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Evolution of TEM-type extended-spectrum β-lactamases in *Escherichia coli* by cephalosporins

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ABSTRACT

Objectives: This study was conducted to examine the molecular mechanisms responsible for the evolution of TEM-type extended-spectrum β-lactamases (ESBLs) following selective pressure from four third-generation cephalosporins, namely ceftazidime, cefotaxime, ceftriaxone and ceftibuten. In addition, selective enrichment for ESBL detection in environmental samples was investigated.
Methods: Using experimental evolution, resistant variants were isolated and mutations in TEM-1 were examined by DNA sequencing. Resistance levels and the development of cross-resistance were determined for ESBL-producing isolates by Etest and disk diffusion assay. Selective plating with or without prior growth in selective broth was used to examine the approach of selective enrichment for ESBL detection.
Results: The third-generation cephalosporins ceftazidime, cefotaxime and ceftriaxone selected for ESBLs, whereas ceftibuten did not. All ESBL variants additionally remained susceptible to ceftibuten. DNA sequencing of the TEM-1 coding sequence of mutants revealed mutations not previously isolated through selection. This indicates that the potential for ESBL evolution is much broader than can be inferred from sequence analysis of clinical samples alone. The results also indicate that selective enrichment for enhanced detection of ESBL-producers may give unreliable results owing to the selection of spontaneous mutations in narrow-spectrum β-lactamases resulting in TEM-type ESBL-producers.
Conclusion: These results help explain the molecular changes responsible for evolution of TEM-type ESBLs and meanwhile question the appropriate use of selective enrichment for detection of ESBLs in environmental samples.

1. Introduction

Bacterial infections are often treated with β-lactam antibiotics. Owing to their high efficacy, low cost and few side effects, they are one of the most utilised classes of antibiotics in human medicine [1]. β-Lactams interfere with synthesis of the bacterial cell wall by binding to penicillin-binding proteins, resulting in stalled cell wall synthesis and subsequent inhibition of growth [2]. The widespread use of β-lactams has resulted in the evolution of β-lactam resistance in pathogenic bacteria [3]. Resistance is frequently conferred by β-lactamases, enzymes able to hydrolyse and inactivate β-lactams [2,4,5]. There are many β-lactamase variants and they have been grouped based on substrate specificity or protein sequence [6–8]. A widely disseminated β-lactamase family is the TEM enzymes that are encoded by TEM-1 and its descendant genes [4,9,10]. TEM-1 is widely disseminated worldwide and has also been found in samples with little exposure to β-lactam antibiotics, including pristine forest soil [11–13]. TEM-1 β-lactamase hydrolyses penicillins and early-generation cephalosporins effectively but is less efficient in degrading later generations of cephalosporins and monobactams [4,10]. However, variants with the ability to degrade the majority of β-lactams have evolved both in vitro and in the clinic owing to extensive β-lactam use [4,14–16], and the TEM β-lactamases now consist of hundreds of variants [10,17]. Single nucleotide polymorphisms (SNPs) in the TEM-1 gene result in amino acid substitutions in the enzyme, and the most common substitutions involved are Glu104→Lys, Arg164→Ser or His, Glu238→Ser and Glu240→Lys (Ambler numbering scheme) [6], increasing the hydrolytic activity of the enzyme and selecting for extended-spectrum β-lactamases (ESBLs) [10]. ESBLs are β-lactamases that are able to hydrolyse oxyimino-β-lactam antibiotics at a rate that is 10% faster than that

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for benzylpenicillin. They show decreased susceptibility to third- and fourth-generation extended-spectrum cephalosporins such as ceftazidime (CAZ) and cefotaxime (CTX) and are inhibited by β-lactamase inhibitors such as clavulanic acid [18,19]. The evolution of ESBL enzymes from TEM-1 has been extensively studied using site-directed mutagenesis and error-prone polymerases, creating artificial ESBL phenotypes with extended substrate spectra and decreased susceptibility to extended-spectrum cephalosporins [20–23]. However, fewer studies [14,24] have investigated the molecular mechanisms of ESBL evolution under selection pressure from third-generation cephalosporins.

The aim of this study was to investigate molecular changes in TEM-1 under selection from four third-generation cephalosporins, namely CAZ, CTX, ceftriaxone (CRO) and ceftibuten (CTB), as well as how these changes relate to the ESBL phenotype. Experimental evolution was used to isolate resistant mutants, and detailed molecular biology was used to identify mutations within the $bla_{\text{TEM-1}}$ gene. Resistance levels of evolved ESBL mutants were evaluated and the development of cross-resistance was investigated. How the common practice of using selective enrichment to isolate bacterial phenotypes affects the ESBL evolutionary process and the selection of ESBL enzymes was also investigated.

2. Methods

2.1. Strains and growth conditions

The bacterial strains used were wild-type strains and derivatives of *Escherichia coli* MG1655. *E. coli* ATCC 25922 and AB604 were used as negative and positive controls for ESBL production, respectively. Strains were grown in Luria–Bertani (LB) broth supplemented with 8 mg/L tetracycline for plasmid maintenance. For selective enrichment, strains were grown in MacConkey broth with or without 1 mg/L CRO.

2.2. Transformation of the pBR322 plasmid

Competent *E. coli* MG1655 cells were prepared using $CaCl_2$. In brief, 50 mL of LB broth was inoculated with 0.5 mL of an *E. coli* MG1655 overnight culture and was incubated with aeration to an optical density at 600 nm (OD_{600}) of 0.5–1.0. Cells were pelleted by centrifugation at 4000 × *g* for 10 min and were re-suspended in 20 mL of ice-cold 0.1 M $CaCl_2$. Following incubation on ice for 30 min, cells were pelleted by centrifugation at 4000 × *g* for 10 min and were re-suspended in 4 mL of ice-cold 0.1 M $CaCl_2$. Competent cells were used for transformation of the pBR322 plasmid encoding TEM-1 (Thermo Fisher Scientific, Waltham, MA, USA). In brief, 2 μL of plasmids was transferred to 200 μL of pre-chilled *E. coli* MG1655 competent cells and was incubated on ice for 15 min. Cells were heat-shocked for 2 min at 42 °C and then 250 μL of LB broth was added and the cells were incubated for 60 min at 37 °C before plating on selective plates containing 8 mg/L tetracycline and incubation overnight at 37 °C. Positive *E. coli* MG1655/pBR322 transformants were stored in 25% glycerol at −80 °C.

2.3. Serial passage experiments

Four third-generation cephalosporins, namely CAZ, CTX, CRO and CTB (Sigma-Aldrich, Copenhagen, Denmark), were used to select resistant mutants. Six parallel lineages of *E. coli* MG1655/pBR322 were propagated in LB medium with increasing concentrations of antibiotics. Cultures were grown with shaking at 37 °C and were submitted to successive daily serial passage by inoculating 50 mL of fresh medium with 50 μL of culture to a 1:10 000 dilution. Cultures were initially grown in the absence of antibiotic for two transfers and were then subjected to the following sub-minimum inhibitory concentrations (sub-MICs) of antibiotics: CAZ, 0.125 mg/L; CTX, 0.03125 mg/L; CRO, 0.0625 mg/L; and CTB, 0.25 mg/L. Antibiotic concentrations were doubled upon observation of vigorous growth up to a concentration corresponding to four times the MIC breakpoint of resistance for the given antibiotic as follows: CAZ, 16 mg/L; CTX, 8 mg/L; CRO, 8 mg/L; and CTB, 4 mg/L. For each serial passage step, 100 μL of culture was plated on LB plates supplemented with the corresponding concentration of antibiotic and the plates were incubated at 37 °C. To ensure plasmid maintenance, single colonies from selective plates were subsequently grown on 8 mg/L tetracycline plates. One clone from each serial passage step and from each linage was stored in 25% glycerol at −80 °C.

2.4. Plasmid extraction

Plasmid DNA was extracted from mutants using a QIAGEN® Plasmid Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's guidelines. Plasmid extraction was verified by enzymatic digestion with *Eco*RI according to the manufacturer's guidelines, followed by checking on an Invitrogen™ E-gel™ 2% agarose gel (Thermo Fisher Scientific) using digested pBR322 as a positive control. Extracted plasmids were subsequently transformed into competent *E. coli* MG1655.

2.5. PCR detection and sequencing of bla_{TEM-1}

Amplification of $bla_{\text{TEM-1}}$ was performed using primers designed by Perilli et al. [14]: TEM-1 FW, ATGAGTATTCAACATTTCCGT; and TEM-1 RV, CCAATGCTTAATCAGTGAGG (Eurofins Scientific, Luxembourg). PCR was performed using Dream*Taq* Mix (Thermo Fisher Scientific) according to the manufacturer's guidelines and with the following cycling conditions: 94 °C for 1 min, 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s, repeated for 30 cycles, followed by a final extension step at 72 °C for 5 min. Amplicons were checked on an Invitrogen™ E-gel™ 2% agarose gel. PCR amplicons were purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's guidelines. DNA concentrations were determined using a DeNovix DS-11+ spectrophotometer (DeNovix, Wilmington, DE, USA) and the purified PCR products were sequenced using an ABI 3730xl DNA Analyzer (GATC Biotech, Konstanz, Germany) using TEM-1 primers. The sequence was determined for both strands, covering the entire coding sequence.

2.6. Determination of minimum inhibitory concentrations

MICs for *E. coli* MG1655 and MG1655/pBR322 were determined by the broth microdilution method. Experiments were performed in Mueller–Hinton broth with an inoculum of 5×10^5 CFU/mL in 96-well microtitre plates incubated at 37 °C for 20 h with shaking at 300 rpm. The OD_{600} was measured on a Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, Vermont, USA). MICs of mutant transformants for CAZ, CTX and CRO were determined by Etest (Thermo Fisher Scientific). MICs for isolates exposed to CTB were determined by disk diffusion assay using CTB disks (30 mg/L) (Thermo Fisher Scientific). *E. coli* ATCC 25922 and AB604 were used as negative and positive controls for the MIC assessment. Cross-resistance to CAZ, CTX, CRO and CTB was investigated for all ESBL-producers by Etest or disk diffusion assay as previously described.

2.7. Detection of extended-spectrum β-lactamase (ESBL)-producing isolates by ESBL confirmation test

For phenotypic confirmation of ESBL production, an overnight culture of transformants was inoculated on a LB agar plate and a combination disk test (ROSCO®, Taastrup, Denmark) was used.

Antibiotic disks containing CTX and CTX + clavulanic acid (CLA) or CAZ and CAZ + CLA were placed on the inoculated agar plate and the inhibition zone diameter was measured following incubation. The isolate was confirmed as positive for ESBL production when the inhibition zone diameter was ≥5 mm larger with CLA than without it. *E. coli* ATCC 25922 and AB604 were used as negative and positive controls, respectively. Initially, mutant endpoints were tested for ESBL production and, if found positive, all isolated mutants from the serial passage experiment were tested.

2.8. Selective enrichment

Twenty individual lineages of MG1655/pBR322 overnight cultures were prepared in LB medium and were incubated overnight at 37 °C. Then, 1 mL of overnight culture of 3×10^9 CFU/mL was spiked into 1 g of pig faeces (provided by DTU Food, Kgs. Lyngby, Denmark) dissolved in 9 mL of MacConkey (Oxoid Ltd., Basingstoke, UK) broth with or without 1 mg/L CRO (Sigma-Aldrich) and was incubated for 16–24 h at 37 °C. Then, 100 μL of culture was plated on selective MacConkey agar (1 mg/L CRO).

Control studies without spiking of MG1655/pBR322 were performed by dissolving 1 g of pig faeces in 9 mL of MacConkey broth with or without 1 mg/L CRO supplement, and 20 biological replicates were made for each study. Samples were incubated for 16–24 h at 37 °C and were subsequently plated on selective MacConkey agar (1 mg/L CRO). All presumptive ESBL-producers were identified on Brilliance™ ESBL agar (Thermo Fisher Scientific). Positive ESBL-producers were stored in 25% glycerol at −80 °C.

2.9. Mutant selection on 1 mg/L ceftriaxone

Twenty individual lineages of MG1655/pBR322 overnight cultures were prepared in 2 mL of MacConkey broth and were incubated overnight at 37 °C. Resistant mutants were selected on selective MacConkey agar (1 mg/L CRO). Presumptive ESBL-producers were identified on Brilliance™ ESBL agar, and positive ESBL-producers were stored in 25% glycerol at −80 °C. Mutation frequencies were calculated as the number of resistant CFU divided by the total CFU determined on selective tetracycline-containing plates.

3. Results

3.1. Cephalosporins select for ESBL production

To understand the evolutionary mechanisms underlying ESBL evolution from TEM-1, the ability of four extended-spectrum cephalosporins to select for ESBLs in an *E. coli* strain encoding TEM-1 was examined. MICs were determined for the host strains *E. coli* MG1655 and MG1655/pBR322 encoding TEM-1. For CAZ, an MIC of 0.25 mg/L was observed for both strains. For CTX, MICs of 0.03 mg/L and 0.06 mg/L were observed for MG1655 and MG1655/pBR322, respectively. For CRO, MICs of 0.06 mg/L and 0.13 mg/L were observed for the two strains, respectively, and for CTB the MIC was 0.5 mg/L for both strains (Table 1). The results demonstrated MIC patterns typical of non-ESBL-producers, with low resistance to third-generation cephalosporins.

Six independent lineages of *E. coli* MG1655/pBR322 were subjected to serial passage in increasing concentrations of each antibiotic. For each passage, one colony was isolated and plasmids were extracted. Plasmids were re-introduced into *E. coli* MG1655 prior to MIC assessment and ESBL confirmation testing. Sequencing of PCR amplicons was performed to identify SNPs in the TEM-1 coding region. ESBL confirmation tests demonstrated that CAZ, CTX and CRO were able to select for ESBL production (Supplementary Tables S1–3), even at concentrations lower than the clinical breakpoint concentration. CTB did not select for ESBL production. Evolution of ESBLs using CAZ produced three types of mutants: R164H (TEM-29); R164S (TEM-12); and D179 G, a previously unidentified TEM ESBL variant (Fig. 1A). Substitution at position 164 by histidine or serine increased the MIC by 32-fold compared with the parental TEM-1 (Fig. 2). The substitution of aspartic acid by glycine at position 179 also positively affected enzyme activity, with a 16-fold increase in the MIC (Fig. 2). Exposure to CTX resulted in the evolution of three types of mutants: G238S (TEM-19); G238S–E104 K (TEM-15); and G238S–E240 K (TEM-71) (Fig. 1B). Acquisition of the G238S substitution increased the MIC by 16-fold compared with TEM-1 (Fig. 2). In four of the six lineages expressing the G238S variant, a subsequent mutation occurred at position 104 or 240, substituting a glutamic acid for a lysine. Acquisition of the second mutation increased the MIC by 8-fold compared with the single mutation and 128-fold compared with TEM-1 (Fig. 2B).

CRO selected five TEM-1 variants: G238S (TEM-19); G238S–E240K (TEM-71); G238S–T265M; R164S–A237T; and R241P (Fig. 1C). Acquisition of the R241P substitution, shifting an arginine for a proline, increased the MIC by 4-fold (Fig. 2). The R164S–A237T and the G238S (TEM-19) substitutions increased the MIC by 8-fold compared with the wild-type. A subsequent mutation occurred in two of the six lineages expressing the G238S variant (TEM-19), resulting in a glutamic acid being substituted for a lysine at position 240 or a threonine being substituted for methionine at position 265. Expression of the G238S–E240K or G238S–T265M variants increased the MIC to a greater value than the Etest strip could measure, resulting in a >256-fold increase (Fig. 2). Exposure to CTB did not select for an ESBL phenotype in MG1655/pBR322, and no mutations in the $bla_{\text{TEM-1}}$ gene were identified (Fig. 1D).

Table 1
Cross-resistance of *Escherichia coli* MG1655, MG1655/pBR322 transformants and extended-spectrum β-lactamase (ESBL) variants.

Substitution(s) in TEM-1	Isolate	MIC (mg/L) [fold change]			
		CAZ	CTX	CRO	CTB
Wild-type	MG1655	0.25	0.0313	0.0625	0.5
None, parental enzyme	MG1655/pBR322	0.25	0.0625	0.125	0.5
R164S (TEM-12)	JCL459	8 [32]	0.5 [8]	0.5 [4]	0.5
R164H (TEM-29)	JCL303	8 [32]	0.25 [4]	0.25 [2]	0.5
D179G (Unknown)	JCL308	4 [16]	0.125 [2]	0.125 [–]	0.5
G238S (TEM-19)	JCL130	1 [4]	1 [16]	>32 [>256]	0.5
G238S–E104K (TEM-15)	JCL328	16 [64]	8 [128]	>32 [>256]	0.5
G238S–E240K (TEM-71)	JCL133	32 [128]	8 [128]	>32 [>256]	0.5
R164S–A237T (unknown)	JCL154	1 [4]	0.5 [8]	1 [8]	0.5
G238S (TEM-19)	JCL150	1 [4]	>32 [>512]	1 [8]	0.5
G238S–E240K (TEM-71)	JCL153	32 [128]	>32 [>512]	>32 [>256]	0.5
G238S-T265M (unknown)	JCL155	1 [4]	>32 [>512]	>32 [>256]	0.5
R241P (unknown)	JCL148	0.5 [2]	0.125 [2]	0.5 [4]	0.5

MIC, minimum inhibitory concentration; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CTB, ceftibuten.

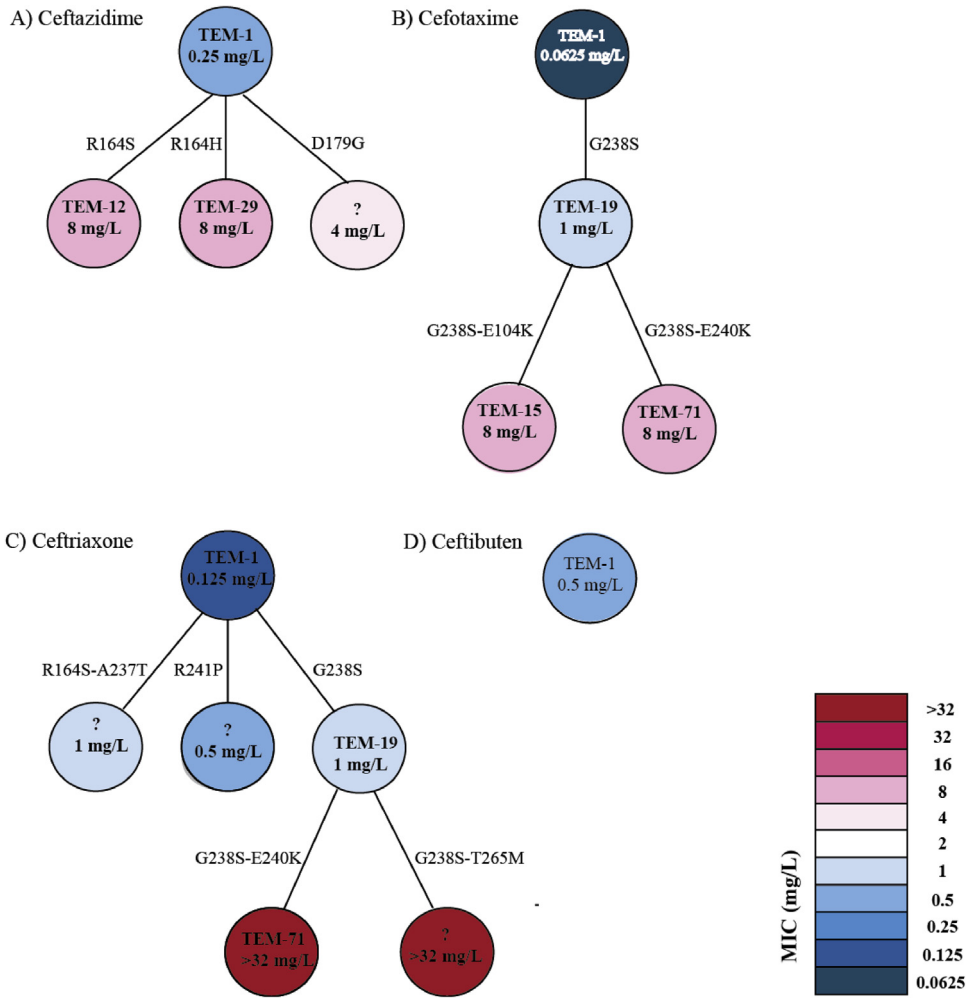

Fig. 1. In vitro evolutionary pathways to TEM-type extended-spectrum β-lactamases (ESBLs) under selective pressure from four third-generation cephalosporins on *Escherichia coli* MG1655 harbouring TEM-1: (A) ceftazidime; (B) cefotaxime; (C) ceftriaxone; and (D) ceftibuten. Color-coded minimum inhibitory concentrations (MICs) of parental TEM-1 for each antibiotic are shown in the upper circle. ESBL variants are shown within circles with MICs and TEM-type (if known), and amino acid substitutions are shown above each ESBL variant.

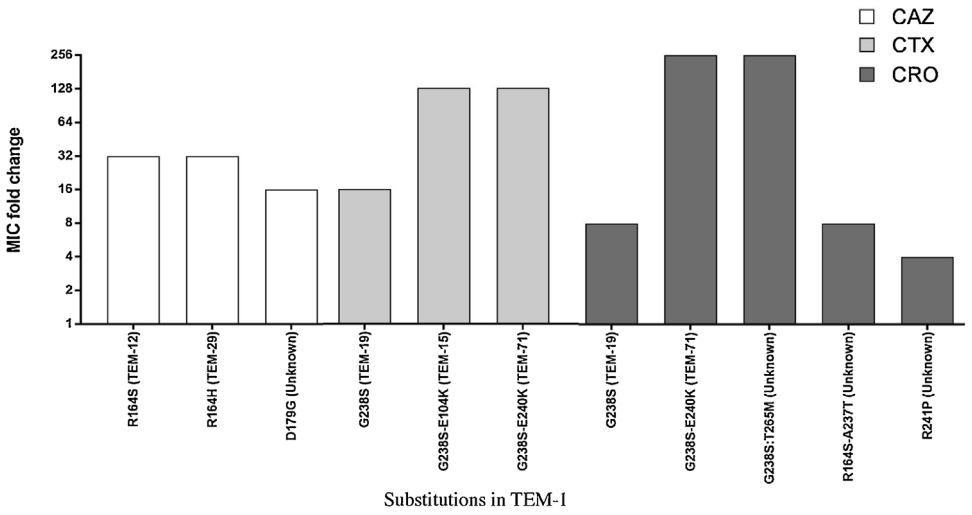

Fig. 2. Fold change in susceptibility of mutated extended-spectrum β-lactamase (ESBL) variants to β-lactams compared with parental TEM-1. Ceftibuten did not select for ESBL-producers. CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone.

3.2. High levels of cross-resistance in extended-spectrum β-lactamase mutants

To investigate the level of cross-resistance of mutants, MICs for CAZ, CTX, CRO and CTB were determined. Substitutions at position 164 were associated with an increase in resistance to both CTX and CRO, with the R164S substitution having a larger effect on the MIC than the R164H substitution (Table 1). Expression of the D179G variant only increased the MIC of CTX by 2-fold, with no effect on CRO resistance. The G238S (TEM-19) substitution demonstrated a high level of resistance to CRO, with a >256-fold MIC increase (Table 1). Resistance to CAZ increased 4-fold. An additional mutation in TEM-19, resulting in the expression of either G238S–E104K (TEM-15) or G238S–E240K (TEM-71), significantly increased the levels of resistance to CAZ with a 64-fold and 128-fold increase, respectively, compared with TEM-1, but had no effect on CRO resistance. ESBL-producers selected on CRO harbouring the G238S substitution (TEM-19) demonstrated a high level of resistance to CTX with a >512-fold increase in MIC, whereas ESBL-producers selected on CTX harbouring the same substitution demonstrated a 16-fold increase in resistance to CRO. In both cases, resistance towards CAZ increased 4-fold. Resistance to CAZ increased 4-fold. Acquisition of an additional mutation in the G238S variant, resulting in expression of G238S–T265M, did not further increase the MIC of CAZ. In contrast, expression of G238S–E240K (TEM-71) led to a 128-fold increase in the MIC of CAZ. CTX resistance was unaffected by the additional mutation. Expression of R241P had a negligible impact on resistance to CAZ and CTX. Expression of R164S–A237T changed the resistance spectrum compared with R164S (TEM-12) by increasing resistance to CRO and decreasing CAZ resistance. Resistance to CTX remained unaltered, and CTB susceptibility remained unchanged in all of the mutant variants.

3.3. Extended-spectrum β-lactamase evolution using selective enrichment

ESBL-producers are sometimes difficult to detect in faecal samples from pigs [25]. To increase the detectable levels of ESBLs, incubation in broth containing antibiotics is performed before selective plating [26]. In the current study, the effect of CRO selective enrichment on ESBL evolution when plasmids harbouring TEM-1 were present was investigated, both in MacConkey broth and MacConkey broth containing pig faecal matter.

When MG1655/pBR322 was grown in MacConkey broth and plated on selective MacConkey agar (1 mg/L CRO), 85% (17/20) of overnight cultures were able to produce resistant colonies positive for ESBL production with a mutation frequency of 1.7×10^{-8}. To examine whether the detection level of ESBL-producers is affected by the selective enrichment procedure, pig faeces was dissolved in MacConkey broth with and without 1 mg/L CRO. Samples were incubated overnight prior to plating on selective MacConkey agar. Without selective enrichment, 30% (6/20) of samples tested positive for ESBL production, whereas 45% (9/20) of samples were positive of ESBL production using selective enrichment (Table 2).

To test whether ESBL evolution is affected by selective enrichment in the presence of a plasmid harbouring TEM-1, an overnight culture of MG1655/pBR322 was spiked into faecal samples dissolved in MacConkey broth with or without 1 mg/L CRO. Following overnight incubation, samples were plated on selective MacConkey agar. Without selective enrichment, spiking of plasmid-encoded TEM-1 resulted in 85% (17/20) of samples positive for ESBL-producers. With selective enrichment, spiking of TEM-1 resulted in 100% (20/20) of samples positive for ESBL-producers (Table 2), indicating de novo evolution of ESBL from the spiked TEM-1 enzymes.

Table 2
Positive extended-spectrum β-lactamase (ESBL)-producers after selective enrichment using pig faeces dissolved in MacConkey broth (F + MB), with and without 1 mg/L ceftriaxone (CRO).

Sample no.	CFU/mL			
	E. coli MG1655		MG1655/pBR322	
	F + MB	F + MB + CRO	F + MB	F + MB + CRO
1	0	7	5	2
2	0	0	10	7
3	1	0	3	6
4	0	9	3	1
5	0	0	9	2
6	0	80	11	2
7	0	11	7	6
8	0	0	3	1
9	18	0	5	6
10	0	16	14	7
11	12	1	0	6
12	0	6	1	3
13	0	0	7	1
14	1	1	5	3
15	7	0	4	1
16	0	0	0	3
17	0	0	9	8
18	1	1	0	3
19	0	0	4	1
20	0	0	1	8

4. Discussion

In this study, the effect of four third-generation cephalosporins (CAZ, CTX, CRO and CTB) on the evolution of TEM-type ESBLs from an *E. coli* MG1655 strain harbouring a plasmid-encoded TEM-1 was examined as well as the consequences of selective enrichment on ESBL evolution. It was shown that CAZ, CTX and CRO selected for multiple TEM ESBL variants (Fig. 1). In addition, CTX and CRO selected for ESBLs at concentrations at the MIC of MG1655/pBR322. Interestingly, CTB did not select for ESBLs, which is in accordance with the work of Perilli et al. [14].

Four TEM-type ESBL variants with amino acid substitutions D179G, R241P, R164S–A237T and G238S–T265M were isolated in this study that previously have only been produced in laboratory experiments. The D179G substitution increased resistance to CAZ by 16-fold compared with the TEM-1, with negligible cross-resistance to CTX and CRO. The R241P substitution resulted in a 4-fold increase in resistance to CRO but had no significant effect on CAZ or CTX resistance. The fact that D179G and R241P ESBLs have not been observed in clinical isolates could illustrate that the potential for ESBL evolution is broader than can be deduced from sequence analysis of clinical samples alone.

In silico approaches have recently been employed to facilitate a deeper understanding of the factors determining TEM specificity [27] and to assist in the prediction of the effects of different mutations. In particular, the effect of the G238S mutation in combination with additional mutations show increased rigidness of the Ω-loop in several cefotaximase variants as well as regions consisting of residues 86–118, 213–229 and 267–271, that upon binding of cefotaxime revealed a hidden potential of mutations yielding increased cefotaximase activity [27–31]. This correlates with the current study where acquisition of the G238S substitution in combination with subsequent additional mutations yielded significantly increased cefotaximase activity compared with TEM-1.

Moreover, advanced sequencing technologies have enabled rapid molecular analysis of genes from unculturable bacteria and revealed a hidden reservoir of uncharacterised β-lactamase sequences [32].

However, owing to the strong epistatic effects of mutations in the TEM-1 sequence [33] and the broad phylogenetic diversity of the β-lactamases in general [32], a computational method that can predict ESBL activity from sequence data alone has yet to be realised.

In this study, a G238S substitution (TEM-19) was selected by CRO that surprisingly resulted in an 8-fold increase in resistance tow CRO but a >512-fold increase in resistance to CTX. CTX also selected a G238S substitution (TEM-19), resulting in a 16-fold increase in resistance to CTX and a >256-fold increase in resistance to CRO, indicating additional factors encoded on the plasmid affecting the resistance spectrum. Clinically isolated ESBL variants frequently harbour more than one amino acid substitution [34]. As the initial substitution offers an expanded substrate spectrum but potentially reduces enzyme stability, a second mutation often compensates for the deleterious effects [35].

Double mutations were observed under CTX and CRO selection, where an initial G238S substitution (TEM-19) occurred early. Continued exposure to CTX resulted in plasmids harbouring TEM-15 (G238S–E104K) or TEM-71 (G238S–E240K). TEM-15 (G238S–E104K) increased the resistance level of CAZ by 64-fold compared with TEM-1 and by 16-fold compared with TEM-19. CTX resistance increased 128-fold compared with TEM-1 and 8-fold compared with TEM-19. Resistance to CRO remained unaltered.

Interestingly, selection of TEM-71 under selective pressure from CTX and CRO shifted the resistance pattern compared with TEM-19. The introduction of TEM-19 significantly increased cross-resistance, but with less of an effect on the level of resistance for the selecting cephalosporins. Selection of TEM-71 did not affect levels of cross-resistance, but a significant increase in the resistance level was observed to the cephalosporin by which it had been selected.

Two ESBL variants harbouring double amino acid substitutions R164S–A237T [22] and G238S–T265M [36] have only previously been isolated in in vitro laboratory experiments using site-directed mutagenesis, and not by selection.

The A237T substitution may work as a modulating substitution, reducing activity against some βlactams while increasing it against others [22]. This was also observed in the current data, where acquisition of the R164S–A237T substitution was associated with an 8-fold decrease in CAZ resistance compared with the R164S background, and a 2-fold increase in CRO resistance. Resistance levels against CTX remained unaltered.

The T265M substitution has previously been described both in clinical and laboratory isolates, but most often in combination with two other mutations. We showed that acquisition of the second T265M substitution in a G238S background increased the MIC against CRO by >32-fold compared with its G238S background. High levels of resistance to CTX remained unaltered compared with the G238S background, as did resistance to CAZ. The role of the T265 M substitution has not yet been fully elucidated but it has been proposed to have a stabilising effect on the enzyme, thereby increasing resistance levels. However, this is in contrast to a study performed by Huang et al. where construction of a G238S–T265M variant using site-directed mutagenesis did not have a significant effect on resistance levels to either CTX or CAZ compared with the wild-type [36]. The clinically important TEM ESBLs variants TEM-12 (R164S) and TEM-29 (R164H) were isolated upon selective pressure from CAZ, and both have been linked to increased resistance to CAZ and CTX [37]. Resistance to CTX and CRO was increased in both mutants with substitutions at position 164, with the serine substitution having the highest impact on resistance levels compared with histidine.

Upon examination of the cross-resistance of selected ESBL mutants, all isolates remained highly susceptible to CTB. This has been reported previously, where the stability of CTB was proposed to be due to the carboxyethilidine moiety at position 7 of the βacyl side chain of the compound [38]. CTB could therefore be considered a therapeutic alternative for use as a resistance management tool to reduce the risk of ESBL evolution in the clinic and as an alternative treatment for infections caused by ESBL-producers. Further investigations of the interaction of CTB and enzymes from other ESBL families such as CTX-M and SHV are required.

The practice of selective enrichment for ESBL detection influences ESBL evolution through the selection of de novo mutations. Selective pre-enrichment is the standard procedure for detection and isolation of ESBL-producing Enterobacteriaceae in clinical samples [25,39–44] and is thought to improve the detection rate of micro-organisms present in low numbers [42]. We show that this procedure may be unreliable owing to a very high frequency of false-negative results (55%) when the same sample was tested multiple times (Table 2). Moreover, we show that the addition of bacteria harbouring the non-ESBL TEM-1 enzyme significantly influenced the number of positive tests results (Table 2). In addition, we clearly show that pre-enrichment increases the number of de novo mutations in non-ESBL enzymes. These data indicate that TEM-type ESBLs are selected for and enriched at two points in this selective enrichment method: (i) plating on plates supplemented with 1 mg/L CRO, as supported by the control study; and (ii) upon incubation of samples with selective enrichment, resulting in 100% of spiked samples being positive for ESBL-producers. The effects of false-negative and false-positive results have been extensively investigated in the context of post-selection ESBL confirmation tests, but the reproducibility of multiple tests of the same sample has been less investigated. The results presented in this study indicate that evolutionary processes such as mutation rates and frequency can strongly affect the outcome of the identification efforts, which ultimately can lead to both overestimation and underestimation of the true prevalence of ESBL-producers in clinical samples.

This concern has been addressed by the European Food Safety Authority (EFSA) who published a technical specification on the use of a selective enrichment approach to increase detection rates [26]. Several studies have shown that the use of selective enrichment significantly increases detectable levels of ESBL in samples [25,39–41,45–47]. However, the use of different antibiotics in varying concentrations makes it difficult to compare data in order to assess the added value of selective enrichment. In the data in the current study, upon spiking samples with TEM-1 85% of samples were positive for ESBL-producers, which increased to 100% of samples with the additional use of selective enrichment (Table 2). These data are therefore of high importance as they suggest that the practice of using selective enrichment could result in an artificially high detection level of TEM-type ESBL-producers in samples. Further investigations are therefore needed to determine the reproducibility of the pre-enrichment procedure in multiple testing and additionally to examine whether other ESBL enzymes are affected by the procedure of selective enrichment to the same extent as the TEM family.

5. Conclusion

This study assessed the ability of four third-generation cephalosporins to select for TEM-type ESBL mutants in vitro. The results showed that CAZ, CTX and CRO selected for ESBLs even at low levels of antibiotic owing to mutations in TEM-1. The majority of the ESBLs demonstrated high-level resistance to the antibiotic by which they had been selected but also demonstrated high levels of cross-resistance. Interestingly, CTB was not able to select for mutations in TEM-1, and all ESBL mutants remained susceptible to CTB. We also propose that the use of selective

enrichment for the detection of ESBLs should be used with caution as there is a risk of overestimating ESBL levels owing to the evolution and selection of TEM-type ESBLs. A high risk of false-negative samples was also observed, which should be kept in mind when establishing whether a sample is negative or positive for ESBL-producers.

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Competing interests

None declared.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.03.010.

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