J Antimicrob Chemother
doi:10.1093/jac/dkz393

Journal of Antimicrobial Chemotherapy

Extended-spectrum resistance to β-lactams/β-lactamase inhibitors (ESRI) evolved from low-level resistant *Escherichia coli*

Ángel Rodríguez-Villodres[1,2]†, María Luisa Gil-Marqués[1,2]†, Rocío Álvarez-Marín[1,2], Rémy A. Bonnin [3], María Eugenia Pachón-Ibáñez[1,2], Manuela Aguilar-Guisado[1,2], Thierry Naas[3], Javier Aznar[1,2,4], Jerónimo Pachón[2,5], José Antonio Lepe[1,2] and Younes Smani [1,2]*

[1]*Clinical Unit of Infectious Diseases, Microbiology and Preventive Medicine, University Hospital Virgen del Rocío, Seville, Spain;* [2]*Institute of Biomedicine of Seville (IBiS), University Hospital Virgen del Rocío/CSIC/University of Seville, Seville, Spain;* [3]*EA7361, Université Paris-Saclay, LabEx Lermit, Bacteriology-Hygiene unit, APHP, Hôpital Bicêtre, EERA 'Evolution and Ecology of Resistance to Antibiotics' Unit, Institut Pasteur-APHP-Université Paris-Sud, Le Kremlin-Bicêtre, France;* [4]*Department of Microbiology, University of Seville, Seville, Spain;* [5]*Department of Medicine, University of Seville, Seville, Spain*

*Corresponding author. Tel: +34-955923100; E-mail: ysmani-ibis@us.es
†These authors contributed equally to this work.

Received 28 April 2019; returned 9 July 2019; revised 29 July 2019; accepted 12 August 2019

Objectives: *Escherichia coli* is characterized by three resistance patterns to β-lactams/β-lactamase inhibitors (BLs/BLIs): (i) resistance to ampicillin/sulbactam and susceptibility to amoxicillin/clavulanic acid and piperacillin/tazobactam (RSS); (ii) resistance to ampicillin/sulbactam and amoxicillin/clavulanic acid, and susceptibility to piperacillin/tazobactam (RRS); and (iii) resistance to ampicillin/sulbactam, amoxicillin/clavulanic acid and piperacillin/tazobactam (RRR). These resistance patterns are acquired consecutively, indicating a potential risk of developing resistance to piperacillin/tazobactam, but the precise mechanism of this process is not completely understood.

Methods: Clinical isolates incrementally pressured by piperacillin/tazobactam selection *in vitro* and *in vivo* were used. We determined the MIC of piperacillin/tazobactam in the presence and absence of piperacillin/tazobactam pressure. We deciphered the role of the bla_{TEM} genes in the new concept of extended-spectrum resistance to BLs/BLIs (ESRI) using genomic analysis. The activity of β-lactamase was quantified in these isolates.

Results: We show that piperacillin/tazobactam resistance is induced in *E. coli* carrying bla_{TEM} genes. This resistance is due to the increase in copy numbers and transcription levels of the bla_{TEM} gene, thus increasing β-lactamase activity and consequently increasing piperacillin/tazobactam MICs. Genome sequencing of two bla_{TEM}-carrying representative isolates showed that piperacillin/tazobactam treatment produced two types of duplications of bla_{TEM} (8 and 60 copies, respectively). In the clinical setting, piperacillin/tazobactam treatment of patients infected by *E. coli* carrying bla_{TEM} is associated with a risk of therapeutic failure.

Conclusions: This study describes for the first time the ESRI in *E. coli*. This new concept is very important in the understanding of the mechanism involved in the acquisition of resistance to BLs/BLIs.

Introduction

To circumvent bacterial resistance to penicillins, β-lactamase inhibitors (BLIs) were developed to be used in combination with penicillins in order to inhibit β-lactamase, thus allowing the penicillins to act unimpeded.[1,2] Among these combinations, ampicillin/sulbactam, amoxicillin/clavulanic acid and piperacillin/tazobactam are efficacious against *Escherichia coli* and other Gram-negative bacteria producing β-lactamases (mainly TEM enzymes).[1] Piperacillin/tazobactam is one of the most important β-lactam (BL)/BLI combinations, and it is widely used in the treatment of severe infections such as bacteraemia and intra-abdominal infections.[3,4] However, the intensive use of BLs/BLIs has contributed to the emergence of resistance mechanisms that now limit their therapeutic use.[5,6]

There are three main mechanisms of *E. coli* resistance to BLs/BLIs dependent on TEMs: TEM-1 hyperproduction, evolution of inhibitor-resistant TEM (IRT) variants and evolution of TEM variants with higher hydrolytic capacities.[5,7,8] Other β-lactamases, OXA-1 and AmpC enzymes, and porin loss are common causes of BL/BLI resistance.[6,9,10] Although BLIs can inhibit the same TEM-type

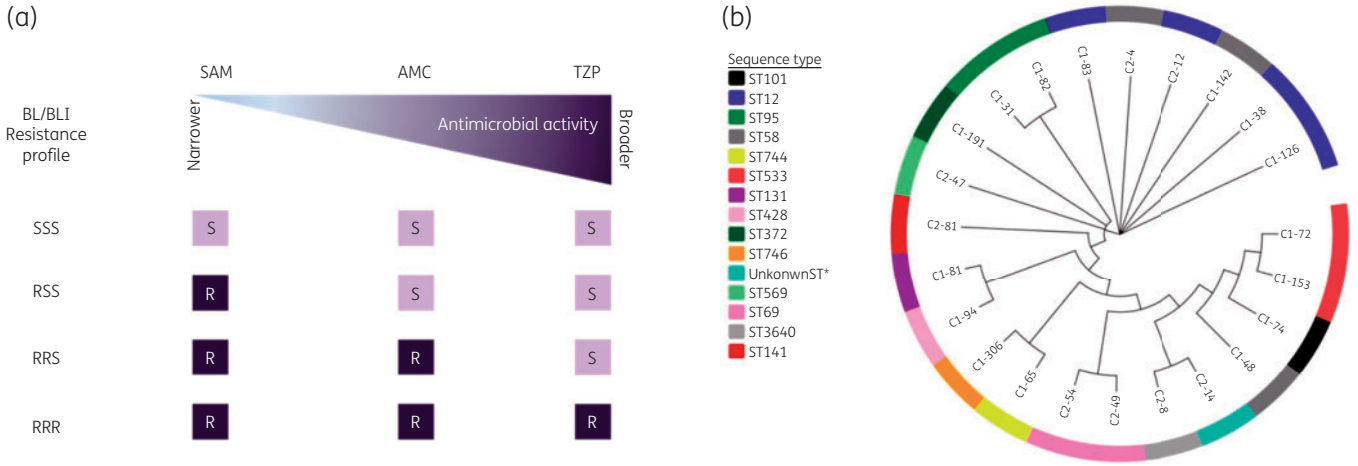

Figure 1. BL/BLI resistance in *E. coli*. (a) Schematic proposal of gradual and unidirectional process of BL/BLI resistance that develops from ampicillin/sulbactam to piperacillin/tazobactam. (b) Phylogenetic tree of the 23 *E. coli* isolates based on MLST gene sequences analysed by the neighbour-joining method with 1000 bootstrap replicates. The asterisk indicates an unknown ST with new alleles, numbered 123, 23, 3, 16, 9, 8 and 6 for the genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*, respectively. SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

enzymes, resistance to BLs/BLIs mediated by these mechanisms has been reported for ampicillin/sulbactam and amoxicillin/clavulanic acid, while piperacillin/tazobactam remains active against the majority of these enzymes.[11,12] This can be explained by the fact that tazobactam exerts the highest inhibitory activity against TEM β-lactamases, followed by clavulanic acid then sulbactam.[2,5]

Therefore, three BL/BLI resistance patterns in *E. coli* clinical isolates are frequently encountered in clinical microbiology laboratories: (i) resistance (R) to ampicillin/sulbactam and susceptibility (S) to amoxicillin/clavulanic acid and piperacillin/tazobactam (RSS); (ii) resistance to ampicillin/sulbactam and amoxicillin/clavulanic acid, and susceptibility to piperacillin/tazobactam (RRS); and (iii) resistance to ampicillin/sulbactam, amoxicillin/clavulanic acid and piperacillin/tazobactam (RRR). Thus, we suggest that BL/BLI resistance in *E. coli* is a gradual and unidirectional process that extends from ampicillin/sulbactam to piperacillin/tazobactam (Figure 1a). However, the role of TEM β-lactamases in extended-spectrum BL/BLI resistance (ESRI) remains unknown, and clinical isolates of *E. coli* with a high level of ampicillin/sulbactam or amoxicillin/clavulanic acid resistance and a low level of piperacillin/tazobactam resistance (RSS or RRS phenotype) may have a potential to acquire high-level piperacillin/tazobactam resistance.

Given that the piperacillin/tazobactam concentration varies from sub- to supra-inhibitory levels during clinical piperacillin/tazobactam treatment, e.g. between the intra-abdominal space and bloodstream, among other body compartments,[13,14] we suggest that sub-inhibitory levels of piperacillin/tazobactam may promote the emergence of resistant *E. coli* isolates.[15,16] Therefore, we treated clinical *E. coli* isolates with increasing sub-inhibitory concentrations of piperacillin/tazobactam to evaluate whether piperacillin/tazobactam can produce high-level resistance to BLs/BLIs (RRR phenotype) from low- or moderate-level resistance to BLs/BLIs (RSS or RRS phenotype). In this study, we elucidate the role of TEM in this process, and define a new concept, ESRI.

Materials and methods

Expanded details of all methods are given in the Supplementary Materials and methods (available as Supplementary data at *JAC* Online).

Bacterial isolates

Twenty-three *E. coli* clinical isolates were obtained from bloodstream and intra-abdominal samples of patients with suspected bacteraemia or intra-abdominal infections, at the University Hospital Virgen del Rocío, Seville (Spain). Two other isolates (PT3 and PT4) from a patient with bacteraemia subsequent to a perianal abscess and treated for 8 days with piperacillin/tazobactam were analysed. *E. coli* ATCC 25922 was used as a control in all the experiments. The study was approved by the Committee of the University Hospital Virgen del Rocio of Seville, Spain (approval number 0023-N-16).

BLI resistance profile

The ampicillin/sulbactam, amoxicillin/clavulanic and piperacillin/tazobactam antimicrobial susceptibility profiles were initially tested by broth microdilution using the MicroScan WalkAway NM44 panels. MICs of piperacillin/tazobactam were subsequently confirmed using the standard broth microdilution method.[17,18] To test whether TEM β-lactamases were affected by variable concentrations in piperacillin/tazobactam combinations, we used a piperacillin/tazobactam combination at an 8:1 ratio. MICs of cefuroxime, cefotaxime and ceftolozane/tazobactam were determined with gradient test strips and the zone diameter of cefazolin was determined by the disc diffusion method.

MLST assay

Seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified by conventional PCR and screened by Sanger sequencing using an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA),[19] and the data were compiled through the website hosted at Enterobase (http://enterobase.warwick.ac.uk).

Phylogenetic analysis or nucleotide sequence analysis

Sequence alignment was performed and phylogenetic trees were constructed using the software MEGA7.[20] Neighbour-joining trees were built from concatenated sequences of MLST loci.[21] The evolutionary distances were computed using Kimura's two-parameter model.[22] A bootstrap consensus tree inferred from 1000 replicates was depicted to represent the evolutionary history of the taxa analysed.[23] All trees were then viewed using the EvolView web application.[24]

Detection and sequencing of bla_{TEM} and bla_{OXA-1} genes

The bla_{TEM} and bla_{OXA-1} genes were analysed in all studied isolates by PCR using specific primers. All positive PCR products were screened using an ABI3500 Genetic Analyzer to determine variant sequences of TEM β-lactamase.

Antimicrobial selection pressure

The diluted bacterial inocula (10^5 cfu/mL) were incubated with sub-inhibitory concentrations of piperacillin/tazobactam corresponding to dilutions 1-fold below the MICs at 37°C for 24 h. Positive bacterial growth was re-adjusted to 10^5 cfu/mL for piperacillin/tazobactam MIC determinations and for further incubation with a 2-fold increased concentration of piperacillin/tazobactam. These steps were repeated until we reached a piperacillin/tazobactam concentration of 256/32 mg/L or a piperacillin/tazobactam concentration that did not allow bacterial growth.

Removal of antimicrobial selection pressure

Daily passages of these isolates on sheep blood agar plates were made for 15 days and the piperacillin/tazobactam MICs were determined at each passage.

Construction of bla_{TEM} mutant strains

bla_{TEM-1}, bla_{TEM-30}, bla_{TEM-40} and $bla_{TEM-135}$ were amplified by PCR with EcoRI-bla_{TEM} primers using C1-153, C1-81, C1-31 and C1-142 chromosomal DNA, with a Taq polymerase. The PCR products were purified using DNA-spin™ Plasmid DNA Purification kits, digested with EcoRI and BamHI and cloned into a linearized EcoRI-BamHI vector pUCp24 using T4 DNA ligase. The constructs were electroporated into *E. coli* ATCC 25922 and transformants (pTEM-1, pTEM-30, pTEM-40 and pTEM-135) were selected on LB agar plates containing gentamicin.

Quantification of β-lactamase activity

The β-lactamase activity was determined using the β-Lactamase Activity Kit (BioVision Inc, Milpitas, CA, USA). Briefly, the bacterial culture pellets were weighed and resuspended in β-lactamase assay buffer. Samples were sonicated and centrifuged. Five microlitres of each sample was incubated with nitrocefin. The OD at 490 nm was immediately measured in kinetic mode.

Plasmid purification

Bacterial plasmids were purified using the QIAGEN Plasmid Mini Kit from overnight cultures. Equal amounts of DNA were loaded in a 1% agarose gel. Fifty nanograms of plasmid sample was used in the qPCR to check the plasmid copy number.

RNA extraction and qRT-PCR

Bacterial RNA was purified using the RNeasy Mini Kit. Specific primers of TEM and *rrsG* (housekeeping gene) were used. The quantitative real-time PCR assay was carried out with SYBR Premix Ex Taq in a MxPro 3005p system. Relative gene expression was quantified using the comparative CT (ΔΔCt) method.

PCR-based replicon typing (PBRT) analysis

Plasmid incompatibility groups were determined according to Carattoli *et al.*[25] This procedure is based on five multiplex and three simplex PCRs using 18 pairs of primers that recognize the FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons.

WGS

The DNA libraries were prepared using the NexteraXT v3 kit and run on the HiSeq System to generate paired-end 150 bp reads. *De novo* assembly of Illumina reads was performed using CLC Genomics Workbench 10.1. The genome was annotated using the Rapid Annotations using Subsystems Technology (RAST) tool.

The gene copy number was determined using the coverage ratio of the $bla_{TEM-like}$ gene (or its genetic context) and the mean of three chromosomally encoded genes (replication initiator protein *dnaA*, the β-subunit of RNA polymerase *rpoB* and the malate dehydrogenase *mdh*). Two incI1 plasmid genes (replicase gene, *repA* and a gene involved in conjugative transfer, *trbC*) were used for the normalization of plasmid copy number in isolate C2-54.

Statistical analysis

The group data are presented as mean ± SEM. Student's *t*-test was used to determine the differences between means. The difference was considered significant at $P < 0.05$. The SPSS (version 21.0) statistical package was used (SPSS Inc., Chicago, IL, USA).

Data availability

Whole-genome sequences were deposited in GenBank under the BioProject accession number PRJNA554906.

Results and discussion

BL/BLI resistance in E. coli

We first selected 23 *E. coli* clinical isolates (2 SSS, 6 RSS and 15 RRS) from intra-abdominal or bloodstream infections that belonged to different STs (Figure 1b and Table S1). All these isolates were carbapenem susceptible. After incrementally increasing the selection pressure (i.e. concentration) of piperacillin/tazobactam, we observed that 17 of 23 (74%) isolates grew at piperacillin/tazobactam concentrations several times higher than the initial MIC. The new piperacillin/tazobactam MIC of these isolates increased ≥8-fold with respect to the initial MIC; these isolates thus became resistant to piperacillin/tazobactam (Table S1). The piperacillin/tazobactam MIC values increased incrementally in parallel with the increased concentrations of piperacillin/tazobactam, suggesting a dose–response relationship between the MIC and the concentrations of piperacillin/tazobactam (Table S1). Next, we addressed whether this effect was due to the combination of piperacillin plus tazobactam, or to either of these compounds alone. Testing

all isolates with piperacillin and tazobactam separately showed that none increased their piperacillin/tazobactam MIC (data not shown).

TEM and IRT are involved in the acquired resistance to piperacillin/tazobactam

Since TEM enzymes in *E. coli* may hydrolyse piperacillin/tazobactam,[5,8] we hypothesized that the increase in piperacillin/tazobactam MIC values of the 17 isolates after exposure to piperacillin/tazobactam was due to the hyperproduction of TEM enzymes. To test this, we detected the presence of bla_{TEM} in these isolates: 13 isolates expressed TEM-1 and the other four isolates expressed TEM-30, TEM-35, TEM-40 and TEM-135, respectively (Table S1). All of these TEM enzymes were efficiently inhibited by tazobactam, thus explaining the susceptibility to piperacillin/tazobactam (RSS and RRS phenotypes) of these isolates. The promoter region of all the bla_{TEM} genes did not acquire mutations after exposure to piperacillin/tazobactam and subsequent removal of antibiotic pressure when compared with the original isolate (Table S2). Notably, after piperacillin/tazobactam exposure, the bla_{TEM} gene was not detected in the five *E. coli* isolates that maintained their susceptibility to piperacillin/tazobactam, and in one isolate (C1-74) that became intermediate according to the 2019 CLSI breakpoint, even though the piperacillin/tazobactam MIC only increased 2-fold (Table S1). It is noteworthy that OXA-1 was not detected in these isolates.

To confirm the role of TEM enzymes in the acquisition of resistance to piperacillin/tazobactam, we cloned different bla_{TEM} genes (bla_{TEM-1}, bla_{TEM-30}, bla_{TEM-40} and $bla_{TEM-135}$) into pUCp24 and introduced the plasmids into *E. coli* ATCC 25922, a strain that does not possess the bla_{TEM} gene. The mutants producing TEM variants increased their piperacillin/tazobactam MIC value from 4 to 512 mg/L (Table S3), suggesting that TEM enzymes are involved in the acquired piperacillin/tazobactam resistance. Since clinical isolates of *E. coli* carrying TEM enzymes can acquire piperacillin/tazobactam resistance by exposure to piperacillin/tazobactam (Table S1), we determined the impact of piperacillin/tazobactam exposure on ATCC 25922, ATCC 25922/pUCp24 and the mutant strains by incubating them with increasing concentrations of piperacillin/tazobactam. The four mutants showed very large increases in their piperacillin/tazobactam MIC values (8- to 32-fold) after incubation with piperacillin/tazobactam. On the other hand, ATCC 25922 or ATCC 25922/pUCp24 showed no change or only a 4-fold increase in their piperacillin/tazobactam MIC (Table S3). Taken together, these data suggest that TEM and IRT are involved in the acquired resistance to piperacillin/tazobactam, and the evolution from low-level (RSS) to high-level resistance (RRR) to BLs/BLIs could be linked to TEM β-lactamases. Hence, ESRI is defined as the acquisition of high-level resistance to BLs/BLIs (RRR) from low- or moderate-level resistance to BLs/BLIs (RSS or RRS).

Although Schechter *et al.*[8] reported that resistance to piperacillin/tazobactam may be due to the genetic amplification of the bla_{TEM-1} gene in one *E. coli* strain, to our knowledge no study has reported on the role of TEM-1 or IRTs in the ESRI of *E. coli*. Here, we show that *E. coli* ATCC 25922 carrying bla_{TEM-1}, bla_{TEM-30}, bla_{TEM-40} or $bla_{TEM-135}$ expresses higher β-lactamase activity compared with the parental ATCC 25922 (Figure 2a). This β-lactamase activation is not restricted to recombinant bla_{TEM} isolates but also extends to clinical isolates carrying bla_{TEM} genes. Indeed, Figure 2(b) shows that most *E. coli* isolates treated with piperacillin/tazobactam showed higher β-lactamase activity than the original isolates. It is noteworthy that the piperacillin/tazobactam resistance acquired by these clinical isolates remained stable when the antibiotic was removed from the medium. When the piperacillin/tazobactam-treated *E. coli* isolates with RRR phenotype were tested after growth in medium free of piperacillin/tazobactam for 15 days, none had returned to their initial phenotype (RRS or RSS), except for one isolate (C1-153) that decreased its piperacillin/tazobactam MIC from 256 to 64 mg/L (which is still classified as resistant by EUCAST and intermediate by CLSI) (Figure 2c). Importantly, in the absence of selective pressure, these isolates maintained higher β-lactamase activity compared with the piperacillin/tazobactam-treated isolates, even when piperacillin/tazobactam was removed from the medium, which may explain the long-term stability of piperacillin/tazobactam resistance in the absence of this antibiotic (Figure 2b). Moreover, the promoter and bla_{TEM} gene of each isolate did not acquire mutations in their sequences after exposure to piperacillin/tazobactam and subsequent removal of antibiotic pressure when compared with the original isolate (Table S2). Therefore, the higher observed TEM activity was likely due to a higher copy number of plasmids carrying bla_{TEM} or to a higher copy number of bla_{TEM} itself in isolates exposed to piperacillin/tazobactam. To confirm this, we analysed the copy number of bla_{TEM} in these isolates, and found that most exposed isolates contained higher levels of copies (Figure 2d) and transcripts of bla_{TEM} (Figure 2e). Moreover, compared with the original isolates, the purified plasmids of those (except for C1-81 and C1-94 isolates) that underwent piperacillin/tazobactam pressure followed by piperacillin/tazobactam removal had higher copy numbers and/or increased size (Figure S1).

Genomic analysis of the region surrounding the $bla_{TEM-like}$ gene in E. coli

To better understand the mechanism of MIC increase, the genome sequences of the original and piperacillin/tazobactam-treated states of two representative clinical isolates, C1-81 carrying bla_{TEM-30} and C2-54 carrying bla_{TEM-1}, were analysed. Results showed two different modes of action: in the original C1-81 isolate, the coverages of *dnaA*, *rpoB* and *mdh* were 181×, 74× and 46×, respectively, providing a mean chromosomal coverage of 100×. The coverage of the bla_{TEM-30} gene was 40×, representing a single copy per cell. Meanwhile, in piperacillin/tazobactam-exposed C1-81S, the coverages of *dnaA*, *rpoB* and *mdh* were 475×, 70× and 54×, respectively, providing a mean chromosomal coverage of 200×. The 1380× coverage of bla_{TEM-30} represents seven copies per cell. Analysis of the coverage of the region surrounding the bla_{TEM-30} gene reveals two copies of IS*26* bracketing the bla_{TEM-30} gene, thus forming a putative class 1 transposon. This putative transposon may duplicate the region through homologous recombination events between the IS*26* copies or by IS*26*-mediated site-specific recombination (Figure 3a). On the other hand, in the original C2-54 isolate the coverages of *dnaA*, *rpoB* and *mdh* were 107×, 93× and 77×, respectively, providing a mean chromosomal coverage of 92×. The coverage of bla_{TEM-1b} was 42×, that of the I1 replicase gene was 51× and that of the transfer gene *trbC* was 58×, thus corresponding to a single copy per cell. In C2-54S exposed to piperacillin/tazobactam, the coverages of *dnaA*, *rpoB*

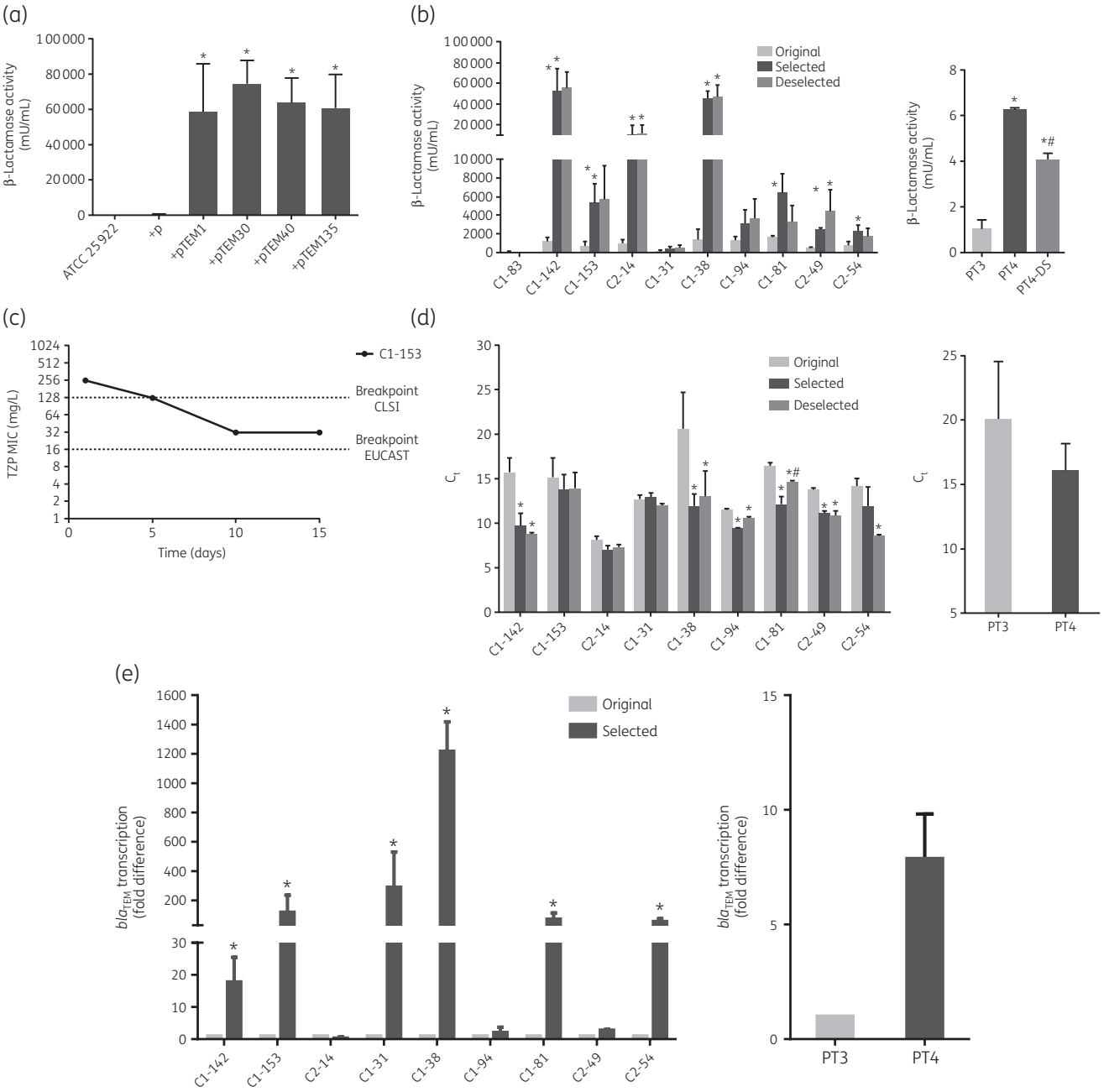

Figure 2. TEM and IRT are involved in the acquired resistance to piperacillin/tazobactam. (a) β-Lactamase activity of the WT strain ATCC 25922, and the same strain carrying the empty plasmid pUCp24 (p) or the pUCp24 harbouring the bla_{TEM-1}, bla_{TEM-30}, bla_{TEM-40} or $bla_{TEM-135}$ gene. (b) β-Lactamase activity of parental *E. coli* isolates in the presence/absence of piperacillin/tazobactam selective pressure. (c) Stability of piperacillin/tazobactam resistance during 15 days in the absence of piperacillin/tazobactam for strain C1-153. (d) qPCR of bla_{TEM} in plasmids purified from parental *E. coli* isolates, in the presence/absence of piperacillin/tazobactam selective pressure, and in PT3 and PT4. (e) bla_{TEM} gene transcription in parental *E. coli* isolates in the presence/absence of piperacillin/tazobactam selective pressure and in PT3 and PT4. *P<0.05 for selected or deselected versus original; PT4 or PT4-DS versus PT3. #P<0.05 for deselected versus selected. Selected indicates an isolate under selective piperacillin/tazobactam pressure. Deselected (DS) indicates an isolate without selective piperacillin/tazobactam pressure. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and *mdh* were 70×, 58× and 54×, respectively, providing a mean chromosomal coverage of 92×. The coverage of bla_{TEM-1b} was 3300×, while that of I1 replicase was 40× and that of transfer gene *trbC* was 31×, suggesting 60 copies of the bla_{TEM} gene/plasmid molecule. Genetic context analysis reveals that the bla_{TEM-1b} gene is part of Tn*2*, as shown in Figure 3(b). Sequence analysis of the boundaries of Tn*2* reveals two different direct repeats: TTTTA or TAGTA. These are only present in C2-54S, indicating a second

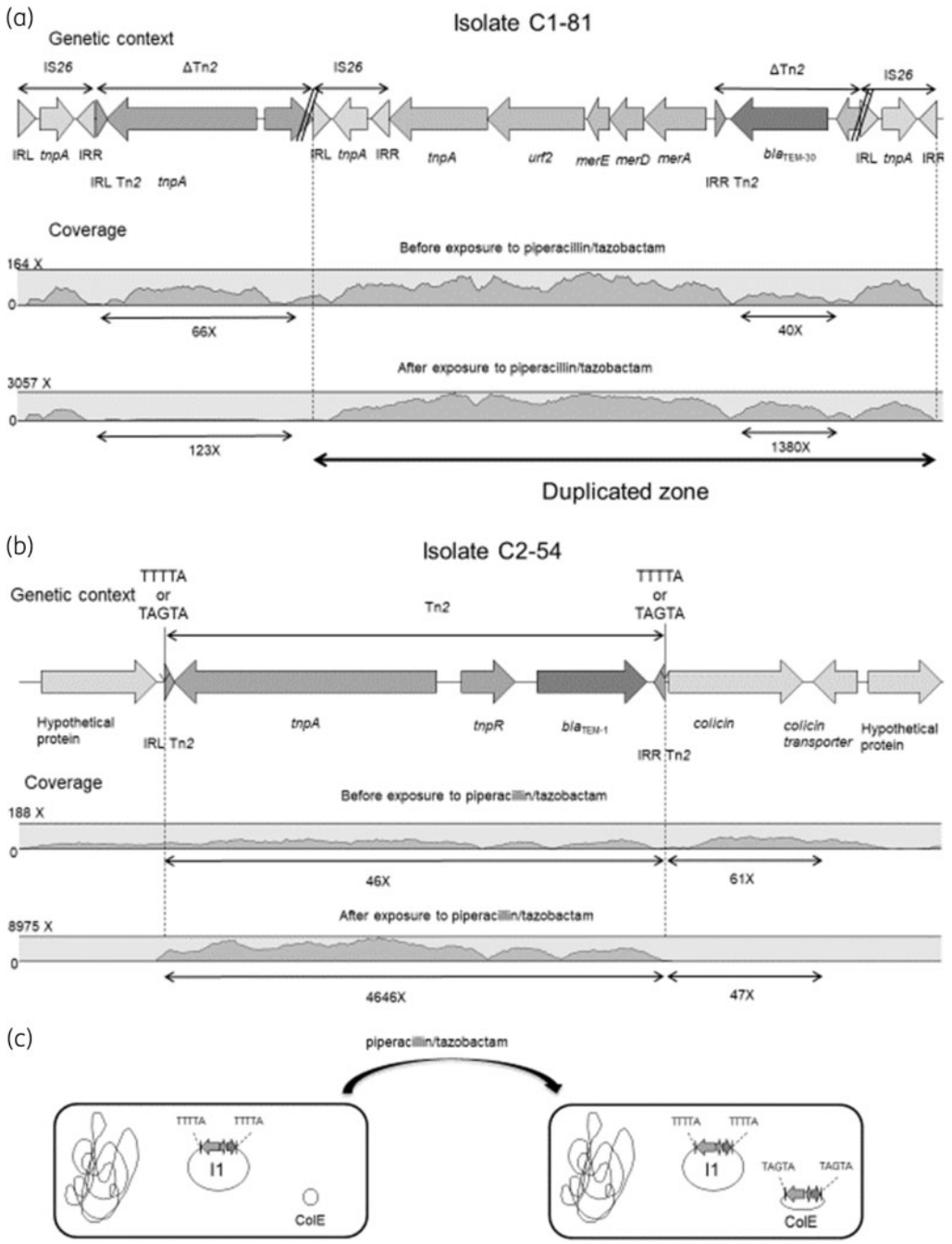

Figure 3. Genomic analysis of the region surrounding the $bla_{\text{TEM-like}}$ gene in isolates C1-81 and C2-54 (a) The upper part represents the genetic context of the $bla_{\text{TEM-30}}$ gene. Coding sequences and their orientations are represented by arrows. Inverted repeat sequences of insertion sequences are indicated by triangles. The lower part represents the coverage of the $bla_{\text{TEM-30}}$ gene region in scale with the genetic context. Coverage is expressed in terms of *x*-fold, which is the mean number of reads covering each nucleotide in the given region. (b) The upper part represents the genetic context of $bla_{\text{TEM-1b}}$. Coding sequences and their orientations are represented by arrows. Inverted repeat sequences of insertion sequences are indicated by triangles. Direct repeat sequences are indicated on the top of the transposon boundaries. The lower part represents the coverage of the $bla_{\text{TEM-1b}}$ region in scale with the genetic context. (c) Schematic representation of transposition event after piperacillin/tazobactam exposure. I1 and ColE correspond to plasmid families.

integration site of Tn*2* (Figure 3b). The first integration site, shared by both isolates, is present on the incI1 plasmid, whereas the second, present only in C2-54S, is on a ColE-family plasmid. In isolate C2-54, the plasmid ColE is present but does not contain a copy of Tn*2*. The ColE family of plasmids are small plasmids with high copy numbers. The duplication of Tn*2* is represented in Figure 3(c).

Thus, we have revealed two modes of action in the acquisition of resistance to piperacillin/tazobactam: one is IS*26*-based

duplication, and the other is based on the transposition of the bla_{TEM} gene onto a high-copy-number plasmid. The latter is in line with a recent study by Schechter *et al.*,[8] in which the presence of the bla_{TEM-1} gene on a 10 kb tandem repeated genomic resistance module was associated with *E. coli* resistance to piperacillin/tazobactam. This kind of genetic event, e.g. a recombination or transposition process occurs randomly but genetic events increasing the MICs may have been selected under antibiotic exposure. In our case, modification of a pre-existing plasmid by transposition, rather than acquisition of foreign DNA, is at the origin of the increased MICs. Selection of this rare genetic event may have occurred during treatment and thus under selective pressure. To explain the two modes of action, we suggest the presence of two different plasmids in C1-81 and C2-54. Indeed, PBRT analysis shows that C1-81 and C2-54 carry IncFIV and IncI1, respectively. More studies are needed to confirm this hypothesis. All these results suggest that TEM hyperproduction is responsible for the ESRI. This property is stable over long periods of time even in the absence of piperacillin/tazobactam selection pressure. Higher expression levels, due to an increase in the numbers of plasmids carrying bla_{TEM} and/or higher bla_{TEM} copy number, may result in clinical failures of piperacillin/tazobactam treatments.

Role of piperacillin/tazobactam treatment in patients in ESRI development

To confirm this hypothesis, clinical and microbiological data from two *E. coli* clinical isolates (C2-49 and C2-54) belonging to ST69 and recovered from intra-abdominal abscesses of the same patient, before and after 10 days of piperacillin/tazobactam treatment without clinical improvement, were analysed. Similarly, two *E. coli* clinical isolates (PT3 and PT4) belonging to ST88 and recovered from blood cultures in a second patient with perianal abscess and persistent bacteraemia, before and after 8 days of piperacillin/tazobactam treatment without clinical improvement, were analysed. The first set of isolates from both patients, C2-49 and PT3, presented an RSS resistance pattern to BL/BLI; and the second set of isolates, C2-54 and PT4, presented with RRS and RRR resistance patterns, respectively. Furthermore, PT4 presented higher numbers of gene copies and transcripts of bla_{TEM}, resulting in a higher β-lactamase activity (Figure 2b, d and e), and a piperacillin/tazobactam MIC of 256 mg/L (Table 1). These data highlight the possible role of piperacillin/tazobactam in ESRI (from RSS to RRR phenotype), especially when the piperacillin/tazobactam concentrations in the abdomen fail to reach a high enough level.[13]

Overall, our findings may have clinical relevance, especially in patients with severe infection by *E. coli* carrying bla_{TEM} and treated with piperacillin/tazobactam at the standard dosing regimen; such a dosage may not provide high enough concentrations at the infection sites (i.e. intra-abdominal infection). These low piperacillin/tazobactam concentrations could instead select for the hyperproduction of TEM, boosting the development of ESRI and consequently leading to therapeutic failure. Our data suggest that the current piperacillin/tazobactam ratio (8:1) could be too low for the treatment of infections by *E. coli*, especially when the drugs could not reach adequate levels at the site of infection. New formulations involving tazobactam, such as ceftolozane/tazobactam and cefepime/tazobactam, have been developed in 2:1 and 1:1 ratios, respectively, which seem to be more appropriate.[26,27]

Three clinical studies are consistent with our observations. Retamar *et al.*[28] analysed the impact of the piperacillin/tazobactam MICs on the outcome of patients with bacteraemia due to ESBL-producing *E. coli* isolates; they found that *E. coli* with intermediate and high piperacillin/tazobactam MIC values were responsible for bacteraemia originating from sources other than the urinary tract and were associated with higher mortality (41.1% versus 0%).[28] Also, piperacillin/tazobactam MIC values on the borderline of the susceptibility breakpoint have been associated with a worse outcome as compared with piperacillin/tazobactam MICs in the susceptibility range in patients with bacteraemia due to Enterobacteriaceae.[29] Recently, results of the MERINO study,[30] an international multicentre trial comparing piperacillin/tazobactam and meropenem treatment

Table 1. MICs of first-, second- and third-generation cephalosporins and cephalosporin/BLI for *E. coli* before and after *in vivo* and *in vitro* treatment with piperacillin/tazobactam

					MIC, mg/L (fold change)[a]			
Isolates	Profile	ST	TEM type	Cefazolin zone diameter, mm (difference)[b]	TZP	CXM	CTX	COZ/TAZ
C2-49	RSS	69	TEM-1	22	8	3	0.032	0.125
C2-54	RRS	69	TEM-1	22 (0)	8 (1)	3 (1)	0.047 (1.5)	0.125 (1)
PT-3	RSS	88	TEM-1	21	8	4	0.047	0.125
PT-4	RRR	88	TEM-1	**14 (6)**	**256 (32)**	4 (1)	0.064 (1.4)	**0.25 (2)**
C1-142	RRS	58	TEM-135	22	4	2	0.047	0.19
C1-142S	RRR	58	TEM-135	**0 (22)**	**1024 (256)**	**24 (12)**	**0.38 (8)**	**1.5 (8)**

Values in bold indicate a difference in susceptibility of ≥2-fold. CTX, cefotaxime; CXM, cefuroxime; COZ/TAZ, ceftolozane/tazobactam; TZP, piperacillin/tazobactam.

S indicates that the isolate was under selective piperacillin/tazobactam pressure.

[a]Fold change between C2-49 and C2-54, PT3 and PT4, and C1-142 and C1-142S.

[b]Difference in zone diameter (mm) between C2-49 and C2-54, PT3 and PT4, and C1-142 and C1-142S.

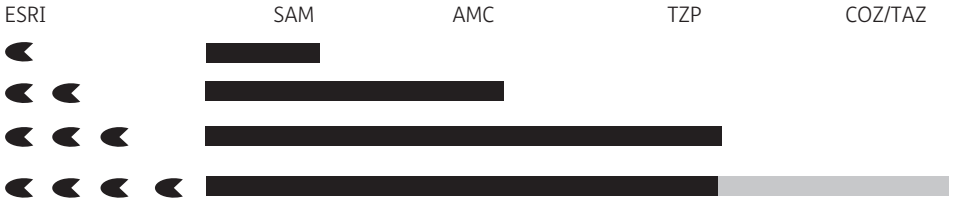

Figure 4. Schematic representation of ESRI as a new concept that affects the susceptibility to BL/BLI and cephalosporins. SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; COZ/TAZ, ceftolozane/tazobactam.

of bacteraemia due to *E. coli* or *Klebsiella* spp. resistant to third-generation cephalosporins, showed a 30 day mortality of 12.3% in patients receiving piperacillin/tazobactam versus 3.7% in patients receiving meropenem.[30] More clinical studies dealing with severe infections such as pneumonia or intra-abdominal infections are needed to confirm the data from these clinical studies.

ESRI affects the susceptibility of E. coli to other β-lactams

Next, we investigated whether ESRI affects the susceptibility of *E. coli* to other β-lactams. Specifically, first-, second- and third-generation cephalosporins (cefazolin, cefuroxime and cefotaxime, respectively) and cephalosporin/BLI (ceftolozane/tazobactam) were tested against the two pairs of clinical isolates, C2-49/C2-54 and PT3/PT4, obtained from two patients before and after unsuccessful piperacillin/tazobactam treatment, and against a representative pair of isolates, C1-142/C1-142S carrying $bla_{\text{TEM-135}}$ and exposed to, then released from piperacillin/tazobactam selection pressure. Bacteriostatic tests showed that after *in vivo* and *in vitro* piperacillin/tazobactam selective pressure on the isolates PT4 and C1-142S, respectively, there were increases in the MICs of the cephalosporins tested, including ceftolozane/tazobactam (Table 1). Isolate C1-142S, carrying $bla_{\text{TEM-135}}$ and presenting higher β-lactamase activity, increased its MIC of ceftolozane/tazobactam by 8-fold as compared with the original isolate (from 0.19 to 1.5 mg/L), thus developing resistance according to EUCAST guidelines.[31] The fold change in MIC observed with cephalosporins is more prominent with the first-generation cephalosporins (12-fold) than with later generations (8-fold) (Table 1). Consistent with these results, Sun *et al.*[32] observed a similar loss of activity of seven different cephalosporins representing the first to fourth generations during progressive exposure of *Salmonella typhimurium* carrying the $bla_{\text{TEM-1}}$ gene to cephalothin and cefaclor (first and second generation, respectively). Thus, ESRI affects the antibacterial activity of BL/BLI, but may also affect cephalosporins with or without tazobactam. Additional studies are needed to confirm this observation in order to determine the real impact of piperacillin/tazobactam on the acquisition of resistance to cephalosporins, thus determining the limits of this therapeutic option for the treatment of severe bacterial infections.

In conclusion, resistance to BLs/BLIs induced by piperacillin/tazobactam and dependent on the TEM-β-lactamases appears to follow a unidirectional and irreversible pathway that comprises a new concept, termed ESRI (Figure 4). This new concept is very important in the understanding of the mechanism involved in the acquisition of resistance to piperacillin/tazobactam, one of the most widely used antibiotics worldwide. Its proper use is essential in the context of the multidrug resistance to antimicrobials, since the inappropriate use of piperacillin/tazobactam may promote the re-emergence of TEM-β-lactamases, which would be a major problem in the clinical setting.

Funding

This study was supported by the Miguel Servet Tipo I Project grant, Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad (CP15/00132), and by Plan Nacional de I + D+i 2013–2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades, Spanish Network for Research in Infectious Diseases (RD16/0016/0009), cofinanced by the European Development Regional Fund 'A Way to Achieve Europe', Operative Program Intelligent Growth 2014–2020. This study was supported by the Assistance Publique – Hôpitaux de Paris (AP-HP), the University Paris-Sud, the Laboratory of Excellence in Research on Medication and Innovative Therapeutics (LERMIT) supported by a grant from the French National Research Agency [ANR-10-LABX-33]. Y. S. is supported by the Subprograma Miguel Servet Tipo I, Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spain (CP15/00132). A. R-V. is supported by the Programa de fomento de investigación en Residentes HUVR/IBIS, Fundación Pública Andaluza Para la Gestión de la Investigación en Salud de Sevilla, Junta de Andalucía, Spain (068/18-HUVR-I). M. L. G-M. is supported by the program FPU (Formación de Profesorado Universitario; FPU13/04545), Ministerio de Educación, Cultura y Deporte, Spain.

Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Figure S1 and Tables S1 to S3 are available as Supplementary data at *JAC* Online.

References

1 Bush K, Bradford PA. β-Lactams and β-lactamase inhibitors: an overview. *Cold Spring Harb Perspect Med* 2016; **6**: a025247.

2 Drawz SM, Bonomo RA. Three decades of β-lactamase inhibitors. *Clin Microbiol Rev* 2010; **23**: 160–201.

3 Solomkin JS, Mazuski JE, Bradley JS *et al*. Diagnosis and management of complicated intra-abdominal infection in adults and children: guidelines by

the Surgical Infection Society and the Infectious Diseases Society of America. *Clin Infect Dis* 2010; **50**: 133–64.

4 Vazquez-Guillamet MC, Vazquez R, Micek ST *et al*. Predicting resistance to piperacillin-tazobactam, cefepime and meropenem in septic patients with bloodstream infection due to gram-negative bacteria. *Clin Infect Dis* 2017; **65**: 1607–14.

5 Chaibi EB, Sirot D, Paul G *et al*. Inhibitor-resistant TEM β-lactamases: phenotypic, genetic and biochemical characteristics. *J Antimicrob Chemother* 1999; **43**: 447–58.

6 Zhou XY, Bordon F, Sirot D *et al*. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 β-lactamase conferring resistance to β-lactamase inhibitors. *Antimicrob Agents Chemother* 1994; **38**: 1085–9.

7 Reguera JA, Baquero F, Pérez-Diaz JC *et al*. Synergistic effect of dosage and bacterial inoculum in TEM-1 mediated antibiotic resistance. *Eur J Clin Microbiol Infect Dis* 1988; **7**: 778–9.

8 Schechter LM, Creely DP, Garner CD *et al*. Extensive gene amplification as a mechanism for piperacillin-tazobactam resistance in *Escherichia coli*. *mBio* 2018; **9**: e00583–18.

9 Jacoby GA. AmpC β-lactamases. *Clin Microbiol Rev* 2009; **22**: 161–82.

10 Pagès JM, Jame CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nat Rev Microbiol* 2008; **6**: 893–903.

11 Waltner-Toews RI, Paterson DL, Qureshi ZA *et al*. Clinical characteristics of bloodstream infections due to ampicillin-sulbactam-resistant, non-extended-spectrum-β-lactamase-producing *Escherichia coli* and the role of TEM-1 hyperproduction. *Antimicrob Agents Chemother* 2011; **55**: 495–501.

12 Cantón R, Morosini MI, Martin O *et al*. IRT and CMT β-lactamases and inhibitor resistance. *Clin Microbiol Infect* 2008; **14**: 53–62.

13 Murao N, Ohge H, Ikawa K *et al*. Pharmacokinetics of piperacillin-tazobactam in plasma, peritoneal fluid and peritoneum of surgery patients, and dosing considerations based on site-specific pharmacodynamic target attainment. *Int J Antimicrob Agents* 2017; **50**: 393–8.

14 Roberts JA, Roberts MS, Robertson TA *et al*. Piperacillin penetration into tissue of critically ill patients with sepsis—bolus versus continuous administration? *Crit Care Med* 2009; **37**: 926–33.

15 Gulberg E, Cao S, Berg OG *et al*. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* 2011; **7**: e1002158.

16 Baquero F. Low-level antibacterial resistance: a gateway to clinical resistance. *Drug Resist Updat* 2001; **4**: 93–105.

17 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing:* Twenty-Ninth Edition: M100-S29. CLSI, Wayne, PA, USA, 2019.

18 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. Approved Standard—Tenth Edition: M07-A10. CLSI, Wayne, PA, USA, 2015.

19 Wirth T, Falush D, Lan R *et al*. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* 2006; **60**: 1136–51.

20 Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016; **33**: 1870–4.

21 Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; **4**: 406–25.

22 Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980; **16**: 111–20.

23 Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985; **39**: 783–91.

24 Zhang H, Gao S, Lercher MJ *et al*. EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. *Nucleic Acids Res* 2012; **40**: W569–W572.

25 Carattoli A, Bertini A, Villa L *et al*. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**: 219–28.

26 Solomkin J, Hershberger E, Miller B *et al*. Ceftolozane/tazobactam plus metronidazole for complicated intra-abdominal infections in an era of multidrug resistance: results from a randomized, double-blind, phase 3 trial (ASPECT-cIAI). *Clin Infect Dis* 2015; **60**: 1462–71.

27 Sader HS, Castanheira M, Mendes RE *et al*. Antimicrobial activity of high-proportion cefepime-tazobactam (WCK 4282) against a large number of gram-negative isolates collected worldwide in 2014. *Antimicrob Agents Chemother* 2017; **64**: e02409–16.

28 Retamar P, López-Cerero L, Muniain MA *et al*. Impact of the MIC of piperacillin-tazobactam on the outcome of patients with bacteremia due to extended-spectrum-β-lactamase-producing *Escherichia coli*. *Antimicrob Agents Chemother* 2013; **57**: 3402–4.

29 Delgado-Valverde M, Torres E, Valiente-Mendez A *et al*. Impact of the MIC of piperacillin/tazobactam on the outcome for patients with bacteraemia due to Enterobacteriaceae: the Bacteraemia-MIC project. *J Antimicrob Chemother* 2016; **71**: 521–30.

30 Harris P, Tambyah PA, Lye DC *et al*. Effect of piperacillin-tazobactam vs meropenem on 30-day mortality for patients with *E. coli* or *Klebsiella pneumoniae* bloodstream infection and ceftriaxone resistance: a randomized clinical trial. *JAMA* 2018; **320**: 984–94.

31 European Committee on Antimicrobial Susceptibility Testing. *Breakpoint Tables for Interpretation of MICs and Zone Diameters*. EUCAST, 2019.

32 Sun S, Berg OG, Roth JR *et al*. Contribution of gene amplification to evolution of increased antibiotic resistance in *Salmonella typhimurium*. *Genetics* 2009; **182**: 1183–95.