The evolution of TEM-1 extended-spectrum -lactamases in *E. coli* by cephalosporins

Clasen, Julie; Birkegård, Anna Camilla; Græsbøll, Kaare; Folkesson, Anders

Published in:
Journal of Global Antimicrobial Resistance

Link to article, DOI:
[10.1016/j.jgar.2019.03.010](https://doi.org/10.1016/j.jgar.2019.03.010)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Clasen, J., Birkegård, A. C., Græsbøll, K., & Folkesson, A. (2019). The evolution of TEM-1 extended-spectrum -lactamases in *E. coli* by cephalosporins. *Journal of Global Antimicrobial Resistance*, 19, 32-39.
<https://doi.org/10.1016/j.jgar.2019.03.010>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Accepted Manuscript

Title: The evolution of TEM-1 extended-spectrum β -lactamases in *E. coli* by cephalosporins

Authors: Julie Clasen, Anna Camilla Birkegård, Kaare Græsbøll, Anders Folkesson

PII: S2213-7165(19)30079-7
DOI: <https://doi.org/10.1016/j.jgar.2019.03.010>
Reference: JGAR 894

To appear in:

Received date: 27 August 2018
Revised date: 7 February 2019
Accepted date: 18 March 2019

Please cite this article as: { <https://doi.org/>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The evolution of TEM-1 extended-spectrum β -lactamases in *E. coli* by cephalosporins

Julie Clasen^{a*}, Anna Camilla Birkegård^b, Kaare Græsbøll^b, Anders Folkesson^a

^aDepartment of Biotechnology and Biomedicine, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

^bDepartment of Applied Mathematics and Computer Science, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

^cNational Veterinary Institute, Technical University of Denmark, Kemitovet, 2800 Kgs. Lyngby, Denmark

***Corresponding author:** Julie Clasen Department of Biotechnology and Biomedicine Technical University of Denmark 2800 Kgs. Lyngby Denmark

Email: jucl@vet.dtu.dk

Highlights

- ESBLs are selected by low concentrations of third-generation cephalosporins
- Ceftazidime, cefotaxime and ceftriaxone selected for multiple ESBL variants
- Ceftibuten did not select for ESBLs
- ESBL variants remained susceptible towards ceftibuten
- Selective enrichment may select for ESBLs due to *de novo* mutations

Abstract:

Objectives: This study was conducted to examine the molecular mechanisms responsible for evolution of TEM-type extended-spectrum β -lactamases (ESBLs), following selective pressure from four third-generation cephalosporins; ceftazidime, cefotaxime, ceftriaxone and ceftibuten. In addition, the approach selective enrichment for ESBL detection in environmental samples was investigated.

Methods: By the use of experimental evolution, resistant variants were isolated and mutations in TEM-1 were examined by DNA sequencing. Using E-tests and disc diffusion assays, resistance levels and development cross-resistance were determined for ESBL producers. Selective plating with or without prior growth in selective broth was used to examine the approach of selective enrichment for ESBL detection.

Results: The third-generation cephalosporins ceftazidime, cefotaxime and ceftriaxone selected for ESBL, while ceftibuten did not select for ESBL. All ESBL variants additionally remained susceptible towards ceftibuten. DNA sequencing of the TEM-1 coding sequence of mutants, revealed mutations that not previously had been isolated through selection. This indicates that the potential for ESBL evolution is much broader than can be inferred from sequence analysis of clinical samples alone. Results also indicate that selective enrichment for the enhanced detection of ESBL producers may give unreliable results due to the selection of spontaneous mutations in the narrow-spectrum β -lactamases such as TEM-type ESBL producers.

Conclusions: These results help explain the molecular changes responsible for evolution of TEM-type ESBLs, meanwhile question the appropriate use of selective enrichment for detection of ESBLs in environmental samples.

Keywords: Extended-spectrum-beta-lactamases; *Escherichia coli*; third generation cephalosporins; evolution; antimicrobial resistance

Introduction

Bacterial infections are often treated with β -lactam antibiotics. Due to their high efficacy, low cost, and few side effects, they are one of the most utilized classes of antibiotics in human medicine [1]. β -lactams interfere with the synthesis of the bacterial cell wall by binding to penicillin-binding proteins (PBPs), resulting in stalled cell wall synthesis and subsequent inhibition of growth [2]. The widespread use of β -lactams has resulted in the evolution of β -lactam resistance in pathogenic bacteria [3]. Resistance is frequently conferred by β -lactamases – enzymes able to hydrolyze and inactivate β -lactams [2, 4, 5]. There are many variants of β -lactamases and they have been grouped based on substrate specificity or protein sequence [6-8]. A widely disseminated β -lactamase family is the TEM enzymes that are encoded by TEM-1 and its descendant genes [4, 9, 10]. TEM-1 is widely disseminated worldwide and has also been found in samples with little exposure to β -lactam antibiotics, including pristine forest soil [11-13]. TEM-1 β -lactamase hydrolyzes penicillins and early-generation cephalosporins effectively but is less efficient in degrading later generations of cephalosporins and monobactams [4, 10]. However, alleles with the ability to degrade the majority of β -lactams have evolved both *in vitro* and in the clinic due to extensive β -lactam use [4, 14-16], and the TEM β -lactamases now consist of hundreds of variants [10, 17]. Single nucleotide mutations in the TEM-1 gene result in amino acid substitutions in the enzyme, and the most common substitutions involved are: 104 (Glu $\rightarrow$ Lys), 164 (Arg $\rightarrow$ Ser or His), 238 (Glu $\rightarrow$ Ser), 240 (Glu $\rightarrow$ Lys) (Ambler's numbering scheme) [6], increasing hydrolytic activity of the enzyme and selecting for extended-spectrum β -lactamases (ESBLs) [10]. ESBLs are β -lactamases that are able to hydrolyze oxyimino- β -lactam antibiotics at a rate that is 10% faster than that for benzylpenicillin.

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

The aim of this study was to investigate molecular changes in TEM-1 under selection from four third-generation cephalosporins CAZ, CTX, ceftriaxone (CRO) or ceftibuten (CTB) and how these changes relates to the ESBL phenotype. We use experimental evolution to isolate resistant mutants, and detailed molecular biology to identify mutations within the TEM-1 gene. Resistance levels of evolved ESBL mutants were evaluated and the development of cross-resistance was investigated. We also investigated how the common practice of using selective enrichment to isolate bacterial phenotypes affects the ESBL evolutionary process and the selection of ESBL enzymes.

Methods

Strains and growth conditions

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

Transformation of pBR322

Competent *E. coli* MG1655 cells were prepared using CaCl_2 . In brief, 50 ml LB broth was inoculated with 0.5 ml *E. coli* MG1655 overnight (ON) culture and incubated with aeration to an OD_{600} of 0.5-1.0. The cells were pelleted by centrifugation at 4000xg for 10 min and re-suspended in 20 ml of ice-cold 0.1 M CaCl_2 . After incubation on ice for 30 min, cells were pelleted by centrifugation at 4000xg for 10 min and re-suspended in 4 ml of ice-cold 0.1 M CaCl_2 . Competent cells were used for transformation of pBR322 plasmid-encoding TEM-1 (ThermoFischer Scientific, Massachusetts, USA). In short, 2 μl plasmids were transferred to 200 μl pre-chilled *E. coli* MG1655 competent cells, and incubated on ice for 15 min. Cells were heat-shocked for 2 min at 42°C. Following this, 250 μl LB broth was added and cells were incubated for 60 min at 37°C, before plating on selective plates containing 8 mg/L Tc and incubated at 37°C ON. Positive *E. coli* MG1655/pBR322 transformants were stored in 25% glycerol at -80°C.

Serial passage experiments

Four third-generation cephalosporins: CAZ, CTX, CRO and CTB (Sigma-Aldrich, Copenhagen, Denmark) were used to select resistant mutants. Six parallel lineages of *E. coli* MG1655/pBR322 were propagated in LB medium, with increasing concentrations of antibiotics. The cultures were grown with shaking at 37°C and submitted to successive daily serial passages by inoculating 50 ml of fresh medium with 50 μl of culture to a 1:10,000 dilution. The cultures were initially grown in the absence of antibiotic for two transfers and then subjected to sub-MIC concentrations of antibiotic: CAZ (0.125 mg/L), CTX (0.03125 mg/L), CRO (0.0625 mg/L) or CTB (0.25 mg/L). Antibiotic concentrations were doubled upon observation of vigorous growth up to a concentration corresponding to four times the MIC breakpoint of resistance for the given antibiotic: CAZ = 16 mg/L, CTX = 8 mg/L, CRO = 8 mg/L and CTB = 4 mg/L.

For each serial passage step, 100 µl culture was plated on LB plates supplemented with the corresponding concentration of antibiotic, and plates were incubated at 37°C. To ensure plasmid maintenance, single colonies from selective plates were subsequently grown on 8 mg/L Tc plates. One clone from each serial passage step and from each lineage was stored in 25% glycerol at -80°C.

Plasmid extraction

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

PCR detection and sequencing of TEM-1

Amplification of TEM-1 was performed using primers designed by Perilli et al. [14]: TEM-1 FW (ATGAGTATTCAACAT TTCCGT) and TEM-1 RV (CCAATGCTT AATCAGTGAGG) (Eurofins Scientific, Luxembourg). PCR was performed using DreamTaq mix (ThermoFischer Scientific, Massachusetts, USA), according to manufacturer's guidelines and with cycling conditions as follows: 94°C for 1 min, 94°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec, repeated for 30 cycles. A final extension step at 72°C for 5 min followed. Amplicons were checked on an E-gel 2% agarose gel (Invitrogen, ThermoFischer Scientific, Massachusetts, USA). PCR amplicons were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer's guidelines. DNA concentrations were determined on a DeNovix DS-11+ Spectrophotometer (Delaware, USA), and purified PCR products were sequenced using an ABI 3730xl

DNA Analyzer (GATC Biotech, Konstanz, Germany) using TEM-1 primers. The sequence was determined for both strands, covering the entire coding sequence.

Determination of MIC

Minimum inhibitory concentrations (MIC) of *E. coli* MG1655 and MG1655/pBR322 were determined by broth microdilution procedure. Experiments were performed in Mueller Hinton (MH) broth with an inoculum of 5×10^5 CFU/mL in 96-well microtiter plates, which were incubated at 37°C for 20 h with 300 rpm shaking. OD₆₀₀ were measured on a Synergy H1 microplate reader (BioTek, Vermont, USA). MIC values of mutant transformants for CAZ, CTX and CRO were determined using E-tests (ThermoFisher Scientific, Massachusetts, USA). MIC values for isolates exposed to CTB were determined by disc diffusion assays using CTB discs (30 mg/L) (ThermoFisher Scientific, Massachusetts, USA). ATCC25922 and AB604 were used as negative and positive controls for the MIC assessment. Cross-resistance to CAZ, CTX, CRO and CTB was investigated for all ESBL producers by E-test or disc diffusion assays, as previously described.

Detection of ESBL producers by ESBL confirmation test

For phenotypic confirmation of ESBL production, ON culture of transformants was inoculated on a LB agar plate and a combination disc test (ROSCO®, Taastrup, Denmark) was used. Antibiotic discs containing CTX and CTX+clavulanic acid (CA) or CAZ and CAZ+CA were placed on the inoculated agar plate and following incubation, inhibition zone diameters were measured. ESBL production was confirmed positive when the inhibition zone diameter was ≥ 5 mm larger with CA than without it. *E. coli* ATCC 259922 and AB604 were used as negative and positive controls, respectively. Initially, mutant

endpoints were tested for ESBL production and if found positive, all isolated mutants from the serial passage experiment were tested.

Selective enrichment

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

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

Mutant selection on 1 mg/L CRO

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

Results: Cephalosporins select for ESBL production

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

Six independent lineages of *E. coli* MG1655/pBR322 were subjected to serial passages in increasing concentrations of each antibiotic. For each passage, one colony was isolated and plasmids were extracted. Plasmids were re-introduced into MG1655 prior to the MIC assessment and ESBL confirmation tests. Sequencing of PCR amplicons was performed to identify single nucleotide mutations in the TEM-1 coding region. ESBL confirmation tests demonstrated that CAZ, CTX and CRO were able to select for ESBL production (Supplementary Tables 1-3), even at concentrations lower than the clinical breakpoint concentrations. CTB did not select for ESBL production. Evolution of ESBLs using CAZ produced three types of mutants: R164H (TEM-12), R164S (TEM-29) and D179G – a previously unidentified TEM ESBL variant (Fig. 1A). Substitution at position 164 by histidine or serine increased the MIC 32-fold compared to the parental TEM-1 (Fig. 2). The substitution of aspartic acid by glycine at position 179 also positively affected enzyme activity, with a 16-fold increase in the MIC (Fig. 2). Exposure to CTX resulted in the evolution of three types of mutants: G238S (TEM-19), G238S-E105K (TEM-15) and G238S-E240K (TEM-71) (Fig. 1B). Acquisition of the G238S substitution increased the MIC 16-fold

compared to TEM-1 (Fig. 2). In four of the six lineages expressing the G238S variant, a subsequent mutation occurred at position 104 or 240, substituting a glutamic acid for a lysine. Acquisition of the second mutation increased the MIC eight-fold compared to the single mutation, and 128-fold compared to TEM-1 (Fig. 2B).

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

High levels of cross-resistance in ESBL mutants

To investigate the levels of cross-resistance of the mutants, MIC values for CAZ, CTX, CRO and CTB were determined. Substitutions at position 164 were associated with an increase in resistance toward both CTX and CRO, with the R164S substitution having a larger effect on MIC than the R164H substitution (Table 1). Expression of the D179G variant only increased the MIC of CTX two-fold, with no effect on CRO resistance. The G238S (TEM-19) substitution demonstrated high levels of resistance toward CRO, with a >256-fold MIC increase (Table 1). Resistance toward CAZ increased four-fold. An additional mutation in TEM-19, resulting in the expression of either G238S-E104K (TEM-15) or G238S-E240K (TEM-71), significantly increased the levels of resistance toward CAZ with a 64-fold and 128-fold

increase, respectively, compared to TEM-1, but had no effect on CRO resistance. ESBL producers harboring the G238S substitution (TEM-19) demonstrated high levels of resistance toward CTX with a >512-fold increase in MIC. Resistance toward CAZ increased four-fold. Acquisition of an additional mutation in the G238S variant, resulting in expression of G238S-T265M, did not further increase the MIC of CAZ. In contrast, the expression of G238S-E240K (TEM-71) led to a 128-fold increase in the MIC of CAZ. CTX resistance was unaffected by the additional mutation. Expression of R241P had a negligible impact on resistance toward CAZ and CTX. Expression of R164S-A237T changed the resistance spectrum compared to R164S (TEM-12) by increasing resistance toward CRO and decreasing CAZ resistance. Resistance toward CTX remained unaltered, and CTB susceptibility remained unchanged in all the mutant variants.

ESBL evolution using selective enrichment

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

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

Without selective enrichment, 30% (6/20) of samples tested positive for ESBL production, whereas 45% (9/20) of samples were positive of ESBL production using selective enrichment (Table 2).

To test whether ESBL evolution is affected by selective enrichment in the presence of plasmid harboring TEM-1, ON culture of MG1655/pBR322 was spiked into fecal samples dissolved in MacConkey broth with or without 1 mg/L CRO. Following ON incubation, samples were plated on selective MacConkey agar.

Without selective enrichment, spiking of plasmid encoded TEM-1 resulted in 85% (17/20) of samples positive for ESBL producers. With selective enrichment, spiking of TEM-1 resulted in 100% (20/20) of samples positive for ESBL producers (Table 2) indicating *de novo* evolution of ESBL from the spiked TEM-1 enzymes.

Discussion

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

Four TEM-type ESBL variants with amino acid substitutions D179G, R241P, R164S-A237T and G238S-T265M were isolated in our study, which previously only have been produced in laboratory experiments. The D179G substitution increased resistance toward CAZ 16-fold compared to the TEM-1, with negligible cross-resistance toward CTX and CRO. The R241P substitution resulted in a four-fold increase in resistance toward CRO but had no significant effect on CAZ or CTX resistance. The fact that D179G

and R241P ESBLs have not been observed in clinical isolates could illustrate that the potential for ESBL evolution is broader than can be deduced from sequence analysis of clinical samples alone.

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

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

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

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

Double mutations were observed under CTX and CRO selection, where an initial G238S substitution (TEM-19) occurred early. Continued exposure to CTX resulted in plasmids harboring TEM-15 (G238S-E104K) or TEM-71 (G238S-E240K). TEM-15 (G238S-E104K) increased the resistance level of CAZ 64-fold compared to TEM-1, and 16-fold compared to TEM-19. CTX resistance increased 128-fold compared to TEM-1 and eight-fold compared to TEM-19. Resistance toward CRO remained unaltered. Interestingly, selection of TEM-71 under selective pressure from CTX and CRO shifted the resistance pattern compared to TEM-19. The introduction of TEM-19 significantly increased cross-resistance, but with less of an effect on the level of resistance for the selecting cephalosporins. Selection of TEM-71 did not affect levels of cross-resistance, but a significant increase in the resistance level was observed toward the cephalosporin by which it had been selected.

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

The A237T substitution may work as a modulating substitution, reducing activity against some β -lactams while increasing it against others (Blazquez et al., 1998). This was also observed in our data, where acquisition of the R164S-A237T substitution was associated with an eight-fold decrease in CAZ resistance compared to the R164S background, and a two-fold increase in CRO resistance. Resistance levels against CTX remained unaltered.

The T265M substitution has previously been described in both clinical and laboratory isolates, but most often in combination with two other mutations. We showed that acquisition of the second T265M substitution in a G238S background increased the MIC against CRO >32-fold compared to its G238S background. High levels of resistance toward CTX remained unaltered compared to the G238S background, as did resistance toward CAZ. The role of the T265M substitution has not yet been fully

elucidated, but it has been proposed to have a stabilizing effect on the enzyme, thereby increasing resistance levels. However, this is in contrast to a study performed by Huang et al., where construction of a G238S-T265M variant using site-directed mutagenesis did not have a significant effect on resistance levels of either CTX or CAZ when compared to the wildtype [36]. The clinically important TEM ESBLs variants TEM-12 (R164S) and TEM-29 (R164H) were isolated upon selective pressure from CAZ, and both have been linked to increased resistance toward CAZ and CTX [37]. Resistance toward CTX and CRO was increased in both mutants with substitutions at position 164, with the serine substitution having the highest impact on resistance levels compared to histidine.

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

The practice of selective enrichment for ESBL detection influences ESBL evolution through to the selection of *de novo* mutations. Selective pre-enrichment is the standard procedure for detection and isolation of ESBL producing *Enterobacteriaceae* in clinical samples [25, 39-44] and is thought to improve detection rate of microorganisms present in low numbers [42]. We show that this procedure may be unreliable, due to a very high frequency of false negative results (55%) when the same sample was tested multiple times (Table 2). Moreover, we show that the addition of bacteria harboring the non-ESBL TEM-1 enzyme, significantly influenced the number of positive tests results (Table 2). In addition, we clearly show that pre-enrichment increases the number of *de novo* mutations in non-ESBL enzymes.

These data indicate that TEM-type ESBLs are selected for and enriched at two points in this selective enrichment method: 1) plating on plates supplemented with 1 mg/L CRO, as supported by our control study, and 2) upon incubation of samples with selective enrichment, resulting in 100% of spiked samples being positive for ESBL producers. The effects of false-negative and false-positive results have extensively been investigated in the context of post-selection ESBL confirmation tests, but the reproducibility of multiple test of the same sample has been less investigated. The results presented in this study indicate that evolutionary processes such as mutation rates and frequency can strongly affect the outcome of the identification efforts which ultimately can lead to both over- and underestimation of the true prevalence of ESBL producers in clinical samples.

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

Conclusion

We assessed the ability of four third-generation cephalosporins to select for TEM-type ESBL mutants *in vitro*. Our results showed that CAZ, CTX and CRO selected for ESBLs even at low levels of antibiotic, due to mutations in TEM-1. The majority of the ESBLs demonstrated high-level resistance toward the antibiotic by which they had been selected, but also demonstrated high levels of cross-resistance. Interestingly, CTB was not able to select for mutations in TEM-1 and all ESBL mutants remained susceptible towards CTB. We also propose that the use of selective enrichment for the detection of ESBLs should be used with caution as there is a risk of overestimating ESBL levels due to the evolution and selection of TEM-type ESBLs. We also observed a high risk of false-negative samples, which should be kept in mind when establishing whether a sample is negative or positive for ESBL producers.

Declarations

Funding: This work was supported by an internal departmental stipend to J.C.

Competing Interests: No conflict of interest.

Ethical Approval: Not required.

Acknowledgments We would like to thank DTU Food for providing the swine fecal samples, and Sophia Rasmussen for technical assistance.

References

1. Bush K, Bradford PA. beta-Lactams and beta-Lactamase Inhibitors: An Overview. Cold Spring Harbor perspectives in medicine. 2016;6(8). Epub 2016/06/23. doi: 10.1101/cshperspect.a025247. PubMed PMID: 27329032; PubMed Central PMCID: PMC4968164.
2. Ghuysen JM. Serine Beta-Lactamases and Penicillin-Binding Proteins. Annual Review of Microbiology. 1991;45(1):37-67. doi: 10.1146/annurev.mi.45.100191.000345.
3. Brakstad OG, Maeland JA. Mechanisms of methicillin resistance in staphylococci. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica. 1997;105(4):264-76. Epub 1997/04/01. PubMed PMID: 9164468.
4. Medeiros AA. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin Infect Dis. 1997;24 Suppl 1(1):S19-45. Epub 1997/01/01 00:00. PubMed PMID: 8994778.
5. Jamin M, Wilkin JM, Frere JM. Bacterial DD-transpeptidases and penicillin. Essays in biochemistry. 1995;29:1-24. Epub 1995/01/01. PubMed PMID: 9189711.
6. Ambler RP, Coulson AF, Frère JM, Ghuysen JM, Joris B, Forsman M, et al. A standard numbering scheme for the class A beta-lactamases. Biochemical Journal. 1991;276(Pt 1):269-70. PubMed PMID: PMC1151176.
7. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrobial agents and chemotherapy. 1995;39(6):1211-33. Epub 1995/06/01. PubMed PMID: 7574506; PubMed Central PMCID: PMC4968164.

8. Giske CG, Sundsfjord AS, Kahlmeter G, Woodford N, Nordmann P, Paterson DL, et al. Redefining extended-spectrum β -lactamases: balancing science and clinical need. *Journal of Antimicrobial Chemotherapy*. 2008;63(1):1-4. doi: 10.1093/jac/dkn444.
9. Brolund A, Sandegren L. Characterization of ESBL disseminating plasmids. *Infectious diseases (London, England)*. 2016;48(1):18-25. Epub 2015/07/03. doi: 10.3109/23744235.2015.1062536. PubMed PMID: 26135711.
10. Bush K. Proliferation and significance of clinically relevant beta-lactamases. *Annals of the New York Academy of Sciences*. 2013;1277:84-90. Epub 2013/01/26. doi: 10.1111/nyas.12023. PubMed PMID: 23346859.
11. Chanal C, Bonnet R, De Champs C, Sirot D, Labia R, Sirot J. Prevalence of beta-lactamases among 1,072 clinical strains of *Proteus mirabilis*: a 2-year survey in a French hospital. *Antimicrobial agents and chemotherapy*. 2000;44(7):1930-5. Epub 2000/06/20. PubMed PMID: 10858357; PubMed Central PMCID: PMCPMC89988.
12. Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110(9):3435-40. Epub 2013/02/13. doi: 10.1073/pnas.1222743110. PubMed PMID: 23401528; PubMed Central PMCID: PMCPMC3587239.
13. Canton R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, et al. Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. 2008;14 Suppl 1:144-53. Epub 2007/12/25. doi: 10.1111/j.1469-0691.2007.01850.x. PubMed PMID: 18154538.

14. Perilli M, Segatore B, Tavio M, Setacci D, Celenza G, De Santis F, et al. In Vitro Selection and Characterization of Mutants in TEM-1-Producing *Escherichia coli* by Ceftazidime and Ceftibuten. *Journal of Chemotherapy*. 2007;19(2):123-6. doi: 10.1179/joc.2007.19.2.123.
15. Barlow M, Hall BG. Predicting Evolutionary Potential: *In Vitro* Evolution Accurately Reproduces Natural Evolution of the TEM β -Lactamase. *Genetics*. 2002;160:823-32.
16. Weinreich DM, Delaney NF, Depristo MA, Hartl DL. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science (New York, NY)*. 2006;312(5770):111-4. Epub 2006/04/08. doi: 10.1126/science.1123539. PubMed PMID: 16601193.
17. Stuttgart Uo. The LAC tamase Engineering Database University of Stuttgart; 2018 [cited 2018 January]. Available from: <http://www.laced.uni-stuttgart.de/>.
18. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. *Antimicrobial agents and chemotherapy*. 2010;54(3):969-76. Epub 2009/12/10. doi: 10.1128/aac.01009-09. PubMed PMID: 19995920; PubMed Central PMCID: PMC2825993.
19. Livermore DM. beta-Lactamases in laboratory and clinical resistance. *Clinical microbiology reviews*. 1995;8(4):557-84. Epub 1995/10/01. PubMed PMID: 8665470; PubMed Central PMCID: PMC172876.
20. Mroczkowska JE, Barlow M. Fitness Trade-Offs in blaTEM Evolution. *Antimicrob Agents Chemother*. 2008;52(7):2340-5.
21. Stapleton PD, Shannon KP, French GL. Construction and Characterization of Mutants of the TEM-1 β -Lactamase Containing Amino Acid Substitutions Associated with both Extended-Spectrum Resistance and Resistance to β -Lactamase Inhibitors. *Antimicrobial agents and chemotherapy*. 1999;43(8):1881-7.

22. Blazquez J, Negri MC, Morosini MI, Gomez-Gomez JM, Baquero F. A237T as a modulating mutation in naturally occurring extended-spectrum TEM-type beta-lactamases. *Antimicrobial agents and chemotherapy*. 1998;42(5):1042-4. Epub 1998/05/21. PubMed PMID: 9593123; PubMed Central PMCID: PMCPMC105741.
23. Palzkill T, Le QQ, Venkatachalam KV, LaRocco M, Ocera H. Evolution of antibiotic resistance: several different amino acid substitutions in an active site loop alter the substrate profile of beta-lactamase. *Molecular microbiology*. 1994;12(2):217-29. Epub 1994/04/01. PubMed PMID: 8057847.
24. Blazquez J, Morosini MI, Negri MC, Baquero F. Selection of naturally occurring extended-spectrum TEM beta-lactamase variants by fluctuating beta-lactam pressure. *Antimicrob Agents Chemother*. 2000;44(8):2182-4. Epub 2000/07/18. PubMed PMID: 10898697; PubMed Central PMCID: PMCPMC90035.
25. Agersø Y, Aarestrup FM, Pedersen K, Seyfarth AM, Struve T, Hasman H. Prevalence of extended-spectrum cephalosporinase (ESC)-producing *Escherichia coli* in Danish slaughter pigs and retail meat identified by selective enrichment and association with cephalosporin usage. *Journal of Antimicrobial Chemotherapy*. 2012;67(3):582-8. doi: 10.1093/jac/dkr507.
26. EuropeanFoodSafety. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food. *EFSA Journal*. 2012;10(6). doi: 10.2903/j.efsa.2012.2742.
27. Hart KM, Ho CM, Dutta S, Gross ML, Bowman GR. Modelling proteins' hidden conformations to predict antibiotic resistance. *Nature communications*. 2016;7:12965. Epub 2016/10/07. doi:

- 10.1038/ncomms12965. PubMed PMID: 27708258; PubMed Central PMCID: PMC5477488.
28. Singh MK, Dominy BN. The Evolution of Cefotaximase Activity in the TEM β -Lactamase. *Journal of Molecular Biology*. 2012;415(1):205-20. doi: <https://doi.org/10.1016/j.jmb.2011.10.041>.
 29. Horn JR, Shoichet BK. Allosteric inhibition through core disruption. *J Mol Biol*. 2004;336(5):1283-91. Epub 2004/03/24. doi: 10.1016/j.jmb.2003.12.068. PubMed PMID: 15037085.
 30. Bowman GR, Bolin ER, Hart KM, Maguire BC, Marqusee S. Discovery of multiple hidden allosteric sites by combining Markov state models and experiments. *Proceedings of the National Academy of Sciences of the United States of America*. 2015;112(9):2734-9. Epub 2015/03/03. doi: 10.1073/pnas.1417811112. PubMed PMID: 25730859; PubMed Central PMCID: PMC4352775.
 31. Bowman GR, Geissler PL. Equilibrium fluctuations of a single folded protein reveal a multitude of potential cryptic allosteric sites. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(29):11681-6. Epub 2012/07/04. doi: 10.1073/pnas.1209309109. PubMed PMID: 22753506; PubMed Central PMCID: PMC3406870.
 32. Brandt C, Braun SD, Stein C, Slickers P, Ehricht R, Pletz MW, et al. In silico serine β -lactamases analysis reveals a huge potential resistome in environmental and pathogenic species. *Scientific reports*. 2017;7:43232-. doi: 10.1038/srep43232. PubMed PMID: 28233789.
 33. Huang W, Palzkill T. A natural polymorphism in β -lactamase is a global suppressor. *Proceedings of the National Academy of Sciences*. 1997;94(16):8801-6. doi: 10.1073/pnas.94.16.8801.

34. Gniadkowski M. Evolution of extended-spectrum β -lactamases by mutation. *Clinical Microbiology and Infection*. 2008;14, Supplement 1:11-32. doi: <http://dx.doi.org/10.1111/j.1469-0691.2007.01854.x>.
35. Raquet X, Vanhove M, Lamotte-Brasseur J, Goussard S, Courvalin P, Frere JM. Stability of TEM beta-lactamase mutants hydrolyzing third generation cephalosporins. *Proteins*. 1995;23(1):63-72. Epub 1995/09/01. doi: 10.1002/prot.340230108. PubMed PMID: 8539251.
36. Huang W, Le QQ, LaRocco M, Palzkill T. Effect of threonine-to-methionine substitution at position 265 on structure and function of TEM-1 beta-lactamase. *Antimicrobial agents and chemotherapy*. 1994;38(10):2266-9. Epub 1994/10/01. PubMed PMID: 7840555; PubMed Central PMCID: PMC284728.
37. Vakulenko SB, Taibi-Tronche P, Toth M, Massova I, Lerner SA, Mobashery S. Effects on substrate profile by mutational substitutions at positions 164 and 179 of the class A TEM(pUC19) beta-lactamase from *Escherichia coli*. *The Journal of biological chemistry*. 1999;274(33):23052-60. Epub 1999/08/07. PubMed PMID: 10438473.
38. Perilli M, Segatore B, Franceschini N, Gizzi G, Mancinelli A, Caravelli B, et al. Ceftibuten stability to active-site serine and metallo-beta-lactamases. *International Journal of Antimicrobial Agents*. 2001;17(1).
39. Jazmati N, Hein R, Hamprecht A. Use of an Enrichment Broth Improves Detection of Extended-Spectrum-Beta-Lactamase-Producing Enterobacteriaceae in Clinical Stool Samples. *J Clin Microbiol*. 2016;54(2):467-70. Epub 2015/11/27 06:00. PubMed PMID: 26607984.
40. Schauss T, Glaeser SP, Gutschow A, Dott W, Kampfer P. Improved detection of extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in input and output samples of

German biogas plants by a selective pre-enrichment procedure. PLoS One. 2015;10(3):e0119791. Epub 2015/03/24 06:00. PubMed PMID: 25799434.

41. Agerso Y, Aarestrup FM. Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing *Escherichia coli* in slaughter pigs. J Antimicrob Chemother. 2013;68(3):569-72. Epub 2012/11/07. doi: dks427 [pii] 10.1093/jac/dks427 [doi]. PubMed PMID: 23129728.
42. van den Bijllaardt W, Janssens MM, Buiting AG, Muller AE, Mouton JW, Verweij JJ. Extended-spectrum beta-lactamase (ESBL) polymerase chain reaction assay on rectal swabs and enrichment broth for detection of ESBL carriage. J Hosp Infect. 2018;98(3):264-9. Epub 2017/10/31 06:00. PubMed PMID: 29080706.
43. Overdeest IT, Willemsen I, Elberts S, Verhulst C, Kluytmans JA. Laboratory detection of extended-spectrum-beta-lactamase-producing Enterobacteriaceae: evaluation of two screening agar plates and two confirmation techniques. J Clin Microbiol. 2011;49(2):519-22. Epub 2010/12/03 06:00. PubMed PMID: 21123527.
44. Willems E, Verhaegen J, Magerman K, Nys S, Cartuyvels R. Towards a phenotypic screening strategy for emerging beta-lactamases in Gram-negative bacilli. Int J Antimicrob Agents. 2013;41(2):99-109. Epub 2013/01/03 06:00. PubMed PMID: 23280443.
45. Reuland EA, Overdeest IT, Al Naiemi N, Kalpoe JS, Rijnsburger MC, Raadsen SA, et al. High prevalence of ESBL-producing Enterobacteriaceae carriage in Dutch community patients with gastrointestinal complaints. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2013;19(6):542-9. Epub 2012/07/05. doi: 10.1111/j.1469-0691.2012.03947.x. PubMed PMID: 22757622.

46. Mesa RJ, Blanc V, Blanch AR, Cortes P, Gonzalez JJ, Lavilla S, et al. Extended-spectrum beta-lactamase-producing Enterobacteriaceae in different environments (humans, food, animal farms and sewage). *The Journal of antimicrobial chemotherapy*. 2006;58(1):211-5. Epub 2006/05/25. doi: 10.1093/jac/dkl211. PubMed PMID: 16720567.
47. Kluytmans-van den Bergh MF, Verhulst C, Willemsen LE, Verkade E, Bonten MJ, Kluytmans JA. Rectal Carriage of Extended-Spectrum-Beta-Lactamase-Producing Enterobacteriaceae in Hospitalized Patients: Selective Preenrichment Increases Yield of Screening. *Journal of clinical microbiology*. 2015;53(8):2709-12. Epub 2015/05/23. doi: 10.1128/jcm.01251-15. PubMed PMID: 25994164; PubMed Central PMCID: PMC4508429.

Figure 1. *In vitro* evolutionary pathways to TEM-type ESBLs under selective pressure from four third-generation cephalosporins on *E. coli* MG1655 harboring TEM-1. A) CAZ, B) CTX, C) CRO and D) CTB. Color-coded MIC values of parental TEM-1 for each antibiotic are shown in the upper circle. ESBL variants are shown within circles with MIC values and TEM-type (if known), and amino acid substitutions are shown above each ESBL variant.

Figure 1. *In vitro* evolutionary pathways to TEM-type ESBLs under selective pressure from four third-generation cephalosporins on *E. coli* MG1655 harboring TEM-1. A) CAZ, B) CTX, C) CRO and D) CTB. Color-coded MIC values of parental TEM-1 for each antibiotic are shown in the upper circle. ESBL variants are shown within circles with MIC values and TEM-type (if known), and amino acid substitutions are shown above each ESBL variant.

Figure 2. Fold changes in susceptibility of mutated ESBL variants to β -lactams, compared to parental TEM-1. CTB did not select for ESBL producers. CAZ= ceftazidime, CTX= cefotaxime, CRO= ceftriaxone

Figure 2. Fold changes in susceptibility of mutated ESBL variants to β -lactams, compared to parental TEM-1. CTB did not select for ESBL producers. CAZ= ceftazidime, CTX= cefotaxime, CRO= ceftriaxone

ACCEPTED MANUSCRIPT

Table 1. Cross-resistance of MG1655, MG1655/pBR322 and ESBL variants. CAZ= ceftazidime, CTX= cefotaxime, CRO= ceftriaxone, CTB= ceftibuten

Substitutions in TEM-1	Isolate	MIC (mg/L) [fold change]			
		CAZ	CTX	CRO	CTB
Wildtype strain	MG1655	0.25	0.0313	0.0625	0.5
None, parental enzyme	MG1655/pBR322	0.25	0.0625	0.125	0.5
R164S (TEM-12)	JCL459	8 [32]	0.5 [8]	0.5 [4]	0.5
R164H (TEM-29)	JCL303	8 [32]	0.25 [4]	0.25 [2]	0.5
D179G (Unknown)	JCL308	4 [16]	0.125 [2]	0.125 [-]	0.5
G238S (TEM-19)	JCL130	1 [4]	1 [16]	>32 [>256]	0.5
G238S-E104K (TEM-15)	JCL328	16 [64]	8 [128]	>32 [>256]	0.5
G238S-E240K (TEM-71)	JCL133	32 [128]	8 [128]	>32 [>256]	0.5
R164S-A237T (Unknown)	JCL154	1 [4]	0.5 [8]	1 [8]	0.5
G238S (TEM-19)	JCL150	1 [4]	>32 [>512]	1 [8]	0.5
G238S-E240K (TEM-71)	JCL153	32 [128]	>32 [>512]	>32 [>256]	0.5
G238S-T265M (Unknown)	JCL155	1 [4]	>32 [>512]	>32 [>256]	0.5
R241P (Unknown)	JCL148	0.5 [2]	0.13 [2]	0.5 [4]	0.5

Table 2. Positive ESBL producers after selective enrichment experiment. F=Fecal sample, MB=MacConkey Broth, CRO= ceftriaxone

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