Purification and characterization of a new β-lactamase OXA-205 from *Pseudomonas aeruginosa*

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**Abstract**

**Background:** We have identified a novel class 1 integron (1503 bp), named In671 in a clinical *Pseudomonas aeruginosa* isolate. Integron sequence analysis revealed two gene cassettes, one coding for a new OXA-type β-lactamase designated as OXA-205 and the other coding for the *aadB* gene that is responsible for aminoglycoside resistance. The 266 amino acid sequence of OXA-205 revealed that this β-lactamase belongs to the Ambler class D showing highest sequence homology to the OXA-2 sub-lineage. Our objective was to purify and characterize β-lactamase OXA-205.

**Methods:** *Escherichia coli* cells were transformed with a plasmid containing cloned *bla*OXA-205 gene from *P. aeruginosa*. Purification of overproduced OXA-205 consisted of a single ion-exchange chromatography step. SDS-PAGE and isoelectric focusing were performed to determine the molecular mass and pI, respectively. Size-exclusion chromatography was undertaken to determine the OXA-205 oligomerization state. Substrate hydrolysis reactions were employed to assess enzyme kinetic parameters.

**Results:** Purification of OXA-205 yielded the enzyme with >95% purity (as verified by SDS-PAGE). Approximate yield of the protein was estimated to be 20 mg per liter of culture. OXA-205 had a pI at 8.1, molecular mass of 26 kDa and a monomeric native structure. Kinetic analysis revealed that OXA-205 hydrolyzed narrow spectrum substrates, including ampicillin, carbenicillin, oxacillin, penicillin G, cefazolin and cefuroxime. Additionally, we observed a substrate inhibition profile towards carbenicillin and oxacillin, but not with ampicillin or penicillin G. Our results also show that OXA-205 conferred unusually high (among class D β-lactamases) resistance towards inhibition by NaCl.

**Conclusions:** OXA-205 can be considered a narrow spectrum monomeric β-lactamase that demonstrates unusually high resistance profile towards inhibition by NaCl.

**Keywords:** *Pseudomonas*, Integron, β-Lactamase, OXA

**Background**

β-Lactamases are enzymes that are capable of degrading β-lactam antibiotics via hydrolysis of the amide bond in their β-lactam ring [1]. Today, these enzymes are of great concern for being directly or indirectly responsible for most of the multi-drug resistance observed in gram-negative bacteria in both hospital and community isolates [1, 2].

OXA-type β-lactamases are distinguished by their ability to hydrolyze cloxacillin or oxacillin at a 50% higher rate than that of penicillin G and are inhibited by NaCl [3]. According to the functional classification, OXA-type enzymes are classified in group 2d which is further subdivided into 2 subgroups: 2de (enzymes that are unable to hydrolyze carbapenems) and 2df (enzymes with carbapenem-hydrolyzing activities) [3]. Structural classification of β-lactamases according to the Ambler assigns OXA enzymes to the D class, which includes a great number
of diverse enzymes [4, 5]. However, it seems that all of them have a conserved carboxylated lysine residue in the active site [6]. Due to a great number of OXA variants and in some cases weak sequence similarity among these enzymes, data concerning their biochemical characteristics are needed to fully understand them.

In this paper, we report biochemical characteristics, substrate hydrolysis and inhibition profiles of OXA-type β-lactamase, designated as OXA-205 from a Pseudomonas aeruginosa imipenem resistant clinical isolate from Lithuania.

Methods

Bacterial isolates

Pseudomonas aeruginosa P16 was recovered from an eye infection of a hospitalized patient in Lithuanian University of Health Sciences Kauno Klinikos Hospital. Species identification of P. aeruginosa P16 isolate was performed with the automated microbiology system Phoenix™(BD) and confirmed by PCR using primers PA-SS-F (5′-GGGGGATCTTCCGACCTCA-3′) and PA-SS-R (5′-CTCCTTAGTGACCACCCCG-3′) [7] and reference strain P. aeruginosa ATCC 27853. For plasmid transfer, OXA-205 cloning and expression, E. coli DH5α harboring an empty vector and vector expressing OXA-205 was determined in Mueller–Hinton broth (Liofilchem), using a final inoculum of 5 × 10⁵ CFU/ml. Antibiotics were obtained from Trek. Testing was performed by the broth microdilution method, as described in the Clinical and Laboratory Standards Institute guidelines [8].

Antimicrobial susceptibility testing

Antibiotic susceptibility for P. aeruginosa P16, E. coli DH5α harboring an empty vector and vector expressing OXA-205 was determined in Mueller-Hinton broth (Liofilchem), using a final inoculum of 5 × 10⁵ CFU/ml. Antibiotics were obtained from Trek. Testing was performed by the broth microdilution method, as described in the Clinical and Laboratory Standards Institute guidelines [8].

Genetic manipulations

The molecular biology tools were purchased from Thermo Fisher Scientific Baltics and used as recommended by the manufacturer. Primers were purchased from Metabion. To clone 801 bp blaOXA-205 gene, the DNA was amplified using primers 205clF6 (5′-GGGGGATCTTCCGACCTCA-3′) and 205clR3 (5′-CTCCTTAGTGACCACCCCG-3′). The former contained fragment of the vector sequence upstream from the specific part to the beginning of blaOXA-205 so that the insert would be cloned in frame. The latter was specific to the 3′ conserved segment of class 1 integrons [9]. For cloning purposes, EcoRI and BamHI restriction sites were introduced into the primers (underlined), respectively. The obtained fragment was digested with EcoRI and BamHI and inserted between the EcoRI and BamHI sites of pUHE 25-(cat) plasmid [10], resulting in plasmid pUHEcat-OXA-205 which was introduced into E. coli DH5α. For the purification of OXA-205, the gene without signal peptide coding region was amplified with primers 205clF5 (5′-GGGGGATCTTCCGACCTCA-3′) and 205clR2 (5′-CTCCTAGTGACCACCCCG-3′). The latter was identical to the primer 205clR3, except it had EcoRI restriction site. The amplified fragment was digested with XbaI and EcoRI and inserted between the XbaI and EcoRI sites of pET-28b, resulting in plasmid pET-OXA-205 which was introduced into E. coli BL21 (DE3). P. aeruginosa P16 genomic DNA was used as the template. pUHEcat-OXA-205 and pET-OXA-205 transformants were selected on chloramphenicol (33 µg/ml) or kanamycin (60 µg/ml) containing LB agar plates, respectively. The accuracy of the cloned DNA inserts was verified by confirmatory sequencing (Macrogen).

Production and purification of OXA-205

E. coli BL21(DE3) cells containing pET-OXA-205 plasmid were grown overnight at 37 °C in LB medium containing kanamycin (60 µg/ml). Culture was diluted 1:100 with fresh LB medium, containing kanamycin (60 µg/ml), grown at 28 °C to an A₆₀₀ = 0.6 and induced with IPTG (Thermo Fisher Scientific Baltics) (final concentration of 1 mM) for 18 h. After induction the cells were harvested by centrifugation at 6000 × g for 15 min at 4 °C, resuspended in 50 mM Tris-H₂SO₄ (pH 7.4) buffer and disrupted by sonication. Debris was eliminated by centrifugation at 12000 × g for 30 min at 4 °C. The extract was filtered through a 0.22 µm membrane filter and then loaded at 2 ml/min on anion-exchange column HiLoad 16/10 Q Sepharose HP (GE Healthcare), previously equilibrated with 50 mM Tris-H₂SO₄ (pH 7.4) which was flowed through a column. HiTrap Desalting column (GE Healthcare) was used for buffer exchange using a 100 mM sodium phosphate buffer (pH 7.0) as an eluent. The fractions of the purified enzyme were stored at –80 °C. All chromatography steps were performed using ÄKTA FPLC system (GE Healthcare).

Protein analysis

The purity of each chromatography step was determined by SDS-PAGE. Protein concentration was assayed by Bradford method using Roti–Quant kit (Roth) with bovine serum albumin (BSA) as a standard. The oligomerization state of OXA-205 was determined as described previously [11], except the Superose 12 10/300 GL column, flow rate of 0.5 ml/min and 60 µg
of the purified protein were used. The column was calibrated with a mixture containing BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) (GE Healthcare) using the same flow rate conditions as mentioned above. Isoelectric focusing (IEF) was performed with IPG gel strips (pH 3–10) with Multiphore II device (GE Healthcare) according to the manufacturer’s protocol. After focusing, β-lactamase bands were detected by overlaying the strips with 0.5 mM nitrocefin. The pl values were determined and compared to those from molecular IEF standards (Bio-Rad).

**Determination of kinetic parameters**

All kinetic measurements were performed at room temperature in 100 mM sodium phosphate buffer (pH 7.0) supplemented with 50 mM NaHCO₃ and 0.2 mg/ml BSA in a total volume of 500 μl, unless specified otherwise. The variations in absorbance were measured using Genesys 10S UV–vis spectrophotometer (Thermo Scientific). The wavelengths and changes in extinction coefficients used in the spectrophotometric assays were ε₄₈₆ = 20,500 M⁻¹ cm⁻¹ with nitrocefin (Calbiochem). For carbapenems and other substrates, parameters were as in [12, 11, 13], respectively. The values of kinetic parameters (Kₘ and kₗcat(i)) were determined by measuring the initial rate (v) of hydrolysis of various different concentrations of β-lactams and fitting the data with non-linear regression to the Michaelis–Menten equation. Kₘ values lower than 20 μM were determined as Kᵢ, in a competition experiment using 0.1 mM nitrocefin as the reporter substrate [14].

For substrates that at high concentrations demonstrated substrate inhibition, data was fitted using kinetic model (Eq. 1) described by LiCata and Allewell [15].

\[ v = \frac{V_{\text{max}} + V_{i}(|S|^x/K_i^x)}{1 + (K_{\text{mi}}/|S|^n_{H}) + (|S|^x/K_i^x)} \]  

(1)

where \( V_{\text{max}} \) and \( V_{i} \) correspond to the catalytic constants \( k_{\text{cat}} \) and \( k_{\text{cat}(i)} \), respectively. \( K_i \) is the Michaelis constant, \( K_m \) is the inhibition constant, \(|S|\) is the substrate concentration, \( v \) is the initial velocity. Exponents \( n_H \) and \( x \) are the Hill’s coefficients allowing for cooperativity of substrate hydrolysis and inhibition modes, respectively. In the case of complete inhibition (\( V_i = 0 \), the Eq. 1 reduces to the Eq. 2.

\[ v = \frac{V_{\text{max}}}{1 + (K_{\text{mi}}/|S|^n_{H}) + (|S|^x/K_i^x)} \]  

(2)

The inhibitory concentration of NaCl that reduced the hydrolysis rate of substrate by 50 % (IC₅₀) was assayed using 200 μM of ampicillin as the reporter substrate, under conditions in which OXA-205 was preincubated with various concentrations of salt for 3 min before the addition of the substrate.

All data fitting in this work was performed using the open source QtiPlot software [16]. Specific activity of OXA-205 was defined as the amount (unit of enzyme) that hydrolyzed 1 μmol of nitrocefin/minute/milligram of protein.

**Bioinformatic analysis**

NCBI BLASTP was used to determine sequence similarities. Multiple amino acid sequence alignments were generated with the Cobalt alignment tool [17]. Sequence alignments and secondary structure information using OXA-46 structure as the reference (PDB:3IF6) were rendered using ESPript online server [18]. Theoretical calculation of protein molecular mass and pl was carried out using the software available at the ExPASy proteomic server (http://www.expasy.org/). Theoretical prediction of the leader peptide size was carried out at with the SignalP 3.0 [19]. OXA-205 model based on OXA-46 structure (PDB:3IF6) was created using SWISS-MODEL online tool [20, 21].

**Results and discussion**

Characterization of integron-encoded OXA-type β-lactamase from the *P. aeruginosa* P16 isolate

During the search for class 1 and 2 integrons of a total of 111 MDR *P. aeruginosa* isolates, obtained from various clinical specimens collected in six regional hospitals in Lithuania during the period of 2005–2007, a novel class 1 integron (1503 bp), named *In671* was identified in multidrug-resistant *P. aeruginosa* isolate P16, recovered from an eye infection of a patient of Lithuanian University of Health Sciences Kauno Klinikos Hospital [22].

Antimicrobial susceptibility testing showed that the isolate was resistant to almost all antibiotics that were tested (Table 1).

Analysis of the novel 1503 bp gene cassette array of the integron revealed two gene cassettes, one coding *aadB* gene that is responsible for aminoglycoside resistance and the other coding for a new OXA-type β-lactamase, which was designated as OXA-205 (Fig. 1). Within the deduced 266 amino acid sequence all the conserved motifs typical for class D enzymes were found, namely, 70STFK73 [23], 118SVX120 [23], 144YGN146 [23], 164 W [24], 216KTVG218 [23], 68P [4], 131G [4], 171L [4], 188L [4], 232 W [4], 235G [4], 247F [4] (residues are numbered according to class D β-lactamase (DBL) numbering scheme [25]) (Fig. 2). Fully matured protein (after the removal of the predicted 21 N-terminal amino acid signal peptide) is predicted to have a molecular weight (Mw) of 28,455 Da.
Comparison of amino acid sequence of OXA-205 with other class D β-lactamases revealed that it belonged to the OXA-2 sub-lineage [11] showing the highest sequence similarity to two OXA-type enzymes: OXA-118 and OXA-119, which were also found in integrons. The first one was found in uncultured bacteria from a wastewater plant and the other in a clinical *Burkholderia cepacia* isolate (97 and 96 % sequence identity, respectively) [11, 22]. OXA-205 exhibited 92 % sequence identity with the well-characterized narrow spectrum class D β-lactamase OXA-46 [11]. The remaining OXA-2 sub-lineage enzymes (OXA-2, OXA-3, OXA-20, OXA-53) showed 80 % sequence similarities to OXA-205.

Although *P. aeruginosa* isolate P16 was resistant to imipenem, OXA-205 sequence shared a weak similarity with carbapenem-hydrolyzing class D β-lactamases (<40 % sequence identity with OXA-23 group, OXA-48 and OXA-58 group enzymes).

### Antimicrobial susceptibility testing

*E. coli* DH5α strain was transformed with the plasmid pUHEcat-OXA-205 and compared against *E. coli* containing empty the vector pUHE 25-2(cat) in terms of susceptibility to various β-lactams (Table 1).

The former strain demonstrated increased resistance to penicillins (ampicillin, penicillin G, piperacillin and carbenicillin), cefazolin and cefotetan. Interestingly, the OXA-205 expressing strain showed no significant increase in the resistance against oxacillin, even though this β-lactam was efficiently hydrolyzed by the purified enzyme (see below). Susceptibility of *E. coli* DH5α (pUHEcat-OXA-205) to aztreonam, cephalosporins (cefuroxime, cefoxitin, ceftazidime, ceftriaxone, cefotaxime, cefepime and cefpodoxime) and carbapenems (imipenem and meropenem) were unaffected. The obtained MIC results data allow for the conclusion that OXA-205, when expressed in *E. coli* DH5α, confers resistance against narrow spectrum β-lactams.

### Expression and biochemical properties of OXA-205

After over-expression and purification, we obtained approximately 20 mg of OXA-205 per liter of culture,

![Fig. 1](image-url)
with the specific activity of ~60 U/mg (measured against nitrocefin). SDS-PAGE revealed that the purified enzyme was >95% homogeneous (Additional file 1: Figure S1). The produced β-lactamase had a pI of 8.1 and molecular weight of ~26 kDa (as judged from SDS-PAGE analysis using Fiji open source software [26]).

In order to determine the oligomerization state of OXA-205, size exclusion chromatography was undertaken. The approximate molecular mass was estimated to be 25 kDa, suggesting a monomeric native form of OXA-205. This result was quite unexpected, because dimerization had been observed among some of the D class β-lactamases, including OXA-10 [23]; OXA-13 [27];
OXA-29 [28] and, most importantly, among the three representatives of OXA-2 sub-lineage [11], namely OXA-46 [11, 29]; OXA-3 [30] and OXA-2 itself [30].

It is known that at least two enzymes, OXA-29 and OXA-46, form dimers independently of divalent cations [11, 28, 29]. The solved quaternary structure of the OXA-46 revealed several amino acid residues important for the cation-independent inter-subunit interactions. These include (according to DBL numbering): β7 strand; Glu89, Asn186, Arg197, Asp201, Arg214 [29], and Glu238 [23]. Interestingly, after a close inspection of OXA-205 sequence we noticed Asp201 substitution by Tyr201. It has been proposed that Asp201 and Arg214 might form a salt bridge which could replace the cation site leading to a dimeric cation-independent structure [23, 31]. We speculate that this could be one of the main reasons why OXA-205 did not dimerize. Indeed, the model of OXA-205 based on the OXA-46 protein quaternary structure showed lack of interactions between the Tyr201 and the Arg214 of OXA-205 (Fig. 3). However, site-directed mutagenesis is needed to fully support this hypothesis.

**Kinetic parameters of OXA-205**

As expected, OXA-205 efficiently hydrolyzed penicillins and 1st generation of cephalosporins, including ampicillin, carbenicillin, oxacillin, penicillin G and cefazolin (Table 2). Penicillin G was the best substrate that showed the highest catalytic efficiency (\( k_{cat}/K_m \)) with the value of \(1.68 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \). Efficiency values for other substrates were similar to those reported for OXA-46 [11]. Interestingly, at high concentrations of carbenicillin and oxacillin (starting at approximately 2 mM and 1.6 mM for carbenicillin and oxacillin, respectively) we observed substrate inhibition kinetics. In order to measure values of constants accurately, we fitted data to the Eq. 2 because the inhibition profile demonstrated complete inhibition (Additional file 2: Figure S2). As already mentioned, the calculated efficiency values were similar to the already reported ones for OXA-46, however OXA-205 efficiency towards oxacillin, compared to OXA-2, was more than one order of magnitude lower [12]. Also, we obtained Hills coefficients (\( n = 2.1 \pm 0.2 \) and \( x = 4.9 \pm 0.7 \) for carbenicillin and \( n = 1.1 \pm 0.2 \) and \( x = 3.6 \pm 0.7 \) for oxacillin) which indicated cooperative effects in both the hydrolysis and inhibition by carbenicillin, while only inhibition by oxacillin demonstrated cooperative manner. To the best of our knowledge, substrate inhibition is not unique among β-lactamases, however it seems that it is the first example among non-metallo-β-lactamases [32, 33]. Currently, the exact mechanism of substrate inhibition for β-lactamases is unknown but it was suggested that it might be linked to the bulky aromatic ring substituents in some of the antibiotics and the peculiarities of the active sites of β-lactamases [33].

Also, we observed that OXA-205 influenced hydrolysis of the 2nd generation cephalosporin cefuroxime. However, a very low turnover number (\( k_{cat} \)) and rather high affinity (\( K_m \)) resulted in poor hydrolysis efficiency (\(6.69 \times 10^2 \text{ M}^{-1} \text{ s}^{-1} \)). Lasty, we did not observe any of OXA-205 influenced hydrolysis of ceftazidime (the 3rd generation cephalosporin), suggesting that OXA-205 could be considered a narrow spectrum β-lactamase towards cephalosporins.

**Fig. 3** Molecular model of OXA-205 based on OXA-46 quaternary structure. D201, R214 and E238 residues forms H-bonds between different subunits in OXA-46 while D201Y substitution in OXA-205 model fails to make H-bonds with R214 residue in different subunit. Substitution also introduces clashes (data not shown). Blue and purple colors of cartoon ribbons denotes different subunits. Aforementioned residues (along with E89, N186, R197 and β7 strand strand in C-terminal end) are thought to be responsible for the formation of dimeric structure in divalent metal ions independent way [23, 29]. GMQE and QMEAN values of the model are 0.71 and −2.13, respectively. Model was created using SWISS-MODEL tool [20, 21] (OXA-46 assembly No. 2 from Protein data bank in Europe (PDB:3IF6) was used as a template) and visualized with the open source software PyMol (v.1.7.1.2)
It has been shown that OXA-2 displays similar catalytic efficiencies towards carbapenems as some of the recognized class D carbapenemases [13]. Strikingly, our data supported this observation—OXA-205 hydrolyzed imipenem and meropenem as efficient as ampicillin and oxacillin, respectively. However, OXA-205 displayed very low turnover numbers, which were approximately one order of magnitude lower when compared to OXA-2. Nevertheless, the enzyme demonstrated a high affinity towards carbapenems. This data support the suggestion that other class D enzymes which currently are regarded as non-carbapenemases may, in fact be carbapenem-hydrolysing class D β-lactamases [12].

Inhibition by sodium chloride (NaCl) is considered one of the useful characteristics for in vitro identification of class D β-lactamases: most enzymes are fully inactivated with ≥100 mM NaCl [34]. In order to determine the pattern of susceptibility to NaCl, we performed inhibition experiments which concluded that OXA-205 was inhibited by salt at extremely high concentration—IC$_{50}$ = 1361 ± 118 mM. Interestingly, OXA-46 also demonstrated reduced susceptibility to NaCl (IC$_{50}$ at ∼230 mM) [11]. These results suggest that low resistance to NaCl may be widespread among the enzymes belonging to the OXA-2 sub-lineage.

### Table 2 Kinetic parameters determined with the purified OXA-205 β-lactamase

| Substrate | $K_m$ (μM) | $V_{max}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $K_i$ (μM) | n | x |
|-----------|------------|---------------------|-------------------------------|-----------|---|---|
| Nitrocefin | 6.9 ± 0.4  | 62.4 ± 3.3          | 9.09 × 10$^5$                 |           |   |   |
| Ampicillin | 18.1 ± 0.6 | 36.4 ± 0.1          | 2.01 × 10$^5$                 |           |   |   |
| Carbenicillin$^a$ | 360 ± 21 | 45.2 ± 1.9          | 1.26 × 10$^5$                 | 2887 ± 56 | 2.1 ± 0.2 | 4.9 ± 0.7 |
| Cefazolin | 4.7 ± 0.3$^b$ | 3.9 ± 0.1         | 8.25 × 10$^4$                 |           |   |   |
| Cefuroxime | NH     | NH                 | –                             |           |   |   |
| Imipenem | 0.02 ± 0.002$^a$ | 0.03 ± 0.001   | 1.39 × 10$^6$                 |           |   |   |
| Meropenem | 0.02 ± 0.002$^a$ | 0.008 ± 0.0005 | 3.08 × 10$^5$                 |           |   |   |
| Oxacillin$^b$ | 1369 ± 87 | 376 ± 25           | 2.75 × 10$^5$                 | 1869 ± 75 | 1.1 ± 0.1 | 3.6 ± 0.2 |
| Penicillin G | 2.3 ± 0.06$^a$ | 39.1 ± 2      | 1.68 × 10$^7$                 |           |   |   |

Reactions were carried out using the concentration of OXA-205 ranging from 2.5 to 814 nM. All values are the means of at least three different measurements.

NH: no hydrolysis observed at a substrate concentration up to 1 mM

$^a$ Measured as an inhibition constant ($K_i$) in competition experiment against nitrocefin [13]

$^b$ Obtained data was fitted to the Eq. 2 because substrate inhibition kinetics were observed

Additional files

**Additional file 1: Figure S1.** SDS-polyacrylamide gel of OXA-205 purification. Lanes: 1 – cell fraction of E. coli BL21 (DE3) (pET-OXA-205) before induction with 1 mM IPTG, 2 – Thermo Scientific PageRuler Prestained Protein ladder (SM0671), 3 – cell fraction of E. coli BL21 (DE3) (pET-OXA-205) after induction with 1 mM IPTG, 4 – purified cell fraction after anion-exchange, using 10 μg of total protein, 5 – purified cell fraction after anion-exchange, using 10 μg of total protein, 6 – purified cell fraction after anion-exchange, using 30 μg of total protein, 7 – purified cell fraction after anion-exchange, using 40 μg of total protein. Relative migrations of Mw marker proteins are indicated on the left.

**Additional file 2: Figure S2.** Inhibition of OXA-205 by carbenicillin and oxacillin. The data (filled square) is plotted as initial velocity (v) versus substrate concentration for oxacillin (upper plot) and carbenicillin (lower plot). Error bars represent standard deviation from three different measurements. The solid line in each plot is a fit to the data using Eq. 2 using kinetic parameters form Table 2.

**Abbreviations**

Mw: molecular weight; MDR: multi-drug resistant; bp: base pairs; LB: Luria-Bertani medium; DBL: Class D β-lactamase; IC$_{50}$: the inhibitor concentration that reduced the hydrolysis rate of substrate by 50 %; MIC: minimal inhibitory concentration.

**Authors’ contributions**

RK, DL, ES designed the study and wrote the manuscript, RK, JP, AM, DL, JA, RP, PK performed experimental work and analyzed data. All authors read and approved the final manuscript.
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Competing interests
The authors declare that they have no competing interests.

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