Chromosomal ampC mutations in cefpodoxime-resistant, ESBL-negative uropathogenic Escherichia coli

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Introduction

AmpC β-lactamase is an enzyme commonly produced by Escherichia coli that causes resistance to cephalosporins and penicillins. 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. Production of the chromosomally-encoded enzyme is constitutive, but at such a low level that clinical failure of β-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.

The common DNA sequence seen in numerous E. coli promoters is a -35 box (TTGACA) separated from a -10 box (TATAAT) by 17 bp. The normal ampC promoter sequence, however, includes a single nucleotide difference in each of these hexameric boxes, together with a spacer difference of 16 bp (Fig. 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.

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. 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. 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.

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ABSTRACT

AmpC β-lactamase is an enzyme commonly produced by Escherichia 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 sets 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 271 bp 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.

KEY WORDS: AmpC beta-lactamase.
Cephalosporins.
Drug resistance, microbial.
Escherichia coli.

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. 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. The CMY-2 plasmid (within the CIT group) is the most common AmpC plasmid encountered to date, and also has the largest geographic spread. Woodford et al. tested 135 strains of E. coli referred from UK laboratories for the investigation of unusual resistance patterns, detecting an ampC-carrying
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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 sets 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. While 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 and methods**

**Strain collection**

Clinical strains of *E. coli* isolated from urine samples in the Gloucestershire laboratories 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 extended-spectrum β-lactamase-(ESBL)-mediated resistance.9 Strains were identified to species level using a chromogenic urine medium plate (257481, Becton Dickinson, Oxford, UK) and API20E identification strips (20100, bioMérieux, 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 (10 μg), cefuroxime (30 μg), cefoxitin (30 μg), cefotaxime (30 μg) and cefepime (30 μg), 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 Iso-Sensitest agar plate (PO0779A, Oxoid). A heavy bacterial suspension, equivalent to MacFarland standard 4.0, was prepared in 100 μL water. Tubes were vortex-mixed for 2 min and then centrifuged for 5 min at 8000 g. 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 min, followed by 35 cycles of 95°C for 10 sec and 60°C for 30 sec. Fluorescence was read after each cycle at the instrument settings for the 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 GTA TTT-3’) and CIT-R (5’-ACA GAC CAA TGC TGG AGT TAG-3’). Primers (Invitrogen, Paisley, UK) were used at a 0.2 μmol/L 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 271 bp 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 0.1 μmol/L concentration.3 PCR protocol parameters were: 95°C for 5 min, followed by 35 cycles of
Results

The 50 collected strains comprised 75% from female patients and 25% from male patients. Mean age was 58.3 years (range: 1–94 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 for Antimicrobial Chemotherapy (BSAC). In contrast, only 98% of strains were resistant to cefuroxime when zone sizes were compared against breakpoints set by the British Society for Antimicrobial Chemotherapy (BSAC).10 In contrast, only 28% of strains demonstrated resistance to cefotaxime. Results also showed 98% of strains were resistant to cefotaxime and there were 22 strains (44%) with resistant or intermediate zone sizes to the fourth-generation cephalosporins. The geometric mean MIC for cefotaxime was 1.2 mg/L for all strains, just above the recommended breakpoint of 1.0 mg/L.10 Those strains with the -32 mutation present had a lower cefotaxime MIC of 0.6 mg/L, compared to 1.2 mg/L for strains with other mutations. In contrast, the 10 strains with a CIT plasmid present had a geometric mean cefotaxime MIC of 7.3 mg/L.

Discussion

In this study, CIT-type plasmids were found in 20% of the 50 strains collected. Although not fully characterised, 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 eight- to 46-fold increase in over-expression when compared to wild-type.
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Table 2. Details of mutations observed at different positions in the amplified 271-bp 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. The control sequence is derived from the GenBank entry for *Escherichia coli* NCTC 12241 (AY899338).

| Genotype | Number 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   | A   |
| G1       | 11                 | A   |     |     |     |     |     |        |     |     |     |     |     |     |     |     | C   |     |
| G2       | 8                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G3       | 2                  | A   |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G4       | 2                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G5       | 2                  | T   |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G6       | 2                  | A   |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G7       | 2                  | A   | A   |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G8       | 1                  | A   |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G9       | 1                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G10      | 1                  | A   |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G11      | 1                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G12      | 1                  | A   | A   |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G13      | 1                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G14      | 1                  | A   | A   |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G15      | 1                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G16      | 1                  | T   | A   |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G17      | 1                  | A   |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |
| G18      | 1                  |     |     |     |     |     |     |        |     |     |     |     |     |     |     |     |     |     |

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

strains. In this study, the geometric mean MIC for cefotaxime in the -32 mutation group was 0.6 mg/L, compared to 0.06 mg/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 have a similar impact on the level of over-expression; 20-fold and 24- to 61-fold, respectively. While we found only three strains with the -42 mutation, other studies have reported a higher prevalence with one study finding 100% of isolates with this mutation. Eight of the 40 strains in the present 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 bp to 17 or 18 bp. 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. demonstrated that these mutations have little actual effect on the level of enzyme production.

Resistance to cefoxitin is proposed as a screening test for AmpC production. In this study, 98% of all strains were resistant by disc susceptibility testing, confirming its utility as such. While 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). AmpC-producing strains are generally considered to have the antibiogram phenotype of cefoxitin-resistant, cefepime-sensitive. Here, only 56% of strains met the criteria for both, with only 42% having a zone size above the breakpoint of 32 mm for cefepime susceptibility.

In this study of 50 uropathogenic strains of cefpodoxime-resistant, ESBL-negative *E. coli*, AmpC β-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 hyperproduction. 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 hyperproduction. 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.

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