**In vivo selection of a complex mutant TEM (CMT) from an inhibitor-resistant TEM (IRT) during ceftazidime therapy**

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**Methods:**
EC1 and EC2 isolates were compared for antibiotic MICs, plasmid content, genotyping, β-lactamase genes and their environment. Both isolates were conjugated with *E. coli* JW4111ΔampC and MICs determined for transconjugants. In addition, ceftazidime-resistant mutants were selected in vitro from EC1.

**Results:**
EC1 and EC2 showed identical patterns for genotyping and resistance plasmids. PCR sequencing of blaTEM in EC1 showed the mutations M69L and N276D corresponding to TEM-35, also called inhibitor-resistant TEM (IRT)-β-lactamase inhibitor combinations (EC1), the relapse *E. coli* isolate showed a similar phenotype but with resistance extended to ceftazidime (EC2). We investigated the molecular mechanisms of β-lactam resistance and sought if EC2 could have been selected in vivo from EC1.

**Conclusions:**
This first known report of *in vivo* selection of CMT from IRT, reproduced in vitro, shows how the evolution of β-lactamase enzymes is easily driven by antibiotic pressure, even during a short antibiotic therapy.

**Keywords:**
antibiotic pressure, β-lactamases, third-generation cephalosporins, neutropenic sepsis, evolution of antibiotic resistance

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**Introduction**

The penicillinase TEM-1 was described early after the clinical introduction of aminopenicillins in the 1960s and spread worldwide.1 It is still the predominant β-lactamase in Gram-negative bacteria.2 After the release of third-generation cephalosporins (3GCs) in the early 1980s, TEM variants carrying mutations conferring resistance to these molecules have been observed and are called extended-spectrum β-lactamases (ESBLs).3 Similarly, the intensive use of β-lactam/β-lactamase inhibitor combinations was followed by the selection of isolates producing inhibitor-resistant TEM (IRT) β-lactamases.4 Since the mid-1990s, TEM β-lactamases combining both ESBL- and IRT-type mutations have emerged, which are commonly referred to as ‘complex mutant TEMs’ (CMTs).5 To date, about 10 CMT alleles have been described (http://www.lahey.org/Studies/temtable.asp), mostly in Europe, but their prevalence is probably underestimated, given the difficulty to detect them with standard phenotypic methods.5,6

Many studies have shown that the selection of point mutations in TEM alleles is driven by antibiotic selection pressure in vitro,5,6 but few reports have described this phenomenon in vivo.5 Here, we investigated the *in vivo* selection of a CMT-type from an IRT-type β-lactamase that occurred in an *Escherichia coli* strain causing
bloodstream infection in a patient treated with ceftazidime for febrile neutropenia.

**Case report**

The patient was hospitalized for consolidation therapy of acute myeloid leukaemia in the haematology ward of Saint Louis University Hospital (Paris, France). The treatment and microbial examinations are detailed in Figure 1(a and b). After 5 days of cancer chemotherapy associated with levofloxacin prophylaxis (500 mg/day), the patient presented neutropenia and fever and received a presumptive treatment of piperacillin/tazobactam (4 g every 6 h) for 2 days. Since the patient remained febrile, antimicrobial therapy was switched to ceftazidime (2 g every 8 h), amikacin (1.5 g/day), vancomycin (2 g/day by continuous infusion) and tinidazole (1 g/day). Blood cultures were positive for an *E. coli* isolate exhibiting an IRT phenotype (EC1). After 7 days of treatment, the patient showed fever again and new blood cultures yielded an *E. coli* isolate with a phenotype similar to that of EC1, but with resistance extended to ceftazidime (EC2) (Figure 1b). Imipenem (1 g every 8 h) replaced ceftazidime for the following 6 days and the patient did not exhibit any signs of infection 21 days after admission.

**Materials and methods**

Antimicrobial susceptibility was determined using the disc diffusion method and MICs by Etest (bioMérieux, Marcy l’Etoile, France) according to French guidelines (http://www.sfm-microbiologie.org/). PCR amplification of genes encoding OXA-1-like *β*-lactamases, SHV and TEM (including its promoter region) was performed as previously described. Genomic

![Figure 1](https://i.imgur.com/3Q5Q5Q.png)

**Figure 1.** Selection of *E. coli* EC2 producing CMT-9 from *E. coli* EC1 producing IRT-4 under antimicrobial therapy. (a) Time course of chemotherapy. The line under the timeline represents the neutropenia period observed for the patient. The two arrows under the timeline indicate the day of blood culture samples yielding the *E. coli* isolates EC1 and EC2, respectively. (b) Antimicrobial susceptibility testing using the diffusion method on MH agar for EC1 and EC2. The increase in ceftazidime resistance is highlighted by circles. (c) Representation of genetic features conferring resistance to *β*-lactams for EC1 and EC2. D, day; CT, cancer chemotherapy; LVX, levofloxacin; AMX, amoxicillin; CAZ, ceftazidime; TIC, ticarcillin; PIP, piperacillin; ATM, aztreonam; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; TIM, ticarcillin/clavulanic acid; NAL, nalidixic acid; FEP, cefepime; TZP, piperacillin/tazobactam; IPM, imipenem; SXT, trimethoprim/sulfamethoxazole; CEF, cefalotin; FOX, cefoxitin; CXM, cefuroxime; TIN, tinidazole; AMK, amikacin; VAN, vancomycin.
DNA from EC1 and EC2 was compared by using the REP-PCR-based Diversilab® system (bioMérieux) according to the manufacturer’s recommendations. The E. coli phylogenetic group was determined by multiplex PCR.9

To investigate plasmid-mediated resistance to β-lactams, EC1 and EC2 were mated with E. coli K12ΔampCkan3 JW4111.11 Transconjugants were selected on Mueller–Hinton (MH) agar plates containing kanamycin (30 mg/L) and amoxicillin (100 mg/L). Plasmids were extracted from the transconjugants with the Qiagen Large-Construct Kit and were compared for EcoRI restriction profiles. The plasmids were assigned to incompatibility groups according to a PCR replicon-typing scheme.11

Ceftazidime-resistant mutants were selected by plating 10⁹ cfu onto MH agar containing from 0.25 to 8 mg/L ceftazidime (Arrow, Lyon, France). To investigate a possible hypermutable character of the strain, mutation frequencies were estimated by monitoring the strain’s capacity to generate mutants conferring resistance to rifampicin in at least six independent cultures.12

Results and discussion
Phenotypic and genotypic comparisons of EC1 and EC2 isolates
E. coli EC1 and EC2 exhibited identical antibiotic susceptibility phenotypes by the disc diffusion method except for ceftazidime resistance in EC2 (Figure 1b). The two isolates belonged to phylogenetic group A and displayed the same REP-PCR pattern (data not shown), suggesting that EC1 and EC2 are the same strain.

EC1 and EC2 were positive for TEM- and OXA-1-type β-lactamase genes. In EC1, the TEM gene harboured the mutations M69L and the deletion of the ampC gene in the recipient E. coli JW4111 and the absence of OXA-1 in the transconjugants, since the OXA-1 gene was not harboured on the plasmid carrying TEM. We observed increased MICs of aztreonam, cefepime and ceftazidime in the TEM-158-producing strains (Table 1), as described previously.6 Nevertheless, we did not find any change in the ceftazidime MIC. This discrepancy with the previous description of TEM-158 can be due to differences in the copy number of the plasmid harbouring TEM, particularly for an enzyme with a low catalytic efficiency against ceftazidime.4

The frequency of rifampicin-resistant mutants was similar for EC1 (4×10⁻¹⁰) and the non-mutator control strain (1×10⁻¹⁰), whereas it was 6×10⁻⁷ for the mutator control strain. This showed that EC1 and EC2 are not hypermutable isolates.

In vitro selection
Ceftazidime-resistant mutants were isolated with frequencies of 10⁻⁹ for E. coli EC1 and 10⁻¹⁰ for E. coli JW4111 (pEC1). Twelve mutants were selected from EC1 and five different single point mutations were observed in blaTEM: E166K (n = 6), R164H (n = 1), R164G (n = 1), R164S (n = 1) and D179G (n = 3) (Table 1). Four mutants were selected from E. coli JW4111 (pEC1) and two point mutations were observed in blaTEM: D163G (n = 3) and D179G (n = 1). No mutation was found in the promoter region.

TEM β-lactamase is composed of two domains delimiting an oxyanion pocket where the main catalytic residue (S70) is located.16 This pocket is particularly lined by the omega loop (R161 to D179) considered to play an important role in the catalytic activity. Interestingly, the six mutations observed in the in vitro mutants were located in this omega loop. The D163G mutation was previously described in one clinical isolate and is supposed to confer resistance to ceftazidime.14 Numerous mutants have previously been described at the R164 residue in both clinical isolates and laboratory-derived mutants (R164C, R164H, R164S, R164G, R164N and R164Y).14 This residue has two salt-bridge interactions in the omega loop with (E171 and D179) and it was suggested that R164 mutation could lead to modification in the omega loop topology, resulting in a higher accessibility of the active site for β-lactams with larger side chains, such as 3GCs.17 In the in vitro mutants, we observed three different mutations (R164H, R164G and R164S) at this position. The D179G mutation, observed in three mutants, is known to increase ceftazidime MICs and also to decrease resistance to penicillins.6,14 This mutation was only described in laboratory-derived mutants, and it has been suggested that fluctuating selective pressure of both penicillins and 3GCs could hinder the selection of such a mutation in clinical isolates.6 We noticed that many in vitro selected ceftazidime-resistant mutants of E. coli EC1 harboured the E166K mutation. To date, mutation at E166 has never been described either in clinical isolates or TEM-1 laboratory-derived mutants. This residue is highly conserved in class A β-lactamases and is described as a key residue in the activation of S70 during catalysis.16 E166K may have a beneficial effect regarding the increase in ceftazidime hydrolysis when it occurs in TEM-35, whereas this mutation could be deleterious in a TEM-1 genetic background, since it has never been described.

In conclusion, we report for the first time the in vivo selection by ceftazidime of a CMT-type β-lactamase from an IRT-producing E. coli strain. Our study shows how the evolution of β-lactamase enzymes is easily driven by antibiotic pressure. Antibiotic therapy, even for 7 days, can lead to the emergence of multiderug-resistant isolates. Given the results we obtained in the in vitro selection experiment and the diversity of alleles observed in clinical isolates, further investigations are required to understand the dynamics of the occurrence of mutations in CMT alleles.
Table 1. MICs of β-lactam antibiotics for E. coli EC1 and EC2 isolates, JW4111, E. coli JW4111(pEC1) and JW4111(pEC2) transconjugants, and mutants obtained by in vitro selection from E. coli EC1 and JW4111(pEC1)

<table>
<thead>
<tr>
<th>β-Lactam antibiotics</th>
<th>MIC (mg/L) for E. coli strains (plasmid)</th>
<th>MIC (mg/L) for in vitro mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC1</td>
<td>EC2</td>
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<tr>
<td>Amoxicillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Amoxicillin+CLA&lt;sub&gt;a&lt;/sub&gt;</td>
<td>64</td>
<td>48</td>
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<tr>
<td>Ticarcillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>Ticarcillin+CLA&lt;sub&gt;b&lt;/sub&gt;</td>
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<td>64</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Piperacillin+TZB&lt;sub&gt;c&lt;/sub&gt;</td>
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<td>24</td>
</tr>
<tr>
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<td>8</td>
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<tr>
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<td>0.19</td>
</tr>
<tr>
<td>Cefotaxime+CLA&lt;sub&gt;c&lt;/sub&gt;</td>
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<tr>
<td>Ceftazidime</td>
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</tr>
<tr>
<td>Imipenem</td>
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<td>0.38</td>
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</tbody>
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CLA, clavulanic acid; TZB, tazobactam.

<sup>a</sup>Amoxicillin:CLA ratio = 2:1.

<sup>b</sup>β-Lactamase inhibitor at 2 mg/L.

<sup>c</sup>β-Lactamase inhibitor at 4 mg/L.
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Transparency declarations
None to declare.

References