TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2018) 48: 1340-1350

doi:10.3906/sag-1803-121

Investigation of the effect of β-lactam antibiotics and serum on growth and gene expression in *Escherichia coli* strain JJ1886

Elif BOZÇAL DAĞDEVİREN*
Section of Basic and Industrial Microbiology, Department of Biology, Faculty of Science, İstanbul University, İstanbul, Turkey

Received: 13.03.2018 • **Accepted/Published Online:** 26.07.2018 • **Final Version:** 12.12.2018

Background/aim: β-Lactamase-producing *Escherichia coli* strain JJ1886 is an epidemic clone with high virulence properties. Because this strain can survive in the bloodstream, we aimed here to understand how β-lactam antibiotics and human serum affect the growth and gene expression of this bacterium.

Materials and methods: We report the time-dependent growth effect of normal human serum and heat-inactivated serum, together with β-lactam antibiotics (including cefotaxime, ceftazidime, and carbenicillin), for *E. coli* strain JJ1886. Relative gene expression of β-lactamase-related genes (encoding the β-lactamase regulator, CTX-M-15, and peptidoglycan glycosyltransferase) and serum survival-associated genes (encoding lipoprotein NlpI, murein lipoprotein, lipopolysaccharide core heptose (I) kinase, lipopolysaccharide biosynthesis protein, capsule synthesis protein, and phosphate transport system) were investigated by RT-qPCR.

Results: Cells proliferated during the exponential growth phase when the bacterium was treated with human serum. However, cefotaxime and ceftazidime together with serum had a bactericidal effect at each of the tested time points. Downregulation was observed in gene-encoding lipoprotein NlpI as a result of treatment with carbenicillin.

Conclusion: Serum plus cefotaxime or ceftazidime had bactericidal activities. When the bacterium was treated with human serum and β-lactam antibiotics, there were no significant changes in relative gene expression, except for the *nlpI* gene.

Key words: *Escherichia coli* JJ1886, β-lactamase, serum, RT-qPCR, cefotaxime, ceftazidime, carbenicillin

1. Introduction

β-Lactam antibiotics are the most commonly used broad-spectrum antibiotics in the treatment of bacterial infections (1,2). β-Lactams interact with penicillin-binding proteins (PBPs), which have a role in the cross-linking of the peptidoglycan component of the bacterial cell wall. β-Lactams inhibit transpeptidation activity of PBSs, leading to bacterial cell lysis (3). β-Lactams with such an effect include penicillins, cephalosporins, carbapenems, and monobactams (2). Cefotaxime (CTX) and ceftazidime (CAZ) are third-generation cephalosporins typically used in the treatment of bacterial infections caused by gram-positive bacteria, but which may also be used against gram-negative bacteria (https://www.fda.gov/). Similarly, carbenicillin (CAR), which belongs to the carboxypenicillin subgroup of the penicillins, has the same mode of action as CTX and CAZ (https://www.drugbank.ca/drugs/DB00578; http://antibiotics.toku-e.com/).

When β-lactams are used in the treatment of bacterial infections, the bacteria protect themselves by producing β-lactamase enzymes. These bacteria produce β-lactamases that hydrolyze β-lactam antibiotics (3). β-Lactamase production occurs in bacteria when antibiotic-associated damage to the cell wall occurs (4). CXT-M-type extended-spectrum β-lactamase (ESBL) enzymes are the most commonly observed β-lactamases and are disseminated rapidly among Enterobacteriaceae members, including *Escherichia coli* strains (5,6). CTX-M-15 and CTX-M-14 are the most common variants of the CTX-M-type ESBLs; CTX-M-15 has catalytic activity against CAZ (6). Dissemination of CTX-M-15 among Enterobacteriaceae and non-Enterobacteriaceae members, including *E. coli* and *Acinetobacter baumannii*, is provided by transferable plasmids (7,8). However, multiple studies have reported that genes encoding CTX-M-15 have integrated into the bacterial chromosome (9,10).

Few studies have addressed the expression of genes encoding the CTX-M-15-type β-lactamase. However, it has been reported that expression of bla_{CTX-M} can be changed according to the CTX concentration, growth

* Correspondence: elif.bozcal@istanbul.edu.tr

phase of *E. coli*, and genetic location of the bla_{CTX-M} gene (11). Similarly, upregulation of the $bla_{CTX-M-15}$ gene has been reported in *Shigella sonneii* in the presence of CAZ (12). Therefore, it can be inferred that genes encoding CTX-M-type β-lactamases can be upregulated or downregulated in the presence of β-lactam antibiotics. Moreover, the control of β-lactamase gene expression is regulated by a histidine kinase/response regulator pair in *Vibrio parahaemolyticus* (4). In addition, AmpE is a putative signaling protein in β-lactamase regulation in *E. coli*, and AmpE might help reveal the effect of β-lactam on peptidoglycan biosynthesis (13).

Rapid dissemination of CTX-M-15-type ESBLs has led to the emergence of highly pathogenic pandemic bacterial clones, including the CTX-M-15-producing *E. coli* O25b:H4-ST131 (14,15). *E. coli* O25b:H4-ST131 causes bloodstream infections, including urinary tract infection, pyelonephritis, and sepsis outside the intestinal tract (16,17). Moreover, this strain is resistant to extended-spectrum cephalosporin and fluoroquinolones (18). Because *E. coli* O25b:H4-ST131 is multidrug-resistant and carries potential virulence determinants, it is considered an emerging pandemic clone of global importance (19). According to phylogenetic analyses, CTX-M-15-producing *E. coli* O25b:H4-ST131 has been defined as an *H*30-R_x subclone. For instance, *E. coli* strain JJ1886, a highly virulent CTX-M-15-producing *H*30-R_x subclone of *E. coli* O25b:H4-ST131, was reported from a urosepsis patient in the United States (16,20).

On the occasions that the CTX-M-15-type β-lactamase-producing *E. coli* ST131 pandemic strain has been found in the blood causing septicemia and bacteremia, it has been resistant to the bactericidal effects of serum (21). Many virulence factors are involved in *E. coli* resistance to the bactericidal activity of serum. To illustrate, the proteins associated with lipopolysaccharide (LPS) biosynthesis and the K capsule have significant contributions to serum survival, as does the outer membrane protein (OmpA) (21,22). Moreover, serum survival factors, including Trat, Iss, and phage proteins encoded by plasmids, are serum survival factors in *E. coli* (23). Other factors or proteins that may be involved in serum resistance have been reported, including NlpI lipoprotein, murein lipoprotein (Lpp), and the phosphate transport system (24–26).

Given that the *E. coli* ST131 strain exhibits resistance to many antibiotics and serum, it is important to study the inhibitory and bactericidal effect of antibiotics and serum on this strain. Here we investigate the effect of β-lactam antibiotics plus human serum on *E. coli* strain JJ1886 (025b:H4-ST131) growth and the gene expression of β-lactamase and serum resistance-associated genes.

2. Materials and methods

2.1. Bacterial strains and normal human serum

The CTX-M-15-type β-lactamase-producing *E. coli* epidemic strain JJ1886 was used as the test microorganism. *E. coli* strain JJ1886 was kindly provided by Professor James R Johnson (University of Minnesota, VA Medical Center, Minneapolis, MN, USA). *E. coli* ATCC 25922 strain was used as a quality control strain for minimum inhibitory concentration (MIC) testing analysis. Normal human serum (NHS) was commercially purchased (Panbiotech, Germany). To inactivate the complement system of NHS, serum was incubated at 56 °C for 30 min, resulting in heat-inactivated serum (HIS) (27). NHS and HIS were stored at −80 °C until further analysis.

2.2. Dimethyl sulfoxide stocks of bacterial strains

Dimethyl sulfoxide (DMSO) stocks of *E. coli* strain JJ1886 and *E. coli* ATCC 25922 were prepared as follows: bacterial cultures were incubated overnight, diluted to 1/100 in Luria Bertani (LB) broth, and grown until the logarithmic growth phase at 37 °C (OD_{600}: 0.8). Next, DMSO was added to the bacterial culture, giving a final concentration of 8%. Bacterial cultures were dispensed in volumes of 120 µL and stored at −80 °C (28).

2.3. Nitrocefin test

Nitrocefin is a chromogenic cephalosporin. Through the procedure provided by the manufacturer (Bioanalyse), we phenotypically tested whether *E. coli* strain JJ1886 is a β-lactamase producer. *E. coli* ATCC 25922 was used as a quality control strain. Nitrocefin disks change from yellow to red within 5 min (as quickly as 15 s) when the β-lactam antibiotic is hydrolyzed by bacterial induction of the β-lactamase enzyme.

2.4. Determination of minimum inhibitory concentration

Carbenicillin (250 mg, Sigma), cefotaxime (100 mg, Sigma), and ceftazidime (50 mg, Sigma) were used for the MIC testing. MIC analysis was performed as previously described (29,30).

2.5. Time-dependent effect of NHS

E. coli strain JJ1886 from DMSO stock was inoculated into LB broth and incubated for 24 h at 37 °C. The concentration of 24-h bacterial culture was adjusted in PBS to the 0.5 McFarland standard (1.5×10^8 CFU/mL). The final bacterial concentration was diluted to 5×10^6 CFU/mL. Next, LB broth, NHS (1:2 v/v), HIS (1:2 v/v), and *E. coli* strain JJ1886 were added to the wells of a 96-well plate. The bacteria were treated with NHS, HIS, and LB by incubation at 37 °C without shaking. Samples were taken at time intervals of 0, 1, 2, and 3 h (31). Serial dilution was performed in a sterile 1X PBS buffer and plated on Luria agar (LA) plates by drop test, as previously described (32). The time-dependent effect of serum on bacterial growth was calculated as $\log_{10}$ CFU/mL after incubation at 37° C for 24 h (n = 3).

2.6. Time-dependent effect of NHS and β-lactam antibiotics

Measurement of the time-dependent effect of NHS and β-lactam antibiotics including CTX, CAZ, and CAR was performed as mentioned in Section 2.5. Briefly, the concentrations of CTX, CAZ, and CAR were diluted in LB broth in 96-well plates as follows: 512–0.125 µg/mL, 5120–2.5 µg/mL, and 20–0.625 mg/mL, respectively. After that, LB broth, NHS (1:2 v/v), HIS (1:2 v/v), and *E. coli* strain JJ1886 were added to each well containing CTX, CAZ, or CAR. Samples were withdrawn at time intervals of 0, 1, 2, 3, and 24 h from the well equal to the MIC value for each antibiotic. Time-dependent effects of NHS and β-lactam antibiotics was calculated as $\log_{10}$ CFU/mL after 24 h of incubation at 37 °C (n = 3).

2.7. Standard PCR

In order to determine the level of expression of serum resistance and β-lactamase-associated genes in the presence of β-lactam antibiotics and NHS, the genes encoding β-lactamase (bla_{CTX-M}), β-lactamase regulator (*ampE*), lipoprotein NlpI (*nlpI*), murein lipoprotein (*lpp*), lipopolysaccharide core heptose (I) kinase (*rfaP*), LPS biosynthesis protein (*wbbJ*), capsule biosynthesis (*kpsS*), peptidoglycan glycosyltransferase (*ftsI*), and phosphate transport system regulator (*phoU*) were confirmed by standard PCR as follows: isolation and purification of bacterial genomic DNA were performed using the GeneALL Genomic DNA Purification Kit (Korea). PCR was carried out in a final volume of 25 µL containing 1.25 U of Taq DNA polymerase (Thermo Fisher Scientific) in 1X PCR buffer, 0.2 µM of each dNTP, 2 mM $MgCl_2$, and 1 µM of each primer (Table 1). The program of PCR included initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95° C for 5 s and 55.5 °C for 1 min, with a final extension step at 72 °C for 2 min. The amplicon size was determined by 1.0% agarose gel electrophoresis (Table 1). The primers for each gene were designed according to the genome-sequenced *E. coli* strain JJ1886 (NCBI Reference Sequence: NC_022648; the reference sequence was derived from CP006784).

2.8. Total RNA isolation and cDNA synthesis

For the examination of the expression of serum resistance and β-lactamase-associated genes, total RNA isolation of *E. coli* strain JJ1886 was performed using the Hybrid-R Total RNA Isolation Kit (Korea). Briefly, the concentration of 24-h bacterial culture was adjusted to OD_{600} of 0.05 with fresh LB medium. Next, bacterial culture was immediately added to the LB medium (control) and LB medium consisting of NHS (v/v 1:2), HIS (v/v 1:2) NHS + CTX, NHS + CAZ, NHS + CAR, HIS + CTX, HIS + CAZ, HIS + CAR, CTX, CAZ, and CAR. Final antibiotic concentrations were calculated according to the MIC values and incubated at 37 °C for 24 h with shaking (50 rpm). The bacterial cultures (24 h) of 12 different conditions were used for total RNA isolation. Isolated mRNA was stored at –80 °C until cDNA synthesis analysis.

cDNA synthesis analysis was carried out, followed by RNA isolation from each condition (LB medium - control; LB medium consisting of NHS [v/v 1:2], HIS [v/v 1:2], NHS + CTX, NHS + CAZ, NHS + CAR, HIS + CTX, HIS + CAZ, HIS + CAR, CTX, CAZ, and CAR). cDNA synthesis was performed using a Biospeedy cDNA synthesis kit (Turkey) as follows: 12 µL of mRNA was mixed with 4 µL of oligo-dT and incubated for 10 min at 70 °C. cDNA synthesis was carried out in a final volume of 40 µL including 8 µL of 5X reaction buffer, 2 µL of dNTP mix, 2 µL of reverse transcriptase, and 12 µL of RNAse and nuclease-free water. The reaction mixture was incubated at 37 °C for 60 min. The obtained cDNA was stored at –20 °C.

2.9. Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (RT-qPCR) was performed using CFX Connect Real-Time PCR equipment (USA). A final volume of 10 µL of reaction mixture consisted of 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1X reaction buffer, 1 U Fast Start Taq DNA polymerase, 0.1X EvaGreen, 4 ng/µL cDNA, and 0.5 µM of each primer. The annealing temperature for each gene ranged from 55 to 57.5 °C. The qPCR program included preincubation (95 °C for 10 min), followed by 40 cycles of amplification (95 °C for 15 min, 55–57.5 °C for 20 s, 72 °C for 40 s) and melting curve (95 °C for 2 s and 65 °C for 1 min). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization of the expression of target genes, including CTX-M-15, *ampE*, *ftsI*, *nlpI*, *lpp*, *rfaP*, *wbbJ*, *kpsS*, and *phoU* (Table 1).

2.10. Statistics

The GraphPad Prism 5.0 (San Diego, CA, USA) program was used for all statistical analyses. Two-way ANOVA was carried out. Bonferroni posttests were applied. P-values of less than 0.0001 were considered significant.

3. Results

E. coli strain JJ1886 was determined to be a β-lactam producer using the nitrocefin phenotypic test. By MIC testing, *E. coli* strain JJ1886 was shown to be resistant to CTX, CAZ, and CAR, with MICs of 1280 µg/mL, 16 µg/mL, and 20 mg/mL, respectively. Because *E. coli* strain JJ1886 was resistant to CAR concentrations between 512 and 0.125 µg/mL, the MIC was detected at a higher concentration (20 mg/mL) for this antibiotic.

During the analysis of the time-dependent effect of NHS on the growth of *E. coli* strain JJ1886 during the exponential growth phase, the bacterium was treated with serum at 37 °C. We found that the growth rate decreased between the 0-h and 1-h time intervals. However, the bacterial cell number increased after 2 h of treatment

Table 1. Primers used in this study.

Gene	Sequence (5′→3′)	Locus_Tag	Size (bp)	Description	Reference
Standard PCR					
bla_{CTX-M}	ATGTGCAGYACCAGTAARGT	NA	593	Internal region of the *blaCTX-M* genes	(42)
	TGGGTRAARTARGTSACCAGA				
ampE	GTGTTAATTTTCGAGCGCCT	P423_RS00600	734	Beta-lactamase regulator AmpE	This study
	ACCTTATCGACATGCGGTTC				
nlpI	TTGCTGGTCCGATTCTGC	P423_RS18500	758	Lipoprotein NlpI	This study
	TGATTCTGGCACGTATGGAA				
lpp	GCGGTAATCCTGGGTTCTACT	P423_RS09290	188	Murein lipoprotein	This study
	ATGTTGTCCAGACGCTGGTT				
rfaP	ATGCTCGGCCTTAACACTTG	P423_RS20930	606	Lipopolysaccharide core heptose (I) kinase RfaP	This study
	GGCACGACCCTGAAAGAG				
wbbJ	TCCCATTGCATACGCTCATA	P423_RS12005	556	LPS Biosynthesis protein WbbJ; catalyzes the transfer of the O-acetyl moiety to the O antigen	This study
	GCCTTTATGGTGGTCTTAGGC				
ftsI	CATGGCGGACTACATCAAAA	P423_RS00470	821	Peptidoglycan glycosyltransferase FtsI, penicillin-binding protein 3	This study
	CGTAGCCGAAAGAGAAGGTG				
phoU	AGTACGCTCGGAATGGTACG	P423_RS21470	546	Phosphate transport system regulator PhoU	This study
	ACATATTTCCGGCCAGTTCA				
qPCR					
CTX-M-15	GCTGAAGAAAAGTGAAAGCGA	P423_07345	174	Class A extended-spectrum beta-lactamase CTX-M-15	This study
	AGCTTATTCATCGCCACGTT				
ampE	GCAGGTAAAGTTCGTCTTCATTATC	P423_RS00600	248	Beta-lactamase regulator AmpE	This study
	GCATACCCCATCAGCGTAAC				
nlpI	ACGTTTGTTCGCTAATGTTGC	P423_RS18500	172	Lipoprotein NlpI	This study
	CAAGACGATCCCAATGATCC				
lpp	GCGGTAATCCTGGGTTCTACT	P423_RS09290	179	Murein lipoprotein (Lpp)	This study
	AGACGCTGGTTAGCACGAG				
rfaP	CAAATCTTTATCCCGCCAAC	P423_RS20930	229	Lipopolysaccharide core heptose (I) kinase	This study
	GACCAACCCACCAGATGTTC				
wbbJ	TCCACACACGCTCTCCAATA	P423_RS12005	231	LPS biosynthesis protein	This study
	TGGATGCCGTAGTTTCCATT				

Table 1. (Continued).

ftsI	GTCGATAGCCAAGGGATTGA	P423_RS00470	216	Peptidoglycan glycosyltransferase FtsI; penicillin-binding protein 3	This study
	GTTAAAGGCCACTGCGTTGT				
kpsS	TACCGTTGAAGAAGGCGG	P423_RS17345	219	Polysialic acid capsule synthesis protein (KpsS)	This study
	TCATACCAGGGGGAAAATGA				
phoU	GTACCGCTTCGTCAATGTCC	P423_RS21470	210	Phosphate transport system regulator (PhoU)	This study
	TGCGTCTGGTGATGGTAATC				
GAPDH	TTTTTCCGAGAATCGACACC	P423_RS08270	249	Type I glyceraldehyde 3-phosphate dehydrogenase	This study
	GGATAGAAGTCGGCACGATG				

with NHS, likely due to its resistance to NHS (Figure 1A). For the quality control strain (*E. coli* ATCC 25922), a rapid decrease in cell number was reported in the first hour of the treatment with NHS (Figure 1B). In addition, we found that HIS-treated *E. coli* strain JJ1886 and *E. coli* ATCC 25922 showed a significant increase in cell numbers (7–8 $\log_{10}$ CFU/mL) after 2 h of treatment at 37 °C.

E. coli strain JJ1886 was treated with NHS or HIS plus CTX (1280 µg/mL), CAZ (16 µg/mL), and CAR (20 mg/mL). During the treatment with CAZ (16 µg/mL), in the exponential growth phase, *E. coli* strain JJ1886 showed resistance to NHS in the first hour. However, compared with HIS + CAZ in LB medium, the number of cells decreased after 2 h. During the 3- to 24-h time interval, NHS + CAZ showed bactericidal activity against *E. coli* strain JJ1886. There was a decrease in cell count after 3 h when HIS and CAZ were present in growth media. However, when only CAZ was present, bacterial growth was inhibited until the third hour of treatment with the serum, but the number of cells doubled after 24 h of culturing compared to the beginning of treatment (0 h) (Figure 2).

Similar results were obtained with CTX when *E. coli* strain JJ1886 was treated with 1280 µg/mL CTX, NHS, and HIS. However, it was observed that the number of cells decreased after 3 h of treatment with NHS + CTX. We found that NHS + CTX had a bactericidal effect at 24 h. When both HIS and CTX were in the growth medium, the cell number did not change until the 3-h time point, but the cell number increased swiftly at 24 h. However, when only CTX was present in the medium, bacterial growth was inhibited until the third hour of treatment, but cell number increased at 24 h (Figure 3).

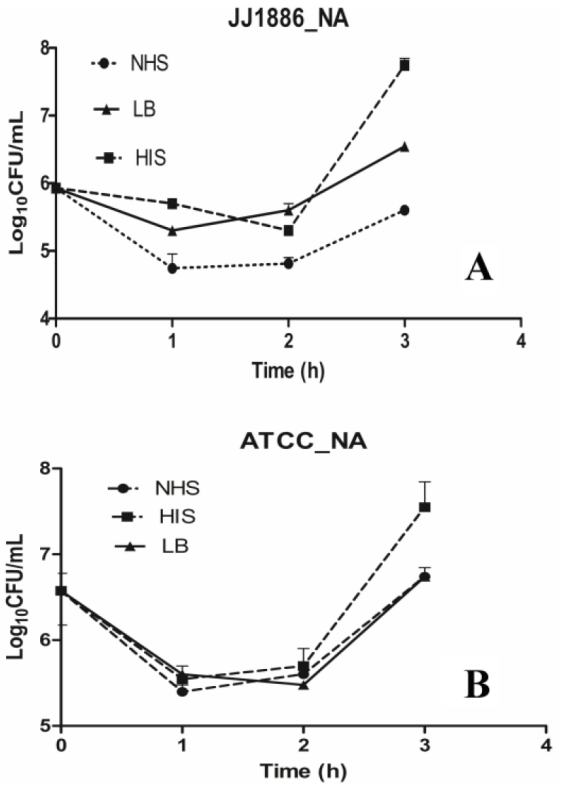

Figure 1. Time-dependent effect of NHS and HIS on bacterial growth. *E. coli* strain JJ1886 was incubated with NHS, HIS, and LB. Log10 CFU/mL was calculated at specified time intervals. JJ1886_NA: *E. coli* strain JJ1886 without antibiotics (A). *E. coli* ATCC 25922 quality control strain was incubated with NHS, HIS, and LB. Log10 CFU/mL was calculated at specified time intervals. ATCC_NA: *E. coli* ATCC 25922 strain without antibiotics (B). Mean and standard deviations were calculated from at least three independent experiments.

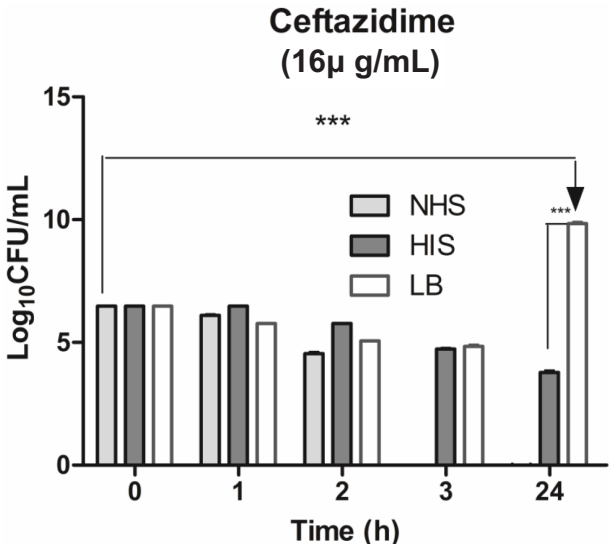

Figure 2. Time-dependent effect of NHS and HIS together with ceftazidime on bacterial growth. *E. coli* strain JJ1886 was incubated with NHS, HIS, and LB together with ceftazidime at 37 °C. The sample was withdrawn from the well corresponding to the MIC value. Log_{10} CFU/mL was calculated at specified time intervals. Mean and standard deviations were calculated from at least three independent experiments. ***: P < 0.0001.

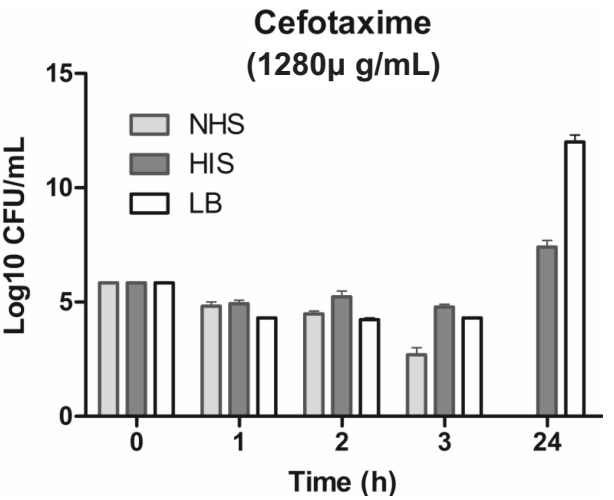

Figure 3. Time-dependent effect of NHS and HIS together with cefotaxime on bacterial growth. *E. coli* strain JJ1886 was incubated with NHS, HIS, and LB together with cefotaxime at 37 °C. The sample was withdrawn from the well corresponding to the MIC value. Log_{10} CFU/mL was calculated at specified time intervals. Mean and standard deviations were calculated from at least three independent experiments.

A decrease in cell number was observed after 1 h of treatment of *E. coli* strain JJ1886 with 20 mg/mL CAR, NHS, and HIS. However, when NHS and CAR were used together in the growth medium, *E. coli* O25b:H4 showed resistance at the second hour and the cell number increased at 24 h (Figure 4).

The quantitative expression of serum resistance and β-lactam-associated genes of *E. coli* strain JJ1886 was investigated by treatment with CAZ, CTX, CAR, NHS, and HIS (LB as a control; NHS, HIS, NHS + CTX, NHS + CAZ, NHS + CAR, HIS + CTX, HIS + CAZ, HIS + CAR, CTX, CAZ, and CAR). Serum resistance and β-lactamase-related genes are shown in Table 1. All relative gene expression was evaluated for downregulation or upregulation according to the control value of 1. A minimum 0.5-fold change was used as the cut-off for downregulation and a minimum 2-fold relative increase for upregulation (Table 2; Figure 5). There was no significant relative gene expression change for CTX-M-15, *ampE*, *ftsI*, *nlpI*, *lpp*, *rfaP*, *wbbJ*, *kpsS*, or *phoU*. Thus, the coexistence of serum and antibiotics in the growth medium did not alter the relative expression of the indicated genes. However, a significant downregulation was observed for the *nlpI* gene following treatment with CAR (Figure 5; Table 2).

4. Discussion

Certain *E. coli* strains present in the bloodstream may lead to serious infections, such as sepsis and urinary tract infections. Surviving in the bloodstream and overcoming host defense mechanisms are necessary virulence traits for *E. coli.* These virulence traits involve iron acquisition systems and serum resistance-associated factors (23). Previous studies have shown that serum survival factors allow pathogenic bacteria to survive in serum. These factors

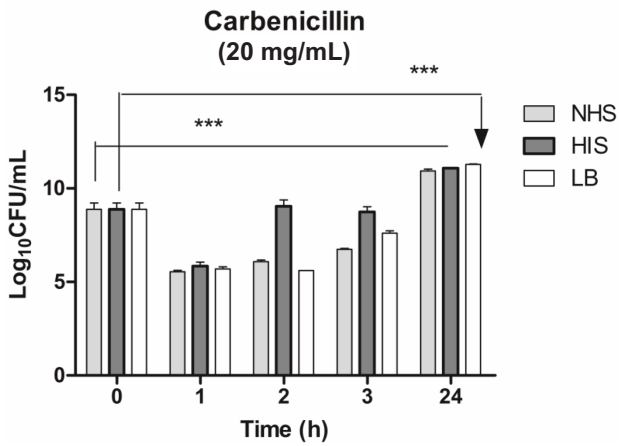

Figure 4. Time-dependent effect of NHS and HIS together with carbenicillin on bacterial growth. *E. coli* strain JJ1886 was incubated with NHS, HIS, and LB together with carbenicillin at 37 °C. Sample was withdrawn from the well corresponding to the MIC value. Log_{10} CFU/mL was calculated at specified time intervals. Mean and standard deviations was calculated from at least three independent experiments. ***: P < 0.0001.

Table 2. Fold change values of *CTX-M-15*, *nlpI*, *ampE*, *lpp*, *rfaP*, *wbbJ*, *ftsI*, *kpsS*, and *phoU* genes.

	Control and genes	NHS	HIS	CTX	CAZ	CAR	NHS + CTX	NHS + CAZ	NHS + CAR	HIS + CTX	HIS + CAZ	HIS + CAR
	CTX-M-15											
Fold change	1.00	0.91	1	0.72	1.27	1.37	1.08	1.18	1.02	0.73	0.8	0.53
St. dev.	0.00	0.16	0.17	0.37	0.05	0.03	0.1	0.53	0.07	0.05	0.1	0.35
	nlpI											
Fold change	1.00	1.05	0.82	0.87	0.58	0.43	1.03	1.12	0.99	0.92	0.96	1.71
St. dev.	0.00	0.27	0.57	0.07	0.7	0.12	0.14	0.1	0.13	0.18	0.11	0.19
	ampE											
Fold change	1.00	0.99	1.08	0.92	1.18	1.14	1.21	1.48	1.1	0.93	1.09	1.26
St. dev.	0.00	0.2	0.2	0.06	0.04	0.07	0.09	0.08	0.05	0.07	0.14	0.23
	lpp											
Fold change	1.00	0.98	1.14	0.92	1.02	1.12	1.03	1.11	0.84	0.88	0.96	0.93
St. dev.	0.00	0.11	0.12	0.11	0.09	0.10	0.06	0.12	0.11	0.08	0.06	0.09
	rfaP											
Fold change	1.00	1.18	1.37	0.82	0.86	0.91	1.19	0.87	0.93	0.75	0.87	0.92
St. dev.	0.00	0.20	0.19	0.03	0.09	0.08	0.10	0.08	0.37	0.39	0.22	0.55
	wbbJ											
Fold change	1.00	1.22	1.37	0.84	0.97	0.83	1.5	1.25	0.61	0.64	0.61	0.9
St. dev.	0.00	0.16	0.21	0.08	0.09	0.16	0.05	0.06	0.12	0.13	0.11	0.24
	ftsI											
Fold change	1.00	1.2	1.21	0.86	1.11	1	1.01	1.33	0.96	0.84	0.95	1.06
St. dev.	0.00	0.16	0.4	0.07	0.02	0.2	0.13	0.04	0.08	0.27	0.35	0.18
	kpsS											
Fold change	1.00	1.18	1.42	0.78	1.14	1.15	1.47	1.41	1.20	1.00	0.97	1.30
St. dev.	0.00	0.12	0.18	0.37	0.12	0.06	0.16	0.03	0.10	0.13	0.12	0.23
	phoU											
Fold change	1.00	1.08	1.31	0.90	1.27	1.19	1.48	1.69	1.32	1.23	1.04	1.31
St. dev.	0.00	0.18	0.23	0.10	0.08	0.07	0.09	0.06	0.09	0.11	0.34	0.10

include outer membrane proteins, capsule, O-antigens of the LPS, periplasmic protease, murein lipoprotein, and the phosphate transport system (21,24,26,33). Many pathogenic *E. coli* pathotypes are known to be serum-resistant, including CFTO73, RS218, 536, CP9, and EC958 (23). Among these, EC958 is a O25b:H4-ST131 strain disseminated globally and a known producer of the CTX-M-15-type ESBL (34). Similarly, the *E. coli* strain JJ1886 is an epidemic clone (O25b:H4-ST131) that is resistant to numerous β-lactams, including cephalosporins (20). In this study, we report that *E. coli* strain JJ1886 is resistant to serum and β-lactams, including CAZ, CTX, and CAR. However, CAZ and CTX together with NHS had bactericidal activity against *E. coli* strain JJ1886 at the tested time points.

A previous investigation reported that the *E. coli* CFTO73 strain survived when treated with serum during the first hour of culture. However, cell number decreased after the first hour of NHS treatment. These findings show that the *E. coli* CFTO73 strain can tolerate serum during the lag phase of growth (28). In our study, we investigated the time-dependent effect of NHS on growth *E. coli* strain JJ1886 in the exponential growth phase when treated with NHS at 37 °C. We found that the cell number decreased between the 0- and 1-h intervals. However, the cell number increased after 2 h of treatment. It can be speculated that the susceptibility and tolerance of different *E. coli* strains vary when *E. coli* strains are treated with NHS.

CAZ is a broad-spectrum cephalosporin used against gram-negative bacteria (35). In our study, only CAZ (16

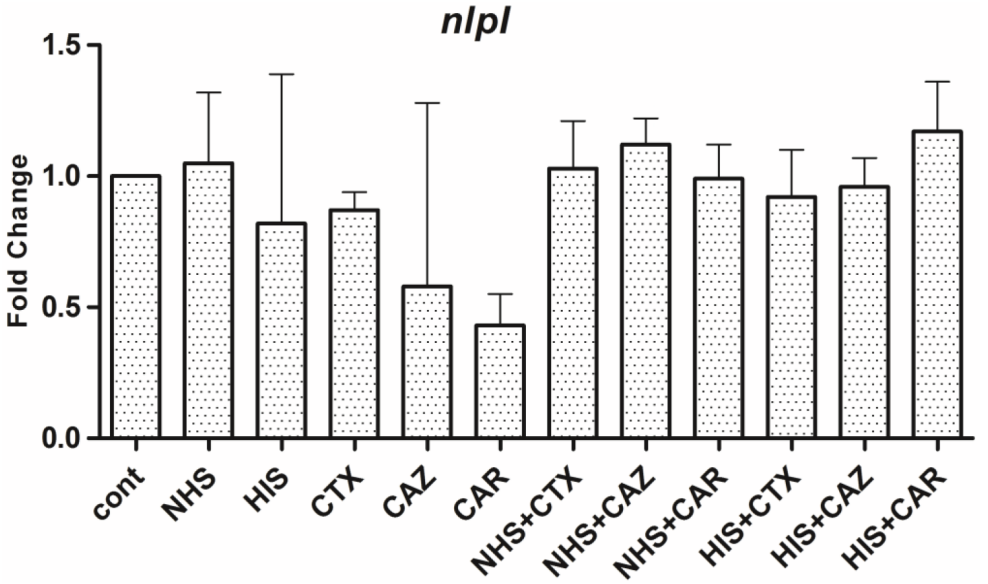

Figure 5. Relative expression of *nlpI* gene. Cultures of *E. coli* strain JJ1886 after 24 h with 12 different conditions—control, NHS, HIS, CTX, CAZ, CAR, NHS + CTX, NHS + CAZ, NHS + CAR, HIS + CTX, HIS + CAZ, and HIS + CAR—were used for RT-qPCR analysis. Final antibiotic concentrations were calculated according to MIC values. Mean and standard deviations were calculated from at least three independent experiments. Fold change: Relative mRNA expression, *nlpI*: a gene encoding lipoprotein NlpI.

μg/mL) in the LB medium showed an inhibitory effect on *E. coli* JJ1886 cells within the first 3 h. However, the number of cells increased at the 24-h time point (Figure 3). Similarly, Keepers et al. reported regrowth of *Enterobacteriaceae* when treated with CAZ and avibactam during 24-h measurement of bactericidal activity (35). Similar results were obtained for CTX (1280 μg/mL) and CAR (20 mg/mL) in this study. More precisely, CTX and CAR had an inhibitory effect until 3 h of culturing. However, the cell number almost doubled at 24 h after treatment. A previous study stated that the death rate of bacteria treated with β-lactam antibiotics was comparable to the growth rate (36). In other words, β-lactam antibiotics inhibit the formation of peptidoglycan synthesis during bacterial growth and cause cell lysis, thus affecting the growth rate and tolerance of the bacterium. Therefore, the inhibitory effect of β-lactam antibiotics can be time-dependent for *E. coli* strain JJ1886 cells until a certain time interval. Afterwards, *E. coli* strain JJ1886 cells proliferated during the 24-h treatment with CAZ, CTX, and CAR. Thus, it can be concluded that *E. coli* JJ1886 cells could tolerate the inhibitory effect of CAZ, CTX, and CAR when exposed to β-lactam antibiotics over a longer period. Another reason why *E. coli* JJ1886 cells regrew at the end of the 24-h culture period is the inoculum effect of β-lactamase-producing strains when compared to non-β-lactamase-producing strains (37). It has been suggested that the starting inoculum (1×10^6 to 5×10^6 CFU/mL) could lead to regrowth of bacteria.

When *E. coli* JJ1886 cells were incubated with β-lactam antibiotics (CAZ and CTX) together with serum, the cell number decreased, and eventually a bactericidal effect of NHS and β-lactam antibiotics (CAZ and CTX) was detected (Figures 2 and 3). However, when NHS and CAR were added together to growth media, cell number increased at the 3- and 24-h time points (Figure 4). A previous investigation of the bactericidal effect of antibiotics in human serum demonstrated that the complement-mediated effect makes bacterial cells more susceptible to antibiotics (38). However, here we found that, even though CAR + NHS were present in growth media, *E. coli* strain JJ1886 was resistant (Figure 4). Based on this finding, it can be inferred that *E. coli* strain JJ1886 is tolerant to both CAR and NHS.

We investigated the relative gene expression of serum resistance and β-lactamase-associated genes in the presence of NHS, HIS, CAZ, CTX, and CAR. A significant downregulation was observed for the *nlpI* gene, which encodes lipoprotein NlpI, when *E. coli* strain JJ1886 was treated with CAR only (Figure 5; Table 2). Lipoprotein NlpI is an outer membrane lipoprotein involved in cell division. It has been reported that NlpI together with periplasmic protease (Prc) controls peptidoglycan synthesis by changing levels of MepS, which is an endopeptidase

that breaks the peptide crosslink of the peptidoglycan component. In other words, NlpI binds to the Prc and MepS in order to degrade MepS via Prc (39). NlpI also contributes to the deposition of the complement regulator C4bp of *E. coli* in order to escape killing by serum (24). In addition to being associated with NlpI, Prc (also known as Tsp) contributes to pathogenicity by improving serum survival. Moreover, Prc processes the carboxy C-terminal region of the periplasmic protein and penicillin-binding protein 3, which is responsible for mediating peptidoglycan synthesis (33). In our study, growth analysis showed that *E. coli* strain JJ1886 was resistant to CAR and NHS during the 24-h incubation, and the *nlpI* gene encoding lipoprotein NlpI was downregulated in the presence of CAR. CAR is a carboxypenicillin, and *E. coli* strains that have surface β-lactamase are resistant to CAR (40). The mode of action of CAR is to inhibit cell wall synthesis during peptidoglycan cross-linking; this could affect the cell wall and periplasm membrane (41). Downregulation of the *nlpI* gene might be related to the peptidoglycan biosynthesis process. However, the exact reason why a downregulation was observed in the *nlpI* gene is unclear. To develop a more accurate conclusion requires further studies on the *nlpI* gene in *E. coli* strain JJ1886.

Taken together, our data show that during the exponential growth phase, *E. coli* strain JJ1886 is resistant to NHS. When the bacterium was treated with NHS together with β-lactam antibiotics, including CAZ and CTX, we detected a bactericidal effect. However, *E. coli* strain JJ1886 cells were resistant to NHS in combination with CAR. Our findings may show that these cells can tolerate the inhibitory effect of CAR and NHS. Moreover, there was no significant change in gene expression for encoding class A extended-spectrum beta-lactamase CTX-M-15, β-lactamase regulator, penicillin-binding protein 3, and serum survival-related factors, except for the *nlpI* gene encoding lipoprotein NlpI.

Acknowledgments

This study was supported financially by the Scientific Research Unit of İstanbul University with project ID FBA-2017-23647. The RT-qPCR analysis was performed by the Bioeksen Research and Development Company (Turkey).

References

1. Qin W, Panunzio M, Biondi S. Beta-lactam antibiotics renaissance. Antibiotics (Basel) 2014; 3: 193-215.
2. Bozcal E, Dagdeviren M. Toxicity of β-lactam antibiotics: pathophysiology, molecular biology and possible recovery strategies. In: Malangu N, editor. Poisoning: From Specific Toxic Agents to Novel Rapid and Simplified Techniques for Analysis. Rijeka, Croatia: InTech Open; 2017. pp. 87-105.
3. Worthington RJ, Melander C. Overcoming resistance to beta-lactam antibiotics. J Org Chem 2013; 78: 4207-4213.
4. Li L, Wang Q, Zhang H, Yang M, Khan MI, Zhou X. Sensor histidine kinase is a beta-lactam receptor and induces resistance to beta-lactam antibiotics. P Natl Acad Sci USA 2016; 113: 1648-1653.
5. D'Andrea MM, Arena F, Pallecchi L, Rossolini GM. CTX-M-type beta-lactamases: a successful story of antibiotic resistance. Int J Med Microbiol 2013; 303: 305-317.
6. Zhao WH, Hu ZQ. Epidemiology and genetics of CTX-M extended-spectrum beta-lactamases in Gram-negative bacteria. Crit Rev Microbiol 2013; 39: 79-101.
7. Shakil S, Khan AU. Detection of CTX-M-15-producing and carbapenem-resistant *Acinetobacter baumannii* strains from urine from an Indian hospital. J Chemother 2010; 22: 324-327.
8. Rogers BA, Sidjabat HE, Paterson DL. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. J Antimicrob Chemoth 2011; 66: 1-14.
9. Coelho A, Gonzalez-Lopez JJ, Miro E, Alonso-Tarres C, Mirelis B, Larrosa MN, Bartolome RM, Andreu A, Navarro F, Johnson JR et al. Characterisation of the CTX-M-15-encoding gene in *Klebsiella pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. Int J Antimicrob Agents 2010; 36: 73-78.
10. Fischer J, Rodríguez I, Baumann B, Guiral E, Beutin L, Schroeter A, Kaesbohrer A, Pfeifer Y, Helmuth R, Guerra B. *bla*CTX-M-15-carrying *Escherichia coli* and *Salmonella* isolates from livestock and food in Germany. J Antimicrob Chemoth 2014; 69: 2951-2958.
11. Kjeldsen TSB, Overgaard M, Nielsen SS, Bortolaia V, Jelsbak L, Sommer M, Guardabassi L, Olsen JE. CTX-M-1 β-lactamase expression in *Escherichia coli* is dependent on cefotaxime concentration, growth phase and gene location. J Antimicrob Chemoth 2015; 70: 62-70.
12. Sabra AH, Araj GF, Kattar MM, Abi-Rached RY, Khairallah MT, Klena JD, Matar GM. Molecular characterization of ESBL-producing *Shigella sonnei* isolates from patients with bacilliary dysentery in Lebanon. J Infect Dev Ctries 2009; 3: 300-305.
13. Lindquist S, Galleni M, Lindberg F, Normark S. Signalling proteins in enterobacterial AmpC beta-lactamase regulation. Mol Microbiol 1989; 3: 1091-1102.
14. Banerjee R, Johnson JR. *Escherichia coli* ST131: variations on a theme of clonal expansion. Enferm Infec Micr Cl 2013; 31: 355-356.

15. Dahbi G, Mora A, Lopez C, Alonso MP, Mamani R, Marzoa J, Coira A, Garcia-Garrote F, Pita JM, Velasco D et al. Emergence of new variants of ST131 clonal group among extraintestinal pathogenic *Escherichia coli* producing extended-spectrum beta-lactamases. Int J Antimicrob Agents 2013; 42: 347-351.

16. Andersen PS, Stegger M, Aziz M, Contente-Cuomo T, Gibbons HS, Keim P, Sokurenko EV, Johnson JR, Price LB. Complete genome sequence of the epidemic and highly virulent CTX-M-15-producing H30-Rx subclone of *Escherichia coli* ST131. Genome Announcements 2013; 1: 1-2.

17. Shaik S, Ranjan A, Tiwari SK, Hussain A, Nandanwar N, Kumar N, Jadhav S, Semmler T, Baddam R, Islam MA et al. Comparative genomic analysis of globally dominant ST131 clone with other epidemiologically successful extraintestinal pathogenic *Escherichia coli* (ExPEC) Lineages. mBio 2017; 8: 1-15.

18. Johnson JR, Porter S, Thuras P, Castanheira M. The pandemic H30 subclone of sequence type 131 (ST131) as the leading cause of multidrug-resistant *Escherichia coli* infections in the United States (2011–2012). Open Forum Infect Dis 2017; 4: ofx089.

19. Shin J, Ko KS. Effect of plasmids harbouring blaCTX-M on the virulence and fitness of *Escherichia coli* ST131 isolates. Int J Antimicrob Agents 2015; 46: 214-218.

20. Owens RC Jr, Johnson JR, Stogsdill P, Yarmus L, Lolans K, Quinn J. Community transmission in the United States of a CTX-M-15-producing sequence type ST131 *Escherichia coli* strain resulting in death. J Clin Microbiol 2011; 49: 3406-3408.

21. Phan MD, Peters KM, Sarkar S, Lukowski SW, Allsopp LP, Gomes Moriel D, Achard ME, Totsika M, Marshall VM, Upton M et al. The serum resistome of a globally disseminated multidrug resistant uropathogenic *Escherichia coli* clone. PLoS Genet 2013; 9: e1003834.

22. Weiser JN, Gotschlich EC. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect Immun 1991; 59: 2252-2258.

23. Miajlovic H, Smith SG. Bacterial self-defence: how *Escherichia coli* evades serum killing. FEMS Microbiol Lett 2014; 354: 1-9.

24. Tseng YT, Wang SW, Kim KS, Wang YH, Yao YF, Chen CC, Chiang CW, Hsieh PC, Teng CH. NlpI facilitates deposition of C4bp on *Escherichia coli* by blocking classical complement-mediated killing, which results in high-level bacteremia. Infect Immun 2012; 80: 3669-3678.

25. Diao J, Bouwman C, Yan D, Kang J, Katakam AK, Liu P, Pantua H, Abbas AR, Nickerson NN, Austin C et al. Peptidoglycan association of murein lipoprotein is required for KpsD-dependent group 2 capsular polysaccharide expression and serum resistance in a uropathogenic *Escherichia coli* isolate. mBio 2017; 8: 1-15.

26. Lamarche MG, Dozois CM, Daigle F, Caza M, Curtiss R 3rd, Dubreuil JD, Harel J. Inactivation of the pst system reduces the virulence of an avian pathogenic *Escherichia coli* O78 strain. Infect Immun 2005; 73: 4138-4145.

27. Liu YF, Yan JJ, Lei HY, Teng CH, Wang MC, Tseng CC, Wu JJ. Loss of outer membrane protein C in *Escherichia coli* contributes to both antibiotic resistance and escaping antibody-dependent bactericidal activity. Infect Immun 2012; 80: 1815-1822.

28. Putrins M, Kogermann K, Lukk E, Lippus M, Varik V, Tenson T. Phenotypic heterogeneity enables uropathogenic *Escherichia coli* to evade killing by antibiotics and serum complement. Infect Immun 2015; 83: 1056-1067.

29. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 2001; 48 (Suppl. 1): 5-16.

30. European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases. EUCAST Definitive Document E.DEF 3.1, June 2000: Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. Clin Microbiol Infect 2000; 6: 509-515.

31. Cremet L, Broquet A, Jacqueline C, Chaillou C, Asehnoune K, Corvec S, Caroff N. Innate immune evasion of *Escherichia coli* clinical strains from orthopedic implant infections. Eur J Clin Microbiol Infect Dis 2016; 35: 993-999.

32. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. J Hyg (Lond) 1938; 38: 732-749.

33. Wang CY, Wang SW, Huang WC, Kim KS, Chang NS, Wang YH, Wu MH, Teng CH. Prc contributes to *Escherichia coli* evasion of classical complement-mediated serum killing. Infect Immun 2012; 80: 3399-3409.

34. Forde BM, Ben Zakour NL, Stanton-Cook M, Phan MD, Totsika M, Peters KM, Chan KG, Schembri MA, Upton M, Beatson SA. The complete genome sequence of *Escherichia coli* EC958: a high quality reference sequence for the globally disseminated multidrug resistant *E. coli* O25b:H4-ST131 clone. PLoS One 2014; 9: e104400.

35. Keepers TR, Gomez M, Celeri C, Nichols WW, Krause KM. Bactericidal activity, absence of serum effect, and time-kill kinetics of ceftazidime–avibactam against beta-lactamase-producing *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2014; 58: 5297-5305.

36. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 2016; 14: 320-330.

37. Burgess DS, Hall RG. In vitro killing of parenteral beta-lactams against standard and high inocula of extended-spectrum beta-lactamase and non-ESBL producing *Klebsiella pneumoniae*. Diagn Microbiol Infect Dis 2004; 49: 41-46.

38. Dutcher BS, Reynard AM, Beck ME, Cunningham RK. Potentiation of antibiotic bactericidal activity by normal human serum. Antimicrob Agents Chemother 1978; 13: 820-826.

39. Singh SK, Parveen S, SaiSree L, Reddy M. Regulated proteolysis of a cross-link-specific peptidoglycan hydrolase contributes to bacterial morphogenesis. P Natl Acad Sci USA 2015; 112: 10956-10961.

40. Neu HC, Swarz H. Resistance of *Escherichia coli* and *Salmonella typhimurium* to carbenicillin. J Gen Microbiol 1969; 58: 301-305.

41. Lee S, Bae S. Molecular viability testing of viable but non-culturable bacteria induced by antibiotic exposure. Microb Biotechnol (in press).

42. Pagani L, Dell'Amico E, Migliavacca R, D'Andrea MM, Giacobone E, Amicosante G, Romero E, Rossolini GM. Multiple CTX-M-type extended-spectrum beta-lactamases in nosocomial isolates of *Enterobacteriaceae* from a hospital in northern Italy. J Clin Microbiol 2003; 41: 4264-4269.