyme (Potz et al. 2006). AmpC beta-lactamase hyper-production, however, is another cause in *E. coli*, with the enzyme normally produced constitutively in small amounts (Caroff et al. 2000). Although *bla*_{AmpC} genes can be acquired on plasmids from other species, chromosomal mutations in the *ampC* promoter region are a more common cause of increased enzyme production (Lewis et al. 2015). Mutations at positions -42, -32 and -13ins in the *ampC* promoter region are considered to be key in affecting the level of enzyme production (Tracz et al. 2007).

In our previous study (Lewis et al. 2015), >70% of cefpodoxime-resistance, ESBL-negative *E. coli* strains in Gloucestershire had an *ampC* promoter mutation present. Unusually, the -32 mutation (T to A) was the most common (63%); whereas other studies have reported a higher prevalence for the -42 mutation (Mulvey et al. 2005; Mammeri et al. 2008; Bogaerts et al. 2010; Jorgensen et al. 2010; Alonso et al. 2016). We therefore set out to investigate whether this was also the case in other parts of the South West of England. Multi-locus sequence typing (MLST) was used to determine the genetic relatedness of *E. coli* strains with *ampC* promoter mutations, with a view to identifying the presence of potentially dominant clones.

Clinical isolates of *E. coli* were collected in five laboratories (Dorchester, Gloucester, Swindon, Taunton and Truro) in the South West of England over a 3-month period in 2013. Isolates were submitted to the study if found to be cefpodoxime-resistant by disc susceptibility testing. On receipt in the testing laboratory (Gloucester), the presence of phenotypic ESBL activity was confirmed using clavulanic acid synergy discs (Mast Diagnostics, UK). DNA was extracted using a mechanical lysis method described previously (Woodford 2010). PCR assays were run on the Smart Cycler II instrument, using the Quantifast SYBR Green master-mix kit (Qiagen, UK). AmpC plasmid groups were detected using two multiplex PCR assays (CIT/ACC/DHA and FOX/MOX/EBC). Most primers (0.2umol/L) were those described previously (Perez-Perez and Hanson 2002), but some were updated to include more recently reported plasmids (Lewis 2016). A PCR protocol of 95°C for 5 mins, followed by 35 cycles of 95°C for 10s and 60°C for 30s was used, with melting curve analysis at the end. A 271bp region of the *ampC* gene, including the promoter region, was amplified using previously published primers (0.1 umol/L) (Caroff et al. 2000) and the same PCR protocol. PCR products were sent to an external company (Eurofins Genomics, Germany) for sequencing, using the forward amplification primer.

For MLST, seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified using the primers (0.15 umol/L) recommended on the *E. coli* MLST website (<u>http://mlst.warwick.ac.uk/mlst/dbs/Ecoli</u>) (Wirth et al. 2006). A PCR protocol of 95°C for 5 mins, followed by 30 cycles of 95°C for 30s, 56°C for 30s and 72°C for 30s was used. PCR products were sent to an external company (Eurofins Genomics) for purification and sequencing, using the forward amplification primer. The allele numbers and sequence type for each isolate was obtained using the online database at <u>http://pubmlst.org/</u> (Jolley and Maiden 2010).

A total of 77 isolates were confirmed as being cefpodoxime-resistant and ESBL-negative. MLST profiles were available for all 77 of the isolates, giving a total of 33 different sequence types (Table 1). ST73 was the most common (n=15), followed by ST12 (n=13) and ST131 (n=9). Over half (53%) of all isolates were allocated to one of just four sequence types. There were two new sequence types found in the group (ST5016 and ST5023), both of which were novel to the study.

AmpC plasmid genes were detected in 19 of the isolates (15 CIT-type and 4 DHA-type). These isolates were allocated to 14 different sequence types (11 of which were as individuals). This

confirms the findings of others, in which AmpC plasmid strains were found to have a high degree of diversity (Naseer et al. 2010). Although *ampC* mutations were found in 27 different positions in the amplified promoter sequence (Table 2), only three (-42, -32, -13ins) are considered to be key in the hyper-production of AmpC beta-lactamase (Tracz et al. 2007). The -32 mutation (T to A) was the most common key mutation found, present in nearly half (47.4%) of isolates. This mutation was seen in isolates mainly assigned to ST73 (n=14) and ST12 (n=12). Although it is possible that these isolates represent the presence of two dominant clones of *E. coli* that have the -32 mutation present, both sequence types (ST12 and ST73) are commonly found in strains without detectable resistance (Gibreel et al. 2012; Day et al. 2016). Mutations were also present in other parts of the promoter region, including the attenuator (+17 to +37). Whilst mutations in the attenuator region can have an impact on the level of enzyme production, this has been shown to be lower than that of the three key mutations (-42, -32 and -13ins) (Tracz et al. 2007).

The -42 mutation was present in 12% of strains. Other studies have found a higher prevalence for this particular mutation (Mulvey et al. 2005; Jorgensen et al. 2010; Mammeri et al. 2010; Alonso et al. 2016), with one reporting a prevalence of 100% (Bogaerts et al. 2010). There were two strains (SW-007 and TR-032) that were found to have insertions (thymine) at position -20. Although a similar mutation has been reported elsewhere (Jorgensen et al. 2010; Alonso et al. 2016), this may be the first report of this mutation in isolates from the UK.

In conclusion, this study confirms the presence of *ampC*-mediated resistance in 91% of cefpodoximeresistant, ESBL-negative *E. coli* isolates from five laboratories in the South West of England. Isolates with key chromosomal *ampC* mutations were more common (67.1%) than those with acquired *ampC* genes (24.7%). The most common *ampC* mutation known to cause an increase in enzyme production was a T to A transition at position -32. MLST revealed not only a high degree of diversity amongst the strains with acquired *ampC* genes, but also a strong association between the -32 mutation and the two sequence types (ST12 and ST73).

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		Laboratory				
ST	No.	DO	GL	SW	ТА	TR
73	15	0	6	3	3	3
12	13	0	9	2	2	0
131	9	2	2	2	2	1
778	4	2	2	0	0	0
38	2	2	0	0	0	0
40	2	0	1	0	0	1
69	2	2	0	0	0	0
88	2	1	1	0	0	0
127	2	0	1	0	1	0
357	2	0	0	1	1	0
648	2	1	0	1	0	0
10	1	0	1	0	0	0
56	1	0	0	0	1	0
80	1	0	0	0	0	1
95	1	0	0	0	1	0
117	1	1	0	0	0	0
200	1	0	1	0	0	0
372	1	1	0	0	0	0
404	1	0	0	1	0	0
405	1	1	0	0	0	0
420	1	0	0	1	0	0
421	1	0	0	0	1	0
448	1	1	0	0	0	0
453	1	0	1	0	0	0
533	1	0	0	1	0	0
624	1	1	0	0	0	0
963	1	0	1	0	0	0
1423	1	0	0	1	0	0
1485	1	1	0	0	0	0
1641	1	1	0	0	0	0
2175	1	0	1	0	0	0
5016*	1	0	1	0	0	0
5023*	1	0	0	0	1	0
Total	77	17	27	13	13	7

* indicates a new sequence type detected in this study
DO, Dorchester; GL, Gloucester; SW, Swindon; TA, Taunton; TR, Truro

Genotype No.	Number of AmpC Plasmids		Mutation Positions*		
	Isolates	-			
G1	16		-73, <u>-32</u> , +58, +63		
G2	11		-73, <u>-32</u>		
G3	3		-73, <u>-32</u> , -28		
G4	1		-73, <u>-32</u> , -28, -20ins		
G5	1		-73, <u>-32</u> , +24, +37		
G6	1		-73, <u>-32</u> , -28, +58		
G7	1		-73, <u>-32</u> , -28, -11		
G8	1		-73, <u>-32</u> , +32, +37		
G9	1		-73, <u>-32</u> , +37		
G10	7		-82, <u>-42</u> , -18, -1, +58, +81		
G11	1		-82, <u>-42</u> , -18, -1, +21, +58, +81		
G12	1		-82, <u>-42</u> , -18, -1, +33, +58, +81		
G13	2		-73, -28, <u>-13ins</u> , +17		
G14	2		-73, -28, <u>-13ins</u>		
G15	1		-73, <u>-13ins</u> , +6		
G16	1		-73, -28, <u>-13ins</u> , +31, +58		
G17	2		-73, -28		
G18	1		-73, -28, -20ins, +17, +34		
G19	1		-73, -28, +17		
G20	1		-73, -28, +34, +58		
G21	1		-76, +22, +26, +27, +32, +70, +71		
G22	11	10x CIT, 1x DHA	+/0, +81		
G23	2	2x CII	-82, -18, -1, +58, +81		
G24	1		-/3, -28,		
G25	1		-/3, +58, +63		
G26	1	1x CIT	-73, -28, +58		
G27	1	1x DHA	-/3, -28, +17		
G28	1	1x DHA	-73, +32, +58, +63		
G29	1	1x DHA	+81		

The reported sequence of each product was compared against the GenBank entry for *E. coli* NCTC12241 (GenBank Accession AY899338).

ins: insertion at position -13 or -20

numbers in bold represent the key promoter mutations described by Tracz et al. (2007).

* positions are numbered according to the method used by Jaurin *et al.* (1981)