Chromosomal *ampC* Mutations in Cefpodoxime-Resistant ESBL-Negative Uropathogenic *Escherichia coli*

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Abstract

AmpC beta-lactamase is an enzyme commonly produced by *E. coli* that causes resistance to cephalosporins and penicillins. Enzyme production is controlled by the strength of the promoter encoded by the chromosomal *ampC* gene, with the level of production affected by the presence of certain mutations in this region. This study set out to determine the prevalence of *ampC* promoter mutations present in a group of uropathogenic *E. coli* strains.

A total of 50 clinical strains of *E. coli* were collected from urine samples between June 2011 and November 2011. Strains were investigated for the presence of mutations in the chromosomal *ampC* promoter region by amplification and sequencing of a 271bp product. The presence of *ampC*-carrying plasmids derived from other species was also determined, to exclude these from further analysis.

ampC-carrying plasmids were found in 10 of the 50 strains, all of which were of the CIT-type. Analysis of the chromosomal *ampC* promoter region in the 40 remaining strains showed mutations at 16 different positions, with 18 different genotype patterns detected overall. The most common *ampC* chromosomal mutation, present in 25 of 40 strains, was a T→A transition at position -32. This mutation has been shown by others to increase enzyme production by up to 46-fold.

Altogether, three separate mutations (-32, -42 and -13ins) were present in 90% of the 40 non-plasmid strains, indicating a strong association with the resistance observed. It appears, therefore, that the majority of AmpC-mediated resistance in *E. coli* can be accounted for by just three point mutations in the chromosome.

Keywords

Antibiotic resistance
Cephalosporin
Escherichia coli
AmpC beta-lactamase

Introduction

AmpC beta-lactamase is an enzyme commonly produced by *Escherichia coli* that causes resistance to cephalosporins and penicillins.[1] All strains of *E. coli* carry the chromosomal *ampC* gene for enzyme production, which is normally weakly expressed and under tight control by regulatory mechanisms.[2] Production of the chromosomally-encoded enzyme is constitutive, but at such a low level that clinical failure of beta-lactam antibiotics is not usually seen. Certain mutations can occur in the promoter region, affecting the level of enzyme production. Strains with chromosomal *ampC* mutations can produce enzymes in higher amounts and are said to hyper-produce the AmpC enzyme, leading to clinical resistance and treatment failures.[3]

The common DNA sequence seen in numerous *E. coli* promoters is a -35 box (TTGACA) separated from a -10 box (TATAAT) by 17bp.[2] The normal *ampC* promoter sequence, however, includes single nucleotide difference in each of these hexameric boxes, together with a spacer difference of 16bp (Figure 1). These small differences are sufficient to affect the function of the promoter and decrease the normal AmpC enzyme production to its

constitutive low level.[4]

Mutations in the *ampC* promoter region can arise, and include transitions and insertions in the -35 or -10 boxes, which create a region more closely related to the standard *E. coli* promoter sequence, and thus a stronger promoter.[2] The most frequently reported promoter mutation (C→T at position -42) is one that creates a displaced -35 box in the promoter sequence, and is associated with a 20-fold increase in enzyme production.[5] Other reported key mutations include substitutions that change the sequence of the wild-type -35 box itself, and insertions in the spacer region between the -35 and -10 boxes. Mutations have also been reported throughout other locations in the promoter, attenuator and coding regions, but these are considered to have a lesser impact on the level of enzyme production.[5]

In addition to chromosomal *ampC* mutations, *E. coli* can also acquire the genes for AmpC enzyme production from other species. First described in 1988, plasmid *ampC* genes are derived from species such as *Enterobacter* spp. and *Citrobacter* spp..[6] Although there are >200 different plasmids reported to carry *ampC* genes, for convenience they are usually classified into six groups based on the species of origin; CIT, ACC, DHA, FOX, MOX, and EBC.[7] The CMY-2 plasmid (within the CIT group) is the most common AmpC plasmid encountered to date, and also has the largest geographic spread.[1] Woodford *et al*. (2007)[8] tested 135 strains of *E. coli* referred from UK laboratories for the investigation of unusual resistance patterns, detecting an *ampC*-carrying plasmid in 49%. The majority were determined to be of the CIT-group, but ACC, FOX and DHA groups were also detected. Strains with plasmids carrying *ampC* genes were found to be more resistant to third-generation cephalosporins than those with *ampC* chromosomal promoter mutations.

This study set out to characterise the chromosomal *ampC* mutations present in a group of uropathogenic *E. coli* strains. Although *E. coli* is one of the most common pathogens isolated in clinical laboratories, there is a lack of data for the UK describing the prevalence and nature of AmpC resistance in clinical isolates. This is particularly the case for the chromosomal mutations responsible for AmpC enzyme hyper-production. Whilst the *ampC*-carrying plasmids can give rise to a higher level of resistance, the chromosomal *ampC* mutations seen in *E. coli* represent a larger overall group of resistant strains.

Materials & Methods

Strain Collection

Clinical strains of *E. coli* isolated from urine samples in the Gloucestershire laboratory between June 2011 and November 2011 were included if disc susceptibility testing indicated cefpodoxime resistance with a subsequent negative result for clavulanic acid synergy; thus excluding the presence of ESBL-mediated resistance.[9] Strains were identified to species level using a chromogenic urine media plate (257481, Becton Dickinson, Oxford, UK) and API20E identification strips (20100, Biomerieux, Basingstoke, UK). Isolates were excluded if the same species had previously been isolated from the patient within a 28-day period. Strains were anonymised before inclusion in the study, and only basic patient demographic data (e.g. age and gender) were collected for each sample. During the collection period, a total of 50 clinical urine strains were included.

Susceptibility Testing

Strains were tested for susceptibility to a range of cephalosporins, including the antibiotics cefpodoxime (10µg), cefuroxime (30µg), cefoxitin (30µg), cefotaxime (30µg) and cefepime (30µg), using a standardised disc susceptibility method.[10] A 0.5 MacFarland suspension was prepared and diluted to a 1:100 concentration. The final suspension was inoculated onto an Isosensitest agar plate (PO0779A, Oxoid, Basingstoke, UK) using a cotton-tipped swab. Antibiotic discs (various, Oxoid, Basingstoke, UK) were applied to the surface of the agar and the plate was incubated for 18-24 hours at 37°C in air. Following incubation, the zone size for each antibiotic disc was recorded.

Strains were also tested to determine the minimum inhibitory concentration (MIC) of cefotaxime. MICE gradient strips (MA0111F, Oxoid, Basingstoke, UK) were used to test the cefotaxime MIC within the range 0.002 to 32mg/L. A 0.5 MacFarland suspension was prepared and inoculated directly onto an Isosensitest agar plate using a cotton-tipped swab. The MICE strip was applied to the surface of the agar and the plate was incubated for 18-24 hours at 37°C in air. Following incubation, the point of intersection of the zone to the strip was recorded as the MIC for the strain.

Detection of ampC Plasmids

DNA templates for PCR amplification were prepared using a crude-lysis method.[11] Strains were incubated overnight on Columbia Horse Blood agar plates (PB0122A, Oxoid, Basingstoke, UK). A heavy bacterial suspension, equivalent to MacFarland standard 4.0, was prepared in 100µl water. Tubes were vortex-mixed for 2 minutes and then centrifuged for 5 minutes at 8,000g. The resulting supernatants were used as the DNA template.

PCR assays were run on the SmartCycler II instrument (Cepheid, Sunnyvale, US), using the DX software (Version 3.0). Assay parameters were those recommended for use with the Quantifast SYBR Green master-mix (204054, Qiagen, Manchester, UK): 95°C for 5 minutes, followed by 35 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Fluorescence was read after each cycle at the instrument settings for FAM dye. A melting curve protocol was run at the end of amplification, with the temperature increasing from 60°C to 95°C at a rate of 0.5°C / sec. The resulting dissociation curve was used to visualise the presence of an amplified product.

The presence of AmpC plasmid groups was determined using two multiplex real-time SYBR Green PCR assays (CIT/ACC/DHA and FOX/MOX/EBC). Primers for five of the plasmid groups were as previously described.[7] The CIT primers were updated to include more recently reported plasmids: CIT-F (5'-TGA TGC AGG AGC AGG CTA TTC-3') and CIT-R (5'-ACA GAC CAA TGC TGG AGT TAG-3'). Primers (Invitrogen, Paisley, UK) were used at a 0.2µM final concentration. Multiplex AmpC plasmid assays with positive dissociation curves were confirmed using the same primers in three separate simplex reactions, to identify the individual plasmid present.

Sequencing the ampC Promoter Region

A 271bp region of the *ampC* gene, including the promoter region, attenuator region and part of the coding region, was amplified for each isolate using previously published primers at 0.1µM concentration.[3] PCR protocol parameters were: 95°C for 5 minutes, followed by 35 cycles of 95°C for 10 seconds and 60°C for 30 seconds. A melting curve protocol was run to

confirm the presence of an amplified product prior to sequencing. PCR products were sent to an external company (Eurofins Genomics, Ebersberg, Germany) for purification and sequencing, using the forward amplification primer. The reported sequence of each product was compared against the GenBank entry for *E. coli* NCTC 12241 (GenBank Accession AY899338), a laboratory control strain. Sequences were aligned using the ClustalW online software (www.ebi.ac.uk/tools/msa/clustalw2). The nature and position of each mutation was noted, and recorded according to the numbering system of Jaurin *et al*. (1981).[12]

Results

The 50 collected strains comprised 75% from female patients, and 25% from male. The mean age of patients was 58.3 years, with a range from 1 to 94 years. The results for disc susceptibility testing are shown in Table 1. The majority (98%) of isolates were resistant to cefuroxime when zone sizes were compared against breakpoints set by the British Society of Antimicrobial Chemotherapy (BSAC).[10] In contrast, only 28% of strains demonstrated resistance to cefotaxime. Results also showed 98% of strains were resistant to cefoxitin, and there were 22 strains (44%) with resistant or intermediate zone sizes to the fourth-generation cephalosporin cefepime.

Ten strains demonstrated an amplification product typical of the CIT group of plasmids. None of the other plasmid groups (ACC, DHA, FOX, MOX, EBC) were detected. As the genes carried on AmpC plasmids can account for enzyme production alone, these strains were excluded from further analysis.

From the remaining 40 strains, sequencing the 271bp region covering the chromosomal *ampC* promoter showed mutations at 16 different positions throughout the amplified region (See Table 2). Overall, 18 different genotype patterns were detected from the 40 strains investigated, with all strains containing at least one polymorphism when compared to the sequence for the *E. coli* control strain. The most common chromosomal *ampC* genotype (n=11) included a T→A substitution at position -32, together with substitutions at positions +58 and +63. The -32 mutation alters the wild-type -35 box from TTGTCA to TTGACA, one that is closer to the *E. coli* standard promoter sequence.[5] The altered -35 box retains its original position in the promoter region. Mutations related to a displaced promoter region were found in three strains, at positions -42 and -18. The -42 (C→T) substitution creates a new -35 box, whereas the G→A mutation at -18 gives rise to a new -10 box region. These mutations are often reported together,[13] and result in two new regions 17bp apart at different locations. The increased distance between the two regions has been reported to affect promoter strength.[5] The -18 mutation was also observed in two strains without the -42 mutation present.

Insertions in the spacer region (position -13) were detected in eight strains, increasing the distance between the -35 and -10 boxes from 16bp through the insertion of 1 or 2 base pairs. Seven strains had mutations in the attenuator region (positions +17, +23 and +37), often present along with promoter mutations. A total of 19 strains were found to have mutations in the coding region. The three mutations at positions +63, +70 and +81 have been reported to alter the amino acid coded for at each of the three respective codons. However, it is not clear whether this has a significant impact on enzyme function.[13]

The geometric mean MIC for cefotaxime was 1.2mg/L for all strains, just above the recommended breakpoint of 1.0mg/L.[10] Those strains with the -32 mutation present had a lower cefotaxime MIC of 0.6mg/L, compared to 1.2mg/L for strains with other mutations. In contrast, the ten strains with a CIT plasmid present had a geometric mean cefotaxime MIC of 7.3mg/L.

Discussion

CIT-type plasmids were found in 20% of the 50 strains collected. Although not fully characterised in this study, these are usually reported to be the CMY-2 plasmid. The presence of CMY-2 plasmids has been reported globally in clinical samples, and has also been associated with foodstuffs and animals.[14–16] Phylogenetic studies have shown that *E. coli* strains carrying AmpC plasmids are more likely to be virulent, pathogenic types, whereas the strains with chromosomal promoter mutations are more likely to be commensal or environmental strains.[17]

A total of 28 (70%) of the remaining 40 strains possessed either the -42 or -32 mutation, both considered to be key factors in creating a stronger *ampC* promoter by creating a -35 box with greater homology to the *E. coli* standard promoter sequence.[5] The -32 mutation was predominant in this study, with 25 strains demonstrating this substitution, either alone or in conjunction with other mutations. This mutation has been reported to result in an 8- to 46-fold increase in over-expression when compared to wild type strains.[5] In this study, the geometric mean MIC for cefotaxime in the -32 mutation group was 0.6mg/L, compared to 0.06mg/L for the *E. coli* control strain (NCTC 12241), showing a ten-fold increase.

The -42 mutation and -13 spacer insertions have been shown to effect a similar impact on the level of over-expression; 20-fold and 24- to 61-fold, respectively.[5] Whilst we found only three strains with the -42 mutation, other studies have reported a higher prevalence,[13,17–19] with one study finding 100% of isolates with this mutation.[20] Eight of the 40 strains in our study had insertions of either one or two bases at position -13. The inserted bases were either adenine or thymine, and increased the spacer region from 16 base pairs to 17 or 18 base pairs. Seven strains were found to have mutations in the attenuator region. Although attenuator region mutations are thought to increase enzyme production through the destabilisation of the stem-loop structure, Tracz *et al*. (2007) demonstrated that these mutations have little actual affect on the level of enzyme production.[5]

Resistance to cefoxitin is proposed as a screening test for AmpC production.[10] In this study, 98% of all strains were resistant by disc susceptibility testing, confirming it's utility as such. Whilst this may represent a good method for detecting AmpC-mediated resistance, the specificity of the method is reduced by other means in which strains can become resistant to cefoxitin (e.g. membrane permeability).[21] AmpC-producing strains are generally considered to have the antibiogram phenotype of cefoxitin-resistant, cefepime-sensitive.[8] Here, only 56% of strains met the criteria for both, with only 42% having a zone size above the breakpoint of 32mm for cefepime susceptibility.

In this study of 50 uropathogenic strains of cefpodoxime-resistant, ESBL-negative *E. coli*, AmpC beta-lactamase resistance was confirmed in 92% of isolates. Although some strains did carry a plasmid *ampC* gene, the majority of strains possessed one of the *ampC*

chromosomal promoter region mutations recognised to cause enzyme hyper-production. The -32 mutation, -42 mutation and -13 insertions accounted for 90% of the resistance in the 40 non-plasmid strains, but were not found together in the same strain. Thus, providing further evidence that these represent the key mutations responsible for enzyme hyper-production. It was of interest to note a predominance of the -32 mutation in the strains, rather than the -42 mutation reported elsewhere. Although unlikely to represent a difference in the level of clinical resistance, strains carrying the -32 mutation may represent a dominant clone in the local population. Further studies are underway to include strains from other laboratories and to utilise molecular typing methods to identify the presence of different resistant strain populations.

Acknowledgments

We are grateful to the laboratory staff at the Department of Microbiology, Gloucestershire Royal Hospital, for assisting with isolate collection.
The study was supported by a research grant from the Institute of Biomedical Science.
Approval from National Research Ethics Service (NRES) was given under reference 11/SW/0224.

References

1. Jacoby GA. AmpC Beta-Lactamases. *Clin. Microbiol. Rev.* 2009; **22** (1): 161-182.

2. Hawley DK, McClure WR. Compilation and Analysis of Escherichia coli Promoter DNA Sequences. *Nucleic Acids Res.* 1983; **11** (8): 2237-2255.

3. Caroff N, Espaze E, Gautreau D, Richet H, Reynaud A. Analysis of the Effects of -42 and -32 ampC Promoter Mutations in Clinical Isolates of Escherichia coli Hyperproducing AmpC. *J. Antimicrob. Chemother.* 2000; **45** (6): 783-788.

4. Corvec S, Caroff N, Espaze E, Marraillac J, Reynaud A. -11 Mutation in the ampC Promoter Increasing Resistance to Beta-lactams in a Clinical Escherichia coli Strain. *Antimicrob. Agents Chemother.* 2002; **46** (10): 3265-3267.

5. Tracz DM, Boyd DA, Hizon R, *et al*. ampC Gene Expression in Promoter Mutants of Cefoxitin-Resistant Escherichia coli Clinical Isolates. *FEMS Microbiol. Lett.* 2007; **270** (2): 265-271.

6. Philippon A, Arlet G, Jacoby GA. Plasmid-Determined AmpC-Type Beta-Lactamases. *Antimicrob. Agents Chemother.* 2002; **46** (1): 1-11.

7. Perez-Perez FJ, Hanson ND. Detection of Plasmid-Mediated AmpC Beta-Lactamase Genes in Clinical Isolates by Using Multiplex PCR. *J. Clin. Microbiol.* 2002; **40** (6): 2153-2162.

8. Woodford N, Reddy S, Fagan EJ, *et al*. Wide Geographic Spread of Diverse Acquired AmpC Beta-lactamases Among Escherichia coli and Klebsiella spp. in the UK and Ireland. *J. Antimicrob. Chemother.* 2007; **59** (1): 102-105.

9. M'Zali FH, Chanawong A, Kerr KG, Birkenhead D, Hawkey PM. Detection of Extended-spectrum Beta-lactamases in Members of the Family Enterobacteriaceae: comparison of the MAST DD test, the double disc and the Etest ESBL. *J. Antimicrob. Chemother.* 2000; **45** (6): 881-885.

10. Andrews JM, Howe RA. BSAC Standardized Disc Susceptibility Testing Method (Version 10). *J. Antimicrob. Chemother.* 2011; **66** (12): 2726-57.

11. Woodford N. Rapid Characterization of Beta-Lactamases by Multiplex PCR. *Methods Mol. Biol.* 2010; **642** : 181-192.

12. Jaurin B, Grundstrom T, Edlund T, Normark S. The E. coli Beta-lactamase Attenuator Mediates Growth Rate-Dependent Regulation. *Nature* 1981; **290** (5803): 221-225.

13. Mulvey MR, Bryce E, Boyd DA, *et al*. Molecular Characterization of Cefoxitin-Resistant Escherichia coli from Canadian Hospitals. *Antimicrob. Agents Chemother.* 2005; **49** (1): 358-365.

14. Dhanji H, Murphy NM, Doumith M, *et al*. Cephalosporin Resistance Mechanisms in Escherichia coli Isolated from Raw Chicken Imported into the UK. *J. Antimicrob. Chemother.* 2010; **65** (12): 2534-2537.

15. Voets GM, Fluit AC, Scharringa J, *et al*. Identical Plasmid AmpC Beta-Lactamase Genes and Plasmid Types in E. coli Isolates from Patients and Poultry Meat in the Netherlands. *Int. J. Food Microbiol.* 2013; **167** (3): 359-362.

16. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-Spectrum Beta-Lactamase-Producing and AmpC-Producing Escherichia coli from Livestock and Companion Animals, and their Putative Impact on Public Health: a global perspective. *Clin. Microbiol. Infect.* 2012; **18** (7): 646-655.

17. Corvec S, Prodhomme A, Giraudeau C, Dauvergne S, Reynaud A, Caroff N. Most Escherichia coli Strains Overproducing Chromosomal AmpC Beta-lactamase Belong to Phylogenetic Group A. *J. Antimicrob. Chemother.* 2007; **60** (4): 872-876.

18. Mammeri H, Nordmann P, Berkani A, Eb F. Contribution of Extended-Spectrum AmpC (ESAC) Beta-Lactamases to Carbapenem Resistance in Escherichia coli. *FEMS Microbiol. Lett.* 2008; **282** (2): 238-240.

19. Jorgensen RL, Nielsen JB, Friis-Moller A, Fjeldsoe-Nielsen H, Schonning K. Prevalence and Molecular Characterization of Clinical Isolates of Escherichia coli Expressing an AmpC Phenotype. *J. Antimicrob. Chemother.* 2010; **65** (3): 460-464.

20. Bogaerts P, Rodriguez-Villalobos H, Bauraing C, *et al*. Molecular characterization of AmpC-producing Escherichia coli clinical isolates recovered at two Belgian hospitals. *Pathol. Biol. (Paris).* 2010; **58** (1): 78-83.

21. Chen HY, Livermore DM. Activity of Cefepime and Other Beta-Lactam Antibiotics against Permeability Mutants of Escherichia coli and Klebsiella pneumoniae. *J. Antimicrob. Chemother.* 1993; **32** (Suppl B): 63-74.

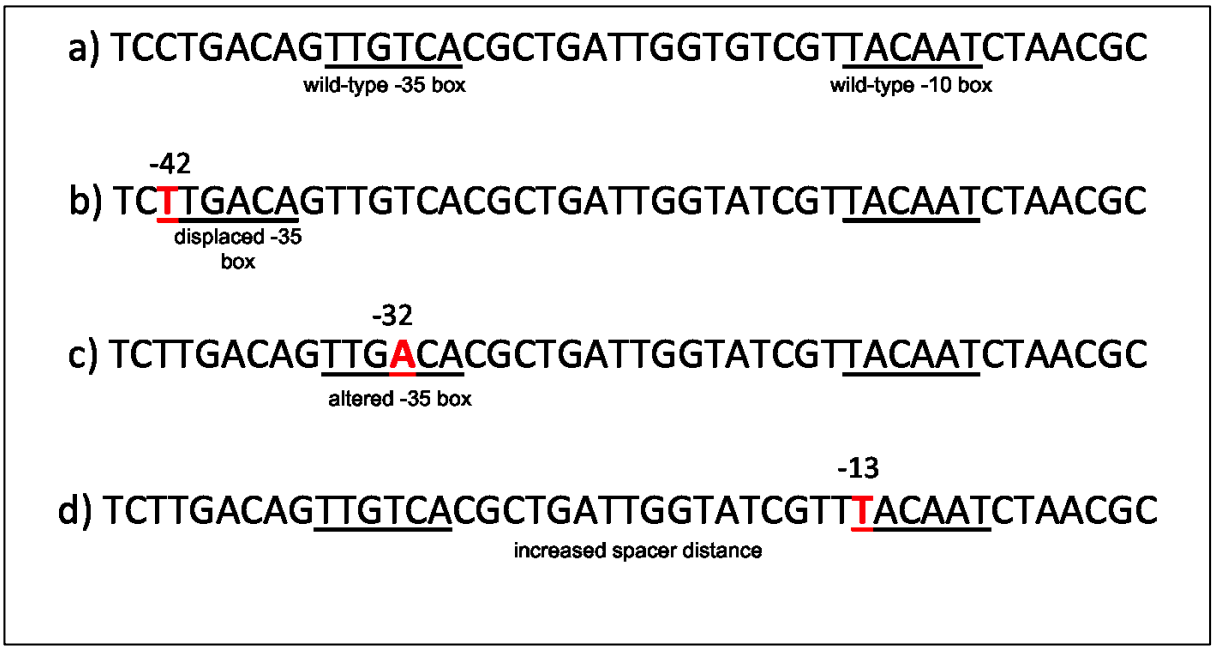

Figure 1: Section of *ampC* promoter region, showing the positions of:

a) The wild-type hexameric -10 and -35 boxes
b) C->T transition at position -42 creates a displaced -35 box with a sequence identical to the *E. coli* standard promoter (TTGACA)
c) T->A transition at position -32 creates an altered wild-type -35 box with a sequence identical to the *E. coli* standard promoter (TTGACA)
d) Insertion of a single nucleotide at position -13 increases the spacer distance from 16bp to 17bp

Numbering of positions is according to Jaurin *et al*. (1981)[12]

	Disc Content (µg)	Zone Diameter Breakpoints (mm)	S (%)	I (%)	R (%)
Cefpodoxime	10	≤19	0 (0)	N/A	50 (100)
Cefuroxime	30	≤19	1 (2)	N/A	49 (98)
Cefotaxime	30	≤23 to ≤29	14 (28)	22 (44)	14 (28)
Cefoxitin	30	≤22	1 (2)	N/A	49 (98)
Cefepime	30	≤26 to ≤31	28 (56)	12 (24)	10 (20)

Table 1: Disc susceptibility results for the 50 strains to a range of five cephalosporins. The zone diameter breakpoints shown are for resistant strains, or for resistant and intermediate strains if a range is given.

S = Resistant, I = Intermediate, R = Resistant

Genotype	No. of Strains	-42	-32	-28	-18	-14	-13	-13ins	-1	+6	+17	+23	+34	+37	+58	+63	+70	+81
Control		C	T	G	G	T	T	-	C	C	C	G	G	G	C	T	C	A
G1	11		A												T	C		
G2	8		A															
G3	2		A														T	G
G4	2							T		T								
G5	2	T			A				T						T			G
G6	2			A				AT										
G7	2			A		A		T			T							
G8	1				A				T						T			G
G9	1			A														
G10	1			A				T										
G11	1						G	TT										
G12	1		A	A							T							
G13	1		A	A														
G14	1		A		A				T			A			T			G
G15	1		A											A			T	G
G16	1	T			A				T			A			T			G
G17	1			A									A		T			
G18	1			A							T							

Table 2: Details of mutations observed at different positions in the amplified 271bp region of the promoter, attenuator and coding regions of the *ampC* gene. Each genotype is shown, with the corresponding numbers of strains allocated to that group. Genotype numbers were allocated within this study. Position numbers for locations on the *ampC* gene were those used by Jaurin *et al.* (1981).[12] The control sequence is derived from the GenBank entry for *E. coli* NCTC 12241 (AY899338).

ins = 1 or 2 bp insertions at position -13.