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# Biochemical characterization of CTX-M-166, a new CTX-M β-lactamase produced by a commensal *Escherichia coli* isolate

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Animals are potential reservoirs of antimicrobial-resistant bacteria.[1,2] Studies have shown that different bacterial species of animal origin carry oxyimino-β-lactam resistance determinants, including CTX-M-type β-lactamases.[3,4] Following the alarming emergence of these enzymes in veterinary isolates, the use of ceftiofur and cefquinome to treat animal infections has become compromised.

Ceftiofur is a third-generation cephalosporin, a critically important class of antibiotics to human health. Nevertheless, in cattle, ceftiofur is the most widely used antibiotic for the treatment of common diseases.[5] Consequently, several studies demonstrated that ceftiofur treatment resulted in increases in resistance to β-lactams and multidrug resistance.[6–8]

In this study, we biochemically characterized the new CTX-M-166 β-lactamase detected in a ceftiofur-resistant *Escherichia coli* recovered in May 2014 from a 6-week-old *Gallus gallus* broiler flock in an industrial poultry unit in the central region of Portugal.

*E. coli* INSLV13072 was non-susceptible to ampicillin (MIC $>64\ mg\,l^{-1}$) and oxyimino cephalosporins ($>32\ mg\,l^{-1}$ for ceftiofur, $8\ mg\,l^{-1}$ for cefotaxime, $4\ mg\,l^{-1}$ for cefepime and $1\ mg\,l^{-1}$ for ceftazidime) but susceptible to carbapenems and colistin. The MICs of ceftazidime and cefotaxime were reduced by clavulanic acid ($\leqslant 0.125$ and $\leqslant 0.06\ mg\,l^{-1}$, respectively).

The $bla_{CTX\text{-}M\text{-}166}$ gene differed from $bla_{CTX\text{-}M\text{-}1}$ by one-point mutation, which led to the amino acid substitution Ala120Val. To our knowledge, this is the first recorded observation of this mutation.

The kinetic parameters of the purified CTX-M enzymes (purity rate $\geqslant 95\%$) (data not shown) and the concentrations of inhibitors required to inhibit enzyme activity by 50% ($IC_{50}$s) are shown in Table 1. CTX-M-166 had strong affinity to penicillin ($K_m$, 14 to 8 μM), piperacillin ($K_m$, 6 to 3 μM), cefotaxime ($K_m$, 127 to 69 μM) and ceftiofur ($K_m$, 46 to 15 μM). However, catalytic efficiency against these antibiotics was lower for CTX-M-166 than for CTX-M-1. Notably, CTX-M-166 had the least decrease in catalytic efficiency against ceftiofur (30.2%) compared with that of CTX-M-1, whose value was set at 100% (Table 1). In contrast, the new enzyme had only 2.7% of catalytic efficiency for amoxicillin in comparison with the parental enzyme. No hydrolysis was detected against ceftazidime or imipenem. Inhibition studies, as measured by determination of the $IC_{50}$s, showed that CTX-M-1 and CTX-M-166 were both inhibited by clavulanic acid (0.031 and 0.030 μM, respectively) and tazobactam (0.007 and 0.005 μM, respectively).

The Ala120Val amino acid substitution, distant to the catalytic site, is located in an α-helix involved in the positioning of the loop harbouring the conserved element Ser-Asp-Asn, which has a major role in proton transfer during the catalytic pocket in class A enzymes.[9] The Ala120 residue is highly conserved in all CTX-M groups, except for CTX-M-25-group, were it is replaced by a glycine.[10] The alanine-to-valine substitution represents an alteration to a non-reactive amino acid that is often associated with binding/recognition of hydrophobic ligands such as lipids and thus involved in increasing the flexibility of protein.[11] The impact of this alteration could become more relevant with the accumulation of mutations affecting enzyme activity and resistance phenotype, which might arise due to antibiotic selection pressure.

## EXPERIMENTAL PROCEDURE

### Antibiotic susceptibility and molecular characterization

MICs of the clinical *E. coli* INSLV13072 isolate were determined by both agar dilution and microdilution methods to: ampicillin, cefotaxime, ceftazidime, cefotaxime/clavulanate, ceftazidime/clavulanate, cefepime, imipenem, meropenem, ertapenem, ciprofloxacin, gentamicin, chloramphenicol, trimethoprim, colistin and tigecycline. The interpretation of susceptibility results was performed

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**Table 1 Kinetic parameters of CTX-M-166 and CTX-M-1 β-lactamases**

| | *CTX-M-1*[a] | | | *CTX-M-166*[a] | | | |
|---|---|---|---|---|---|---|---|
| *Substrate* | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M$) | $k_{cat}/K_m$ ($\mu M^{-1}\ s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M$) | $k_{cat}/K_m$ ($\mu M^{-1}\ s^{-1}$) | *Efficiency*[b] *(%)* |
| Penicillin G | 87.7±1.8 | 14±0.5 | 6.453 | 8.2±0.2 | 8±0.03 | 0.996 | 15.4 |
| Amoxicillin | 31.4±0.6 | 10±0.3 | 3.097 | 3.1±0.1 | 37±0.6 | 0.084 | 2.7 |
| Ticarcillin | 7.3±0.4 | 21±0.1 | 0.354 | 0.5±0.002 | 21±0.03 | 0.024 | 6.8 |
| Piperacillin | 32.7±1.2 | 6±0.5 | 5.512 | 2.4±0.01 | 3±0.2 | 0.685 | 12.4 |
| Cephalothin | 598.4±95.1 | 57±3.0 | 10.683 | 81.1±1.4 | 85±2.3 | 0.954 | 8.9 |
| Cefuroxime | 77.6±2.7 | 17±0.5 | 4.543 | 8.0±0.7 | 36±0.5 | 0.225 | 5.0 |
| Cefotaxime | 129.9±0.6 | 127±1.9 | 1.021 | 8.3±0.3 | 69±1.8 | 0.124 | 12.2 |
| Ceftazidime | <0.01 | 170±2.5 | 0.000 | <0.01 | ND | ND | ND |
| Ceftiofur | 5.5±0.4 | 46±1.1 | 0.120 | 0.6±0.004 | 15±0.3 | 0.036 | 30.2 |
| Cefepime | 2.3±0.6 | 26±0.6 | 0.089 | 1.6±0.2 | 102±3.0 | 0.015 | 17.3 |
| Aztreonam | 2.1±0.006 | 29±0.7 | 0.073 | 0.2±0.005 | 41±0.1 | 0.005 | 7.0 |
| Imipenem | <0.01 | 107±8.7 | <0.001 | <0.01 | ND | ND | ND |

Abbreviation: ND, not determinable because the hydrolysis rates were too low.
[a]Values are means±s.d.
[b]Efficiency of CTX-M-166 compared with that of CTX-M-1, which was set at 100%.

according to the epidemiological cut-off values of the European Committee on Antimicrobial Susceptibility Testing.[12]

β-Lactamase-encoding genes were identified by PCR and confirmed by sequencing, as previously described.[13]

### Cloning experiments

For comparison, CTX-M-166 (from INSLV13072) and CTX-M-1 (from INSLV21400) were expressed in an isogenic background. The Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA) was used to clone CTX-M-type PCR fragments into plasmid *pCR-Blunt*. Recombinant pCR-CTX-M-type plasmids were transformed by heat-shock transformation of chemically competent *E. coli* One Shot TOP10 cells. *E. coli* transformants were selected on MacConkey agar supplemented with 30 mg $l^{-1}$ of kanamycin and 2 mg $l^{-1}$ of cefotaxime. The presence and orientation of the inserted genes was confirmed by PCR as above described.

### Purification of β-lactamases

CTX-M-166 and CTX-M-1 β-lactamases were produced overnight, at 37 °C, from *E. coli* One Shot TOP10 in LB broth, supplemented with 2 mg $l^{-1}$ cefotaxime. Both enzymes were extracted by ultrasonic treatment and the clarified supernatant was purified by ion exchange and gel filtration chromatography as described elsewhere.[14]

### Determination of β-lactamase kinetic constants

$K_m$ and catalytic activity ($k_{cat}$) of CTX-M-1 and CTX-M-166, and the concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% ($IC_{50}$) were determined by a computerized microacidimetric method, as described elsewhere.[14] Specific activity and $IC_{50}$ were monitored with penicillin G (200 μM) as the reporter substrate.

### Nucleotide sequence accession number

The $bla_{CTX\text{-}M\text{-}166}$ nucleotide sequence was submitted to DDBJ/EMBL/GenBank with accession number NG_048951.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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