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Evaluation of CTX-M steady-state mRNA, mRNA half-life and protein production in various STs of *Escherichia coli*

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Objectives: High levels of β-lactamase production can impact treatment with a β-lactam/β-lactamase inhibitor combination. Goals of this study were to: (i) compare the mRNA and protein levels of CTX-M-15- and CTX-M-14-producing *Escherichia coli* from 18 different STs and 10 different phylotypes; (ii) evaluate the mRNA half-lives and establish a role for chromosomal- and/or plasmid-encoded factors; and (iii) evaluate the zones of inhibition for piperacillin/tazobactam and ceftolozane/tazobactam.

Methods: Disc diffusion was used to establish zone size. RNA analysis was accomplished using real-time RT–PCR and CTX-M protein levels were evaluated by immunoblotting. Clinical isolates, transformants and transconjugants were used to evaluate mRNA half-lives.

Results: mRNA levels of CTX-M-15 were up to 165-fold higher compared with CTX-M-14. CTX-M-15 protein levels were 2–48-fold less than their respective transcript levels, while CTX-M-14 protein production was comparable to the observed transcript levels. Nineteen of 25 *E. coli* (76%) had extended CTX-M-15 mRNA half-lives of 5–15 min and 16 (100%) CTX-M-14 isolates had mRNA half-lives of <2–3 min. Transformants had mRNA half-lives of <2 min for both CTX-M-type transcripts, while transconjugant mRNA half-lives corresponded to the half-life of the donor. Ceftolozane/tazobactam zone sizes were ≥19 mm, while piperacillin/tazobactam zone sizes were ≥17 mm.

Conclusions: CTX-M-15 mRNA and protein production did not correlate. Neither *E. coli* ST nor phylotype influenced the variability observed for CTX-M-15 mRNA or protein produced. mRNA half-life is controlled by a plasmid-encoded factor and may influence mRNA transcript levels, but not protein levels.

Introduction

In Gram-negative bacteria, β-lactamase production is the most common mechanism identified conferring resistance to β-lactams.1 Development of β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam provides an effective method for evading this resistance mechanism. These inhibitors have minimal antibiotic activity against enteric bacilli when used alone; however, a synergistic effect is created when administered in combination with a penicillin or cephalosporin. Currently there are four penicillin/inhibitor combinations approved for clinical use in the USA, including ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin/clavulanate and piperacillin/tazobactam.2 Recently, the FDA approved the use of ceftolozane/tazobactam for the treatment of complicated urinary tract infections. By irreversibly binding to the enzyme, the β-lactamase inhibitor protects the β-lactam antibiotic from being hydrolysed by the β-lactamase. Such β-lactamase inhibitor combinations are highly active against most class A β-lactamases, but are poorly active against classes C and D, and inactive against class B β-lactamases.2 The clinical efficacy of a β-lactamase inhibitor/β-lactam combination depends on many factors, including concentration of inhibitor used in the
formulation, amount of β-lactamase produced by the bacterial cell and the concentration of antibiotic that enters the periplasmic space. Emergence of resistance to β-lactam/β-lactamase inhibitor combinations can severely impact the ability to treat serious respiratory tract, urinary tract and bloodstream infections. Therefore, the ratio of β-lactam/β-lactamase inhibitor used in combinations is critical because use of an inappropriate amount of inhibitor may impact the therapeutic value of the drug.

CTX-M-producing Escherichia coli are predominately the pandemic ST131 clone and frequently cause urinary tract infections. The rapid spread of these strains has led to the CTX-M pandemic. Two major genotypes of CTX-M have become established worldwide, CTX-M-15 and CTX-M-14. These CTX-M producers have contributed to both hospital- and community-acquired urinary tract infections. E. coli represents 50% of infections leading to uroseptic shock in hospitalized patients and the majority of uroseptic infections in these patients originate from the community. β-Lactam/β-lactamase inhibitor combinations can be an effective treatment for infections caused by CTX-M-producing organisms.

Recently, our laboratory has documented elevated levels of CTX-M-15 mRNA, in comparison with CTX-M-14 mRNA levels, in E. coli from human urine samples. This difference in steady-state mRNA expression between CTX-M-15 and CTX-M-14 producers was observed from isolates collected from various geographical locations worldwide indicating that this observation was not due to a local clonal population of isolates. Because different primer sets were used for expression of CTX-M-14 and CTX-M-15 structural genes and promoter regions by PCR, ligation of the PCR products into vector MDR009 (chloramphenicol promoter removed) and transformation into MG1655, J53 and HFM16 E. coli. The structural genes encoding CTX-M-14 and CTX-M-15 were sequenced from various clinical isolates along with their upstream promoter regions using primers listed in Table S2. All clones and subclones were sequenced. To create the CTX-M MG1655 transformants, pMRD009 was the final destination vector. For the luciferase clones, the CTX-M promoter regions were fused to the luciferase gene and cloned into vector pMP220, while CTX-M genes driven by the chloramphenicol or lacZ promoter were cloned into pACYC184 (ATCC) and pUCP26, respectively.

Methods

Bacterial isolates and susceptibility testing

The study population comprised 57 CTX-M-producing E. coli clinical isolates chosen from 18 geographic locations to ensure that the data did not represent a local point-source clonal outbreak (Table 1). In addition to harbouring a CTX-M-14 or CTX-M-15 β-lactamase, most isolates possessed other β-lactamases such as TEM-1-like or OXA-1, as determined by family-specific PCR. Twenty-five CTX-M-15-producing and 16 CTX-M-14-producing E. coli were used to evaluate mRNA half-life. The donor strains used in conjugation studies included XQ12 (CTX-M-15), XQ25 (CTX-M-15), C15 (CTX-M-15), D14 (CTX-M-14) and XQ10 (CTX-M-14) were conjugated with J53 E. coli using brain heart infusion (Difco, Thermo Scientific) broth mating. A donor to recipient ratio of 1:2.5 was used in all conjugation experiments. Transconjugants were selected on LB agar with sodium azide (NaN₃) 100 mg/L and cefotaxime 24 mg/L. J53 transconjugants were mated with SalLT2 and plated on MacConkey agar with cefotaxime at 24 mg/L. Following transfer into SalLT2, the CTX-M-harbouring plasmid was moved by filter mating into MG1655 and FHM16 with transconjugants selected using MacConkey agar supplemented with 24 mg/L cefotaxime. Plasmid transfer was confirmed via agarose gel electrophoresis (data not shown).

Cloning and sequencing

Transformants were constructed by amplifying the blaCTX-M-14 and blaCTX-M-15 structural genes and promoter regions by PCR, ligation of the PCR products into vector MDR009 (chloramphenicol promoter removed) and transformation into MG1655, J53 and HFM16 E. coli. The structural genes encoding CTX-M-14 and CTX-M-15 were sequenced from various clinical isolates along with their upstream promoter regions using primers.

Primer efficiency evaluations and determination of copy number using PCR

Because different primer sets were used for expression of CTX-M-14 and CTX-M-15, comparisons of the data obtained using these primers could not be made unless the primers amplified with the same efficiency. Total DNA was extracted using the Qiagen DNeasy Blood and Tissue kit. Ten-fold serial dilutions were prepared ranging from 250 ng to 0.025 ng. The master mix included 1x Rotorgene SYBR green buffer (Qiagen, Valencia, CA, USA), 10 pmol of each forward and reverse CTX-M-specific real-time primer and RNA-free H₂O for a total reaction volume of 40.5 µL. A separate master mix was prepared using the endogenous control primer set (frr or 16S rRNA) and these genes were used to normalize the expression data. A standard curve was constructed for both the CTX-M and endogenous control primer sets for each isolate. The C₅₀ values were plotted against the logarithm of the DNA concentration. Each standard curve was generated from a linear regression of the plotted points. Using the slope of each standard curve, the PCR amplification efficiency (E) was calculated using the equation E = 10⁻¹/slope - 1, while the efficiency percentage was calculated using the equation E = (10⁻¹/slope - 1)×100. The R² value ranged from 0.97 to 0.99. The targets CTX-M-15, CTX-M-14
Table 1. Characteristics, expression data and susceptibility data (zone sizes in mm) for CTX-M-15- and CTX-M-14-producing E. coli isolates used in this study

| Strain | Geographical location of isolation | bla<sub>CTX-M</sub> allele | ST | Phylotype | Relative fold change in expression ± SD | Relative fold change in protein ± SD | Zone diameter (mm) |
|--------|-----------------------------------|--------------------------|----|-----------|----------------------------------------|-------------------------------------|-------------------|
| D14    | Omaha, NE                         | CTX-M-14                 | ST405 | D2       | 1                                     | 1 ± 0.15                           | 22 21             |
| CUMC 247 | Omaha, NE                        | CTX-M-15                 | ST131 | B2       | 72 ± 12.56                           | 2.5 ± 0.39                         | 23 21             |
| XQ12   | Seattle, WA                       | CTX-M-15                 | ST131 | B2       | 21 ± 5.12                           | 10.3 ± 3.6                         | 22 22             |
| XQ35   | Seattle, WA                       | CTX-M-15                 | ST131 | B2       | 8 ± 0.87                            | 1.06 ± 0.87                        | 20 25             |
| A15    | Omaha, NE                         | CTX-M-15                 | ST44  | D2       | 15 ± 1.28                           | ND                                 |                   |
| C15    | Omaha, NE                         | CTX-M-15                 | ST405 | B1       | 48 ± 0.70                           | 3.9 ± 2                            | 23 22             |
| H15    | Omaha, NE                         | CTX-M-15                 | ST205 | B2       | 20 ± 0.68                           | 12.2 ± 5.0                         | 17 23             |
| W15    | Omaha, NE                         | CTX-M-15                 | ST131 | B2       | 29 ± 1.43                           | 2.6 ± 0.25                         | 17 21             |
| FH6M   | India                             | CTX-M-15                 | ST131 | B2       | 48 ± 8.41                           | 3.2 ± 1.21                         | 21 19             |
| RS059  | UK                                | CTX-M-15                 | ST131 | B2       | 77 ± 32.47                         | 10.0 ± 2.43                        | 23 20             |
| RS061  | UK                                | CTX-M-15                 | ST131 | B2       | 52 ± 3.65                           | 7.7 ± 2.33                         | 24 20             |
| RS007  | UK                                | CTX-M-15                 | ST131 | B2       | 1 ± 0.09                           | ND                                 |                   |
| RS120  | UK                                | CTX-M-15                 | ST131 | B2       | 1 ± 0.14                           | ND                                 |                   |
| F010   | UK                                | CTX-M-15                 | ST69  | D1       | 26 ± 2.51                           | 3.5 ± 1.5                         | 20 25             |
| F024   | UK                                | CTX-M-15                 | ST2076 | D1       | 46 ± 2.30                           | ND                                 |                   |
| F076   | UK                                | CTX-M-15                 | ST182 | D1       | 74 ± 6.92                           | ND                                 |                   |
| RS135  | UK                                | CTX-M-15                 | ST131 | B2       | 165 ± 27.76                        | 22.3 ± 10.7                        | 18 20             |
| RS153  | UK                                | CTX-M-15                 | ST617 | A2       | 37 ± 2.75                           | 8.1 ± 1.27                         | 25 30             |
| CM8106 | Minneapolis, MN                   | CTX-M-15                 | ST131 | B2       | 51 ± 8.17                           | 2.1 ± 0.46                         |                   |
| J22052 S | Evanston, IL                    | CTX-M-15                 | ST90  | B1       | 32 ± 1.58                           | 2.9 ± 1.97                         |                   |
| J22052 F | Evanston, IL                    | CTX-M-15                 | ST90  | B1       | 32 ± 3.90                           | 2.4 ± 1.80                         |                   |
| J22053 | Evanston, IL                      | CTX-M-15                 | ST131 | B2       | -14 ± 0.04                           | UD                                 |                   |
| J22131 | Minneapolis, MN                   | CTX-M-15                 | ST167 | A2       | 47 ± 10.83                           | 2.9 ± 1.79                         |                   |
| J2235 S | Houston, TX                      | CTX-M-15                 | ST167 | A2       | 39 ± 11.27                           | 15.4 ± 2.75                        |                   |
| J2235 F | Houston, TX                      | CTX-M-15                 | ST167 | A2       | 62 ± 17.32                           | 4.4 ± 2.41                         |                   |
| J2236  | New York, NY                      | CTX-M-15                 | ST648 | D2       | 65 ± 10.88                           | 14.1 ± 4.86                        |                   |
| J2241  | New Brunswick, NJ                 | CTX-M-15                 | ST131 | B2       | 14 ± 3.97                           | -2 ± 0.10a                        |                   |
| J2242  | Rochester, NY                     | CTX-M-15                 | ST131 | B2       | 6 ± 0.44                           | UD                                 |                   |
| J2243  | Milwaukee, WI                     | CTX-M-15                 | ST131 | B2       | 1 ± 0.90                           | UD                                 |                   |
| J2244  | Detroit, MI                       | CTX-M-15                 | ST131 | B2       | 29 ± 4.22                           | 1.7 ± 0.62                         |                   |
| J2246 S | Ewa Beach, HI                     | CTX-M-15                 | ST410 | A2       | 60 ± 15.83                           | 5.1 ± 1.50                         |                   |
| J2246 F | Ewa Beach, HI                     | CTX-M-15                 | ST410 | A2       | 155 ± 41.89                         | 28.1 ± 2.48                        | 20 22             |
| J2247  | Galveston, TX                     | CTX-M-15                 | ST131 | B2       | 48 ± 7.02                           | -1.3 ± 0.42a                       | 20 22             |
| J2251  | New York, NY                      | CTX-M-15                 | ST131 | B2       | 49 ± 5.06                           | 1.6 ± 0.93                         | 20 26             |
| J2253 SW | Minneapolis, MN                  | CTX-M-15                 | ST410 | A2       | 36 ± 5.94                           | 2.4 ± 0.57                         |                   |
| J2253 RG | Minneapolis, MN                  | CTX-M-15                 | ST410 | A2       | 34 ± 5.91                           | 12.1 ± 3.10                        |                   |
| J2267  | Salt Lake City, UT               | CTX-M-15                 | ST648 | D2       | 42 ± 25.83                           | 9.5 ± 1.50                         |                   |
| J2431  | New York, NY                      | CTX-M-15                 | ST131-like | B2       | 39 ± 9.42                           | 23.8 ± 3.15                        |                   |
| MHHB54 | Spain                             | CTX-M-15                 | ST131 | B2       | 49 ± 2.40                           | 6.2 ± 2.10                         |                   |
| MHWl2  | France                            | CTX-M-15                 | ST131 | B2       | 59 ± 15.05                           | 16.1 ± 3.49                        |                   |
| QO15   | Queensland, Australia             | CTX-M-15                 | ST131 | B2       | 14 ± 3.85                           | 1.9 ± 0.55                         |                   |
| C14    | Omaha, NE                         | CTX-M-14                 | ST648 | D2       | 5 ± 2.08                            | 5.0 ± 0.82                         | 21 21             |
| Lo14   | Omaha, NE                         | CTX-M-14                 | ST648 | D2       | 4 ± 1.68                            | 6.2 ± 0.10                         | 26 28             |
| N14    | Omaha, NE                         | CTX-M-14                 | ST405 | D2       | 5 ± 0.39                            | 3.1 ± 0.81                         |                   |
| F044   | UK                                | CTX-M-14                 | ST131 | B2       | 1 ± 0.15                            | -1.4 ± 0.09a                       | 25 24             |
| NL217  | UK                                | CTX-M-14                 | ST131 | B2       | 1 ± 0.19                           | 1.6 ± 0.75                         | 26 23             |
| XQ10   | Seattle, WA                       | CTX-M-14                 | ST38  | D        | 1 ± 0.40                           | ND                                 |                   |
| XQ13   | Seattle, WA                       | CTX-M-14                 | ST68  | D        | 2 ± 0.72                            | 1.5 ± 0.90                         | 23 21             |
| XQ22   | Seattle, WA                       | CTX-M-14                 | ST156 | B2       | 1 ± 0.73                           | ND                                 | 23 23             |

Continued
Table 1. Continued

| Strain      | Geographical location of isolation | blaCTX-M allele | ST | Phylotype | Relative fold change in expression + SD | Relative fold change in protein + SD | Zone diameter (mm) |
|------------|-----------------------------------|-----------------|----|-----------|----------------------------------------|--------------------------------------|--------------------|
| XQ24       | Seattle, WA                       | CTX-M-14        | ST10 | A         | 1 ± 0.17                               | ND                                   | 22                 |
| JJ2339     | Ewa Beach, HI                     | CTX-M-14        | ST38 | D2        | 2 ± 0.64                               | 2.1 ± 0.66                           | 22                 |
| JJ2354     | Orange County, CA                 | CTX-M-14        | ST354 | D1        | 2 ± 0.76                               | 1.4 ± 0.47                           | 23                 |
| JJ2356     | Seattle, WA                       | CTX-M-14        | ST46 | A1        | 1 ± 0.26                               | 3.7 ± 1.17                           | 23                 |
| FS-ESBL013 | Denmark                           | CTX-M-14        | ST10 | D2        | 2 ± 0.53                               | 1.3 ± 0.42                           | 23                 |
| FS-ESBL014 | Denmark                           | CTX-M-14        | ST38 | A2        | 3 ± 1.55                               | 13.9 ± 4.32                          | 23                 |
| FS-ESBL062 | Denmark                           | CTX-M-14        | ST10 | D1        | 2 ± 0.47                               | 11 ± 1.06                            | 23                 |

ND, not determined; UD, undetected.
All transcript levels are relative to D14, which was used as the comparator and set to 1.
Fold changes in protein are normalized values.
*Down-regulation of gene or protein production.

or luciferase (luc) and reference (frf, 16S rRNA) primer sets did not vary more than 10% from each other.

**blaCTX-M-15 and blaCTX-M-14 relative copy number determination**
Relative gene copy number quantification was calculated using the equation of Skulj et al.23, copy number = (Ec average) C(Frfr average) C(luc), where E is the primer efficiency, C is the normalization gene and p is the gene of interest. Copy number studies were completed on select CTX-M-14- and CTX-M-15-producing clinical isolates in Tables 2 and 3.

**MLST and phylotyping**
Seven-locus MLST was done according to the Achtman system (http://mlst.ucc.ie/mlst/dbs/Ecoli). Major E. coli phylogenetic groups (A, B1, B2 or D) were determined using an established triplex PCR method.23

**RNA isolation and mRNA expression assays**
RNA was isolated using TRIZol® Max™ (Invitrogen) from 1.5 mL of mid-logarithmic phase culture grown in Mueller–Hinton broth (OD600 of ~0.5).24,25 Genomic DNA was removed by RQ1 DNase (Promega, Madison, WI, USA) treatment. 250 ng of DNA-free RNA was used in 50 μL PCRs that consisted of 1x concentration of QuantitTect® SYBR® Green RT–PCR master mix, RT mix (Qiagen, Hilden, Germany) and 25 pmol of each primer. RT–PCR was performed on the Rotor Gene Q Splex high resolution melt system (Qiagen, Valencia, CA, USA) that included RT activation at 50°C for 40 min, HotStart Taq DNA polymerase activation at 95°C for 15 min and three step cycling conditions for 40 cycles. Cycling parameters included denaturation at 95°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 30 s. Relative mRNA expression for CTX-M-14 and CTX-M-15 was calculated using the 2^ΔΔct method with D14 as the comparator.26,27 The single copy gene, frf was used to normalize the data. Three independent RNA isolations and three individual RT–PCR assays were completed with a coefficient of variation of <10%.

**Evaluation of mRNA half-lives**
Cultures were grown to mid-logarithmic phase (OD600 of ~0.5) in Mueller–Hinton broth and treated with 200 mg/L rifampicin (Sigma Aldrich, St Louis, MO, USA). Cells were harvested by centrifugation at 0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min post-rifampicin addition and RNA was isolated using TRIZol® Max™. Real-time RT–PCRs were done using 250 ng of DNA-free RNA. Data were normalized to the 16S rRNA gene of E. coli. The equation by Pfaffl28 was used to determine the ratio of transcript that remained at each timepoint.

**Immunoblot for CTX-M-14/CTX-M-15 detection**
The linear response range of the Stain-Free fluorescence and the anti-CTX-M antibody for bacterial lysates was determined by performing a western blot on a dilution series of total protein ranging from 40 to 0.625 μg as previously described.27 The protein linearity ranged from 5 to 40 μg (Figure S1) and the linear range of the anti-CTX-M antibody was from 2.5 to 40 μg (Figure S2). Therefore, 10 μg of total protein and an antibody dilution factor of 1:45 000 were used for immunoblot analyses.

Whole cell protein lysate was prepared from clinical isolates listed in Table 1 as previously described.29 Stain-Free SDS-PAGE, imaging and total protein normalization were carried out as previously described.29 A custom polyclonal antibody specific for CTX-M-14 and CTX-M-15, directed toward the peptide sequence CAIPGDPRDTT was generated by GenScript (Piscataway, NJ, USA) and the secondary antibody (horseradish peroxidase-goat anti-rabbit IgG) was used with a dilution factor of 1:50 000. The chemiluminescent signal intensity of CTX-M was normalized to the Stain-Free fluorocent signal intensity of total protein for each isolate. K12 MG1655 (WT E. coli) and FHMI61 (clinical isolate without CTX-M) lysates were used as controls for cross-reactivity to proteins other than CTX-M. To ensure that the antibody recognized each protein with the same affinity, E. coli transformants expressing each CTX-M gene (blaCTX-M-14 and blaCTX-M-15) from a common plasmid driven by the same promoter element were used to compare the efficiency of antibody detection (data not shown). Three biological replicates of each isolate were independently collected and the means of the normalized data sets were used to calculate the relative amount of CTX-M production.28,29 Statistical significance of CTX-M protein levels between isolates was evaluated using a t-test (double-sided and paired) performed with GraphPad Prism software, version 4.0.

**Results**

**Relative levels of blaCTX-M-14 and blaCTX-M-15 transcripts**
The CTX-M β-lactamases originated from the Klyuyeva species, a group of environmental, non-pathogenic organisms that contain...
Table 2. Upstream region and mRNA half-life of selected strains of CTX-M-15 and CTX-M-14 producers

| Strain   | bla<sub>CTX-M</sub> allele | Upstream region | mRNA half-life (min) |
|----------|-----------------------------|------------------|----------------------|
| XQ12     | CTX-M-15                    | ISEcp1<sup>a</sup> | 8 or 10<sup>c</sup>  |
| C15      | CTX-M-15                    | ISEcp1<sup>a</sup> | 5                    |
| XQ35     | CTX-M-15                    | ISEcp1<sup>a</sup> | <2                   |
| JJ2053   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | 9                  |
| JJ2241   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | 8                  |
| JJ2242   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | <2                   |
| JJ2243   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | 12                  |
| JJ2244   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | 15                  |
| JJ2247   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | 2                    |
| JJ2131   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | <2                   |
| JJ2235S  | CTX-M-15                    | ISEcp1-like<sup>b</sup> | <2                   |
| JJ2235F  | CTX-M-15                    | ISEcp1-like<sup>b</sup> | <2                   |
| JJ2236   | CTX-M-15                    | ISEcp1-like<sup>b</sup> | 10                  |
| JJ2253RG | CTX-M-15                    | ND                | 11                   |
| MHHS4    | CTX-M-15                    | non-ISEcp1<sup>a</sup> | <2, 7 or 12<sup>c</sup> |
| MHvab2   | CTX-M-15                    | non-ISEcp1<sup>a</sup> | 9                    |
| QU015    | CTX-M-15                    | non-ISEcp1<sup>a</sup> | <2                   |
| JJ2251   | CTX-M-15                    | non-ISEcp1<sup>a</sup> | 9                    |
| JJ2052S  | CTX-M-15                    | non-ISEcp1<sup>a</sup> | 2                    |
| JJ2052F  | CTX-M-15                    | non-ISEcp1<sup>a</sup> | 9                    |
| JJ2246S  | CTX-M-15                    | non-ISEcp1<sup>a</sup> | <2 or 7<sup>c</sup> |
| JJ2246F  | CTX-M-15                    | non-ISEcp1<sup>a</sup> | 7                    |
| JJ2267   | CTX-M-15                    | ND                | 9                    |
| JJ2253SW | CTX-M-15                    | ND                | 5                    |
| JJ2243   | CTX-M-15                    | ND                | <2                   |
| D14      | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| XQ10     | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| C14      | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| Lo14     | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| Lo14     | CTX-M-14                    | ISEcp1<sup>a</sup> | 3                    |
| N14      | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| F044     | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| XQ22     | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| XQ24     | CTX-M-14                    | ISEcp1<sup>a</sup> | <2                   |
| FS-ESBL014 | CTX-M-14               | ND               | <2                   |
| JJ2339   | CTX-M-14                    | ND                | <2                   |
| JJ2354   | CTX-M-14                    | ND                | <2                   |
| JJ2356   | CTX-M-14                    | ND                | <2                   |
| NL217    | CTX-M-14                    | ND                | <2                   |
| FS-ESBL013 | CTX-M-14            | ND               | 3                    |
| FS-ESBL062 | CTX-M-14         | ND               | 3                    |

Each mRNA half-life represents one experiment. Studies were not completed in triplicate due to cost.
Half-life calculated using the 2001 method of Pfaffl.26
<sup>a</sup>Confirmed by sequence analysis.
<sup>b</sup>Upstream region PCR mapped.
<sup>c</sup>Indicates the graph intersects the 50% transcript remaining line at more than one timepoint.

Table 3. Steady-state mRNA expression and mRNA half-lives for CTX-M-14 and CTX-M-15 transconjugants in different E. coli backgrounds

| Clinical isolate donor/ transconjugant | bla<sub>CTX-M</sub> allele | Relative mRNA levels ± SD | mRNA half-life (min)<sup>a</sup> |
|---------------------------------------|-----------------------------|---------------------------|----------------------------------|
| XQ12                                 | CTX-M-15                    | 20 ± 5.12                 | 8 – 10                           |
| XQ12-J53                             | CTX-M-15                    | 23 ± 10.58                | 7                                |
| XQ12-K12 MG1655                      | CTX-M-15                    | 31 ± 4.41                 | 9                                |
| XQ12-FHM16                           | CTX-M-15                    | 26 ± 3.63                 | 9                                |
| XQ35                                 | CTX-M-15                    | 4 ± 1.60                  | <2                               |
| XQ35-J53                             | CTX-M-15                    | 7 ± 3.47                  | <2                               |
| XQ35-K12 MG1655                      | CTX-M-15                    | 26 ± 7.21                 | <2                               |
| XQ35-FHM16                           | CTX-M-15                    | 16 ± 3.12                 | <2                               |
| C15                                  | CTX-M-15                    | 56 ± 17.52                | 5                                |
| C15-J53                              | CTX-M-15                    | 62 ± 6.89                 | 5                                |
| C15-K12 MG1655                       | CTX-M-15                    | 25 ± 12.73                | 5                                |
| C15-FHM16                            | CTX-M-15                    | 59 ± 28.98                | 5 or 11                          |
| D14                                  | CTX-M-14                    | 1 ± 0.20                  | <2                               |
| D14-J53                              | CTX-M-14                    | 1 ± 0.46                  | <2                               |
| XQ10                                 | CTX-M-14                    | 0.73 ± 0.48               | <2                               |
| XQ10-J53                             | CTX-M-14                    | 0.76 ± 0.39               | <2                               |

All fold changes are relative to D14.
<sup>a</sup>Calculated using the 2001 method of Pfaffl.26

with the other study isolates was used as the comparator (Table 1). mRNA analysis of CTX-M-specific transcripts relative to isolate D14 showed that E. coli isolates expressing CTX-M-15 had mRNA transcript levels that ranged from 14-fold lower to 165-fold higher than the comparator. However, most CTX-M-15 producers (32 of 40; 80%) showed 20–165-fold increases in bla<sub>CTX-M-15</sub> transcripts. When mRNA levels of the CTX-M-14 producers were compared with each other relative to the comparator strain, D14, the range in expression among these isolates was only 5-fold (Table 1). Among all the isolates evaluated, the trend observed for the elevated expression of CTX-M-15 transcripts and the lower level of CTX-M-14 transcripts were consistent among the isolates regardless of the geographic location from which the isolates were collected.

**Relationship between CTX-M mRNA expression and E. coli ST and phylotype**

Various STs and phylotypes were evaluated for CTX-M gene expression and protein production. No correlation was observed with respect to specific ST and/or phylotype of E. coli expressing CTX-M-14 or CTX-M-15 genes (Table 1). For example, strains RS135 and JJ2053 were both identified as ST131 phylotype B2 isolates. However, RS135 expressed the bla<sub>CTX-M-15</sub> gene 165-fold higher than the comparator D14 strain whereas strain JJ2053 expressed the bla<sub>CTX-M-15</sub> gene 14-fold less than the comparator. Twenty-one of 41 isolates were ST131 phylotype B2 with bla<sub>CTX-M-15</sub> gene expression ranging from a 14-fold decrease to a 165-fold increase in CTX-M-15 mRNA levels relative to the comparator strain, D14. Three ST405 isolates were evaluated for CTX-M gene expression. Two strains expressed CTX-M-14 and were both phylotype D2. Strain Lo14 expressed the bla<sub>CTX-M-14</sub>

chromosomally encoded CTX-M β-lactamase genes.30 Therefore, to evaluate the relative expression of both mRNA and protein levels among the E. coli CTX-M-producing isolates, an E. coli isolate (D14) that produced a low amount of CTX-M transcript compared

with respect to specific ST and/or phylotype of E. coli expressing CTX-M-14 or CTX-M-15 genes (Table 1). For example, strains RS135 and JJ2053 were both identified as ST131 phylotype B2 isolates. However, RS135 expressed the bla<sub>CTX-M-15</sub> gene 165-fold higher than the comparator D14 strain whereas strain JJ2053 expressed the bla<sub>CTX-M-15</sub> gene 14-fold less than the comparator. Twenty-one of 41 isolates were ST131 phylotype B2 with bla<sub>CTX-M-15</sub> gene expression ranging from a 14-fold decrease to a 165-fold increase in CTX-M-15 mRNA levels relative to the comparator strain, D14. Three ST405 isolates were evaluated for CTX-M gene expression. Two strains expressed CTX-M-14 and were both phylotype D2. Strain Lo14 expressed the bla<sub>CTX-M-14</sub>
gene 5-fold higher than the comparator strain, D14. The CTX-M-15-producing ST405 strain was of phylotype B1 and expressed bla_{CTX-M-15} 48-fold higher than the comparator, D14. Four strains were phylotype D1, but were all different STs (ST69, ST2076, ST183 and ST354). ST354 expressed the bla_{CTX-M-15} gene only 2-fold higher than the bla_{CTX-14} mRNA expression of D14, but the bla_{CTX-M-15} mRNA expression observed in the D1 phy- lotypes ranged from 26- to 74-fold higher compared with D14.

**Determination of CTX-M-14/CTX-M-15 mRNA half-lives from clinical isolates**

The differential expression of mRNA was not attributed to the ST or the phylotype of the organism. Therefore, we wanted to determine whether the differential expression between these two types of CTX-M transcripts was occurring at the initiation of transcription or post-transcriptionally due to mRNA stability differences. The promoter regions of 29 strains encoding CTX-M-14 or CTX-M-15 were sequenced and most of the strains had identical upstream sequences that housed the insertion sequence IS_{Ecp1} (Table 2). When PCR mapping data were used to define the promoter, there was no difference in the trends of CTX-M-15 expression for IS_{Ecp1}-like upstream elements versus those strains that did not generate a specific amplicon for the IS_{Ecp1} element (for example, strains JJ2236 and JJ2246F; Table 2). Taken together, these data indicated that transcription initiation was probably not the cause of the differences observed in the mRNA levels.

mRNA half-life, i.e. the degradation rate of a transcript, can impact steady-state mRNA levels. Therefore, mRNA half-life studies were performed. The average half-life of an E. coli transcript is \( \sim 2-3 \text{ min} \).16 Sixteen CTX-M-14-producing clinical E. coli isolates were evaluated for mRNA half-life. Each isolate had a half-life of \( \leq 3 \text{ min} \) (Table 2 and Figures S3–S7). However, 16 of 25 CTX-M-15-producing E. coli isolates had an extended half-life of between 5 and 15 min. The remaining nine CTX-M-15 clinical E. coli isolates had mRNA half-lives of \( \leq 2 \text{ min} \). The difference in mRNA half-life observed for these isolates could be the result of chromosomal- or plasmid-encoded factors.

**Factors controlling CTX-M mRNA half-life: chromosomal or plasmid encoded**

To determine the contribution of chromosomal- or plasmid-encoded factors to the observed differences in the half-lives of the bla_{CTX-M} transcripts, candidate strains were chosen and transconjugants and transformants constructed (Table 3). DNA from isolates D14 and XQ12 were used as a template to construct the transformants. For the CTX-M-15-producing isolates, three clinical isolates representing three distinct mRNA half-lives were selected to be conjugation donors and included XQ12 (half-life of 8–10 min), XQ35 (half-life of \( < 2 \text{ min} \)) and C15 (half-life of 5 min). Three different recipient strains were used for the conjugations for the CTX-M-15-containing plasmid: J53, MG1655 and FHM16 (Table 3). For the CTX-M-14-producing isolates, two clinical isolates both having an mRNA half-life of \( < 2 \text{ min} \) were selected for conjugation studies and included D14 and XQ10.

Before the half-lives of the CTX-M transconjugant transcripts were determined, the gene copy number and CTX-M steady-state expression were evaluated. CTX-M gene copy number was determined for the D14, XQ10 and XQ12 transconjugants. The copy number of the CTX-M genes for the transconjugants tested was identical to the gene copy number determined for the clinical donor strains, which was 1. D14 was used as the comparator strain when evaluating steady-state expression of the transconjugants to remain consistent with the initial expression data (Table 3). For most of the transconjugants, the mRNA expression levels were similar to those of the clinical isolate from which they were created (Table 3). Compared with D14, CTX-M-15 mRNA expression in the XQ12 clinical isolate was 20-fold higher and in the XQ12-J53, XQ12-MG1655 and XQ12-FHM16 transconjugants was comparable at 23-, 31- and 26-fold higher (Table 3). The steady-state mRNA expression level for the XQ35 clinical isolate was 4-fold higher than the comparator strain D14 and expression in the J53, MG1655 and FHM16 transconjugants was 7-, 26- and 16-fold higher, respectively (Table 3). Interestingly, the mRNA expression level of the XQ35-MG1655 and XQ35-FHM16 transconjugants was \( \sim 2-6 \text{ fold} \) higher compared with the XQ35 clinical isolate and the J53 transconjugant. Steady-state mRNA expression levels for the C15 clinical isolate and its J53, MG1655 and FHM16 transconjugants were 56-, 62-, 25- and 59-fold higher, respectively when compared with D14. The CTX-M-15 expression level from the C15-MG1655 transconjugant was \( 2-3 \text{ fold} \) lower compared with the J53 and FHM16 transconjugants (Table 3). The CTX-M-14 steady-state mRNA levels for XQ10 and D14 and their J53 transconjugants were both similar to those of the D14 clinical isolate (Table 3).

Variation in steady-state levels of mRNA expression was observed between some of the CTX-M transconjugants, but not all, when compared with the clinical isolate. These differences were not due to gene copy number (all isolates had one copy of the gene), but could be attributed to transcription initiation events or transcript half-life in these different genetic backgrounds. To test the influence of these genetic backgrounds on mRNA stability, the mRNA half-life of each of the transcripts in the transconjugants was measured (Table 3 and Figures S3–S7). Despite the difference in genetic background (J53 versus MG1655 versus ST131), each of the transconjugant’s mRNA half-lives reflected the mRNA half-life of the clinical isolate from which the plasmid was obtained. Specifically, for transconjugant XQ12 the CTX-M-15 transcript had an extended half-life of 7–9 min, the XQ35 transconjugant had a bla_{CTX-M-15} mRNA half-life of \( < 2 \text{ min} \) and the C15 transconjugant had a bla_{CTX-M-15} half-life of 5 min (Table 3). The same trend was seen with the CTX-M-14 transconjugants, each of which had a bla_{CTX-M-14} mRNA half-life of \( < 2 \text{ min} \), similar to the parent strain. These data indicated that plasmid-encoded factors and not chromosomally encoded factors were contributing to the difference in mRNA half-life observed between bla_{CTX-M-15} and bla_{CTX-M-15}.

To verify further that a clinical plasmid-encoded factor(s) was responsible for mRNA stability, the mRNA half-lives of the CTX-M-14 and CTX-M-15 K12 transformants were evaluated. To create these constructs, the structural CTX-M-14 or CTX-M-15 gene and upstream promoter regions were amplified, cloned into pMDR009 and transformed into K12 MG1655 or FHM16 (ST131).13 When mRNA half-lives were evaluated in the MG1655 transformants, both the CTX-M-14 and CTX-M-15 transcripts were \( < 2 \text{ min} \) (Figure S7). To determine whether the ST131 genetic background would influence the CTX-M-15 transcript half-life, the mRNA was evaluated in the transformant of ST131 E. coli strain, FHM16. The mRNA half-life of the CTX-M-15 transcript in
this transformant was also <2 min (Figure S7). These data substantiated that a plasmid-encoded factor(s), rather than the chromosomal background was responsible for the extended half-lives of the CTX-M-15 transcripts.

To rule out any contribution of the CTX-M native promoters or the structural genes themselves on CTX-M half-life, the half-lives of CTX-M promoter/luciferase fusions, lacZ promoter/CTX-M clones and chloramphenicol promoter/CTX-M clones were evaluated. When the promoter regions of the CTX-M-14 and CTX-M-15 genes were fused to luciferase, the half-life of the luciferase gene was <2 min for all clones (data not shown). Furthermore, the half-lives of the CTX-M-14 and CTX-M-15 transcripts driven by heterologous promoters (lacZ or chloramphenicol) were <2 min (data not shown). All clones discussed above included either the CTX-M promoter or structural gene, which had been removed from its native clinical plasmid and transformed into a K12 E. coli host.

**Relative levels of CTX-M-14 and CTX-M-15 protein**

The elevated levels of the CTX-M-15 mRNA transcripts suggested that the level of CTX-M-15 enzyme produced by those E. coli isolates would also be elevated, perhaps jeopardizing the effectiveness of β-lactam/β-lactamase inhibitor combinations. Therefore, western blots were used to evaluate the relative level of CTX-M β-lactamase produced by the study isolates listed in Table 1. An ~1:1 relationship between mRNA and protein production was observed for the CTX-M-14-producing isolates (Table 1 and Figure 1a and b). For example, E. coli isolate C14 expressed both its CTX-M-14 mRNA and protein at levels 5 times higher than the comparator isolate, D14. This trend in mRNA and protein production was seen with all of the CTX-M-14-producing isolates with levels ranging between 1- and 6-fold higher compared with isolate D14 (Table 1). In contrast, this direct relationship was not observed for CTX-M-15-producing isolates. While mRNA levels for the CTX-M-15 transcript ranged from 14-fold lower to 165-fold higher than the CTX-M-14 mRNA levels of isolate D14, the corresponding protein levels ranged from undetected to 10-fold higher (Table 1 and Figure 1a and b). In addition, the higher levels of CTX-M-15 mRNA did not always correlate with the highest levels of CTX-M-15 protein production. For example, isolate RS153 had an mRNA level 37-fold higher than the comparator strain, D14, but its protein level was only 8-fold higher. While a 77-fold increase in mRNA levels for strain RS059 was observed, only a 10-fold increase in protein production was noted. Isolate C15 had a 48-fold increase in mRNA compared with only a 2-fold increase in protein. Moreover, isolates J12246F and RS135 had the most pronounced increases for both mRNA and protein production of CTX-M-15 with a 155-fold and 165-fold increase in mRNA and a 28-fold and 22-fold increase in protein, respectively.

**Susceptibility to β-lactam/β-lactamase inhibitor combinations**

Protein production among the test isolates ranged from no detectable level of protein observed to a 28-fold increase in protein production for strain J12246F. The level of β-lactamase production is a key contributor to the β-lactam-resistant phenotype. Therefore given the range of CTX-M production in these isolates a subset of 23 isolates were tested to determine the impact CTX-M β-lactamase production had on piperacillin/tazobactam and

![Figure 1](image-url)

**Figure 1.** (a) Immunoblots for CTX-M-14 and CTX-M-15 from 15 representative clinical isolates. Protein levels were compared and analysed relative to E. coli strain D14. Lane 1, D14 (CTX-M-14); lane 2, CUMC 247 (CTX-M-15); lane 3, W15 (CTX-M 15); lane 4, C15 (CTX-M-15); lane 5, FHM6 (CTX-M-15); lane 6, XQ12 (CTX-M-15); lane 7, RS059 (CTX-M-15); lane 8, C14 (CTX-M-14); lane 9, NL217 (CTX-M-14); lane 10, F044 (CTX-M-14); lane 11, XQ13 (CTX-M-14); lane 12, RS061 (CTX-M-15); lane 13, RS061 (CTX-M-15); lane 14, RS153 (CTX-M-15); lane 15, La14 (CTX-M-14). (b) Relative fold changes in CTX-M-15 and CTX-M-14 protein from the 15 isolates from (a) normalized to total protein using Stain-Free technology. All protein levels are relative to D14. Statistical significance of the isolates relative to D14 was evaluated using a t-test (two-tailed and paired) performed with GraphPad Prism 6.0.
Disc diffusion breakpoints for ceftolozane/tazobactam are currently set by the FDA and available for only *Pseudomonas aeruginosa*; therefore, susceptibility data for this drug combination for *E. coli* could not be determined. Only zone size comparisons could be made. In spite of the high-level expression from the CTX-M-15 gene, 7 of 15 isolates were susceptible to the piperacillin/tazobactam. Isolates W15 and H15 were resistant to piperacillin/tazobactam, while the remaining isolates were intermediate (Table 1). All of the CTX-M-14-producing isolates listed in Table 1 were susceptible to piperacillin/tazobactam. With respect to ceftolozane/tazobactam, all the isolates listed in Table 1 regardless of the level of protein production observed had zone sizes of ≥19 mm. An additional 126 isolates not evaluated for protein production, but possessing either CTX-M-14 or CTX-M-15 β-lactamases, were also tested against ceftolozane/tazobactam. All 50 of the CTX-M-14-like-producing isolates had zone diameters of ≥21 mm. Of the 76 additional isolates producing CTX-M-15-like enzymes, 73 showed zone diameters to piperacillin/tazobactam. With respect to ceftolozane/tazobactam, all 50 of the possessing either CTX-M-14 or CTX-M-15 β-lactamase-producing isolates listed in Table 1 were susceptible to piperacillin/tazobactam. Isolates W15 and H15 were resistant to piperacillin/tazobactam, while the remaining isolates were intermediate (Table 1). All of the CTX-M-14-producing isolates listed in Table 1 were susceptible to piperacillin/tazobactam. With respect to ceftolozane/tazobactam, all the isolates listed in Table 1 regardless of the level of protein production compared to the corresponding donor strains (Table 3). Second, transformants of cloned CTX-M-14 and CTX-M-15 genes into identical plasmid vectors had blaCTX-M mRNA half-lives of <2 min regardless of the genetic background, including a ST131 *E. coli* (FH16). Third, native or heterologous promoters expressing blaCTX-M alleles did not influence the mRNA half-life. These data also indicated that the plasmid backbones of CTX-M-15-encoding isolates were not identical since some of these isolates had mRNA half-lives of ≥2 min, whereas others had half-lives of ≥5 min. We speculate that the CTX-M-15-encoding plasmids that exhibited shorter half-lives have a similar composition to the CTX-M-14-encoding plasmids with respect to the encoded factor that determines mRNA half-life.

**Discussion**

Previous studies evaluating the effectiveness of ceftolozane/tazobactam against isolates that produced CTX-M-14 or CTX-M-15 indicated that the concentration of tazobactam may play a role in the overall efficacy of this combination when used against these ESBL-producing bacteria. When a breakpoint of 1 mg/L of ceftolozane plus 4 mg/L of tazobactam was used, 12% of isolates evaluated by Titelman et al. were non-susceptible. A similar finding for ESBL-producing Enterobacteriaceae and ceftolozane/tazobactam has been reported by Livermore et al. It is possible that the level of CTX-M production was responsible for the lack of susceptibility to ceftolozane/tazobactam in these isolates. Given our observation that CTX-M-15 isolates in general transcribed more mRNA than CTX-M-14 isolates, we wanted to evaluate the impact that this discrepancy in mRNA expression would have on CTX-M protein production and ultimately susceptibility to β-lactam/β-lactamase inhibitor combinations. To our surprise, the level of CTX-M protein production for most of the isolates evaluated was similar for CTX-M-14 and CTX-M-15 producers despite the differences in mRNA expression. Therefore, the level of CTX-M production was most likely not responsible for the resistance observed in the 12% of isolates evaluated by Titelman. The choice of breakpoint for ESBL-producing bacteria is controversial and EUCAST and CLSI have set lower breakpoints for these ESBL producers for ceftolozane/tazobactam are currently set by the FDA and available for only *Pseudomonas aeruginosa*; therefore, susceptibility data for this drug combination for *E. coli* could not be determined. Only zone size comparisons could be made. In spite of the high-level expression from the CTX-M-15 gene, 7 of 15 isolates were susceptible to the piperacillin/tazobactam. Isolates W15 and H15 were resistant to piperacillin/tazobactam, while the remaining isolates were intermediate (Table 1). All of the CTX-M-14-producing isolates listed in Table 1 were susceptible to piperacillin/tazobactam. With respect to ceftolozane/tazobactam, all the isolates listed in Table 1 regardless of the level of protein production observed had zone sizes of ≥19 mm. An additional 126 isolates not evaluated for protein production, but possessing either CTX-M-14 or CTX-M-15 β-lactamases, were also tested against ceftolozane/tazobactam. All 50 of the CTX-M-14-like-producing isolates had zone diameters for ceftolozane/tazobactam of ≥21 mm. Of the 76 additional isolates producing CTX-M-15-like enzymes, 73 showed zone diameters to piperacillin/tazobactam of ≥19 mm, while 2 of 76 isolates had zone diameters of 18 mm and 1 of 76 had a zone diameter of 14 mm.

Steady-state levels of mRNA expression in the transconjugants indicated that for two of the three CTX-M-15-producing isolates (XQ12 and C15) evaluated, the genetic background did not influence promoter usage, plasmid copy number or transcript half-life. However, the steady-state level of mRNA for the CTX-M-15-producing XQ35 transconjugants in the FH16 and MG1655 backgrounds was 4–6.5-fold higher compared with blaCTX-M-15 expression in the XQ35 clinical isolate. It is possible that when blaCTX-M-15 was expressed in the FH16 and K12 MG1655 backgrounds, the copy number of the plasmid or transcription initiation events were altered compared with expression in the XQ35 and J53 backgrounds. Copy number has been shown to change even when the same plasmid vector is used, but transformed into different hosts (genetic backgrounds) of Enterobacteriaceae. Sequence analysis of CTX-M-14/CTX-M-15-containing plasmids has shown multiple open reading frames of hypothetical proteins that could encode for a factor that influences the CTX-M transcript half-life. A protein that can modify mRNA half-life and is encoded by a plasmid has multiple implications. This factor could not only prolong the half-life of antibiotic resistance gene transcripts, as demonstrated in this study, but also influence the production of factors responsible for virulence or metabolic pathways, leading to increased fitness for strains that carry blaCTX-M-15. Identification of this factor should be a priority, since it could serve as a potential target for the development of new antimicrobial agents to limit the production of proteins benefiting from a prolonged mRNA half-life.

The ability to evaluate β-lactamases in clinical isolates using hydrolysis data is difficult for isolates that carry multiple β-lactamases as in the ones evaluated in this study. Therefore, to evaluate the level of protein production of CTX-M β-lactamases only, immunoblot analysis was used. The use of an internal control to normalize the data is difficult using clinical isolates, as expression of any internal housekeeping protein may fluctuate in response to various selective pressures faced by a given organism. Therefore, the use of Stain-Free technology allows for a more accurate comparison of relative amounts of protein among clinical isolates.

The difference observed between the levels of CTX-M-15 mRNA and protein suggests that these isolates are inefficient in translating all the transcribed mRNA into functional β-lactamase. Although some of the mRNA is translated, as evident from the immunoblot data, the level of CTX-M-15 β-lactamase did not result in decreased zone sizes to either tested inhibitor combination. This suggests that the rate of penetration of antibiotics through the outer membrane may be important when evaluating CTX-M-producing resistant isolates. Even though there are no disc diffusion breakpoints for Enterobacteriaceae currently available
for ceftolozane/tazobactam, comparisons between MIC data and zone size suggest that a zone size of ≥19 mm could be considered susceptible (Cubist data on file). However, those data still contain some major errors between some isolates regarding MIC versus disc zone size that need to be resolved (Cubist data on file). None the less, most of the isolates in this study (126 of 129) had zone sizes to ceftolozane/tazobactam of ≥19 mm despite the elevated levels of protein production observed. Notably, isolate RS135 produced the highest level of mRNA and the second highest level of CTX-M-15 protein, yet showed a zone size of 19 mm for ceftolozane/tazobactam. In contrast, the highest protein production was noted for strain JJ2246F, but the ceftolozane/tazobactam zone of inhibition was 30 mm indicating that the level of β-lactamase did not contribute to smaller zone sizes for these isolates.

It was surprising that the levels of mRNA expression observed for most CTX-M-15-producing isolates were disproportional to protein production. This disproportional correlation between mRNA and protein production could be the result of a translational block due to an RNA binding protein or small RNA interference, or the result of decreased CTX-M-15 protein stability compared with CTX-M-14. The differential levels of expression between mRNA and protein production did not seem to be influenced by phylogenetic background since no correlations could be determined with ST or phylotype. As the selective pressure increases with β-lactam/β-lactamase inhibitor combinations or carbapenems, a future concern is the selection of genomic or plasmidic mutations that will allow increased translatability of the CTX-M transcripts resulting in increased β-lactamase production. These events could lead to an emergence of CTX-M-producing organisms resistant to β-lactam/β-lactamase inhibitor combinations and may increase the number of isolates resistant to the carbapenems through porin down-regulation. Fortunately, our current findings suggest that resistance in \textit{E. coli} to β-lactam/β-lactamase inhibitor combinations including ceftolozane/tazobactam is not dependent on the type or level of CTX-M β-lactamase produced by the \textit{E. coli} isolate, but requires as yet unidentified resistance mechanisms.

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**Supplementary data**

Tables S1 and S2 and Figures S1 to S7 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**

1 Harris PNA. Clinical management of infections caused by Enterobacteriaceae that express extended-spectrum β-lactamase and AmpC enzymes. Semin Respir Crit Care Med 2015; 36: 56 – 73.

2 Harris PNA, Tambyah PA, Paterson DL. β-Lactam and β-lactamase inhibitor combinations in the treatment of extended-spectrum β-lactamase producing Enterobacteriaceae: time for a reappraisal in the era of few antibiotic options? Lancet Infect Dis 2015; 15: 475–85.

3 Draws SM, Bonomo RA. Three decades of β-lactamase inhibitors. Clin Microbial Rev 2010; 23: 160 – 201.

4 Rogers BA, Sidjabat HE, Paterson DL. Escherichia coli O25b-ST131: a pandemic, multiresistant, community-associated strain. J Antimicrob Chemother 2011; 66: 1 – 14.

5 Nicolas-Chanoine MH, Bertrand X, Madec JY. Escherichia coli ST131, an intriguing clonal group. Clin Microbial Rev 2014; 3: 543 – 74.

6 Pitout JD, Gregson DB, Campbell L et al. Molecular characteristics of extended-spectrum-β-lactamase-producing \textit{Escherichia coli} isolates causing bacteremia in the Calgary Health Region from 2000 to 2007: emergence of clone ST131 as a cause of community-acquired infections. Antimicrob Agents Chemother 2009; 53: 2846 – 51.

7 Ensor VM, Shahid M, Evans JT et al. Occurrence, prevalence and genetic environment of CTX-M β-lactamases in Enterobacteriaceae from Indian hospitals. J Antimicrob Chemother 2006; 58: 1260 – 3.

8 Chanawong A, M’Zali FH, Heritage J et al. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among \textit{Enterobacteriaceae} in the Republic of China. Antimicrob Agents Chemother 2002; 46: 630 – 7.

9 Dhanji H, Patel R, Wall R et al. Variation in the genetic environments of \textit{bla}_{CTX-M-15} in \textit{Escherichia coli} from the faeces of travellers returning from the United Kingdom. J Antimicrob Chemother 2011; 66: 1005 – 12.

10 Falogas ME, Karageogeopoulos DE. Extended-spectrum β-lactamase producing organisms. J Hosp Infect 2009; 73: 345 – 54.

11 Hawkey PM, Jones A. The changing epidemiology of resistance. J Antimicrob Chemother 2009; 64 Suppl 1: 13 – 10.

12 Pitout JD, Church DL, Gregson DB et al. Molecular epidemiology of CTX-M producing \textit{Escherichia coli} in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob Agents Chemother 2007; 51: 1281 – 6.

13 Wagenlehner FM, Pilat A, Naber KG et al. Therapeutic challenges of urosepsis. Eur J Clin Invest 2008; 38 Suppl 2: 45 – 9.

14 Giske GC, Monnet DL, Cars O et al. Clinical and economic impact of common multidrug-resistant Gram-negative bacilli. Antimicrob Agents Chemother 2008; 52: 813 – 21.

15 Geyer C, Fowler R, Hanson N. The discordant relationship between CTX-M-15 mRNA and protein production does not interfere with β-lactam/β-lactamase inhibitor combination susceptibilities in \textit{Escherichia coli}. Poster P1106. In: Abstracts of the Twenty-fourth European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 2014, European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland.

16 von Gebain A, Belasco JG, Schottel JL et al. Decay of mRNA in \textit{Escherichia coli}: investigation of the fate of specific segments of transcripts. Proc Natl Acad Sci USA 1983; 80: 653 – 7.

17 Farrell DJ, Flamm RK, Sader HS et al. Antimicrobial activity of ceftolozane-tazobactam tested against Enterobacteriaceae and \textit{Pseudomonas aeruginosa} with various resistance patterns isolated in U.S. Hospitals (2011 – 2012). Antimicrob Agents Chemother 2013; 57: 6305 – 10.

18 Geyer CN, Hanson ND. Rapid PCR amplification protocols decrease the turn-around time for detection of antibiotic resistance genes in Gram-negative pathogens. Diagn Microbial Infect Dis 2013; 77: 113 – 7.
19 Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-S21. Wayne, PA, USA, 2011.

20 Sinnett D, Montpetit A. Isolation of cosmid and BAC DNA from E. coli. Methods Mol Biol 2003; 235: 99 – 102.

21 Lee C, Kim J, Shin SG et al. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. J Biotech 2006; 123: 273 – 80.

22 Skulj M, Okrslar V, Jalen S et al. Improved determination of plasmid copy number using quantitative real-time PCR for monitoring fermentation processes. Microb Cell Fact 2008; 7: 6.

23 Clermont O, Christenson JK, Denamur E et al. The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep 2013; 5: 180 – 90.

24 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 2001; 25: 402 – 8.

25 Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29: 2001 – 7.

26 Fowler RC, Hanson ND. The OpdQ porin of Pseudomonas aeruginosa is regulated by environmental signals associated with cystic fibrosis including nitrate-induced regulation involving the NarXL two-component system. MicrobiologyOpen 2015; doi:10.1002/mbo3.305.

27 Livermore DM, Mushtaq S, Ge Y. Chequerboard titration of cephalosporin CXA-101 (FR264205) and tazobactam versus β-lactamase-producing Enterobacteriaceae. J Antimicrob Chemother 2010; 65: 1972 – 4.

28 Livermore DM, Andrews JM, Hawkey PM et al. Are susceptibility tests enough, or should laboratories still seek ESBLs and carbapenemases directly? J Antimicrob Chemother 2012; 67: 1569 – 77.

29 Roth A, Kurpiel P, Lister PD. blaKPC RNA expression correlates with two transcriptional start sites but not always with gene copy number in four genera of Gram-negative pathogens. Antimicrob Agents Chemother 2011; 55: 3936 – 8.

30 Woodford N, Caratoli A, Karisik E et al. Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M-enzymes in three major Escherichia coli lineages from the United Kingdom, all belonging to the international clone O125:H4-ST131 clone. Antimicrob Agents Chemother 2009; 53: 4472 – 82.

31 Woodford N, Caratoli A, Karisik E et al. Complete genome sequencing of the FII plasmid pHK01, encoding CTX-M-14, and molecular analysis of its variants among Escherichia coli from Hong Kong. J Antimicrob Chemother 2011; 66: 752 – 6.