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Conference report

ampD homologs in biotypes of *Yersinia enterocolitica*: Implications in regulation of chromosomal AmpC-type cephalosporinases

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ABSTRACT

Inducible 'AmpC-type' chromosomal cephalosporinases have been reported to be differentially expressed in different biotypes of *Yersinia entercolocolitica*. AmpD amidases are key regulators of the expression of *ampC* genes in *Y. entercolocolitica* as their inactivation results in hyper production of AmpC. To understand the differences in regulation of *ampC* expression in different biotypes of *Y. enterocolitica,* characteristics of *ampD* homologs were studied in strains of *Y. enterocolitica* belonging to five biotypes namely 1A, 1B, 2, 3 and 4. Our results indicated that the mechanisms which regulate expression of *ampC* might differ in different biotypes. While a three-step regulation mechanism seemed to be functional in biotypes 2, 3 and 4, a two-step regulation mechanism using other AmiD like proteins might be functional in biotypes 1A and 1B. The existence of *ampD* homolog(s)-mediated expression of *ampC* in other members of the family *Enterobacteriaceae may provide further credence to our findings.*

1. Introduction

Yersinia enterocolitica, a Gram negative enteropathogen, can be divided into six biotypes (1A, 1B, 2–5) and more than fifty serotypes. It produces two chromosomal β-lactamases named as BlaA and BlaB. BlaA is a penicillinase (functional group 2 and molecular class A β-lactamase), while BlaB is an AmpC-type cephalosporinase (functional group 1 and molecular class C β-lactamase). In most bacterial species, the AmpC β-lactamases are constitutively expressed at low levels (repressed state). Of the genes that regulate the expression of *ampC*, *ampR* encodes a transcriptional regulator from the LysR family, *ampD* encodes a cytoplasmic *N*-acetyl-anhydromuarmoyl-L-alanine amidase and *ampG* encodes a transmembrane permease. The AmpD amidases play a dual role in bacteria, recycling the products of peptidoglycan catabolism and preventing the induction of *ampC*. Exposure of bacteria to antibiotics like imipenem and cefoxitin results in generation and accumulation of large quantities of muropeptides in the cytoplasm (beyond the recycling capability of AmpD) which bind to *ampR,* converting it into an activator of *ampC* (derepressed state) (Normark, 1995; Dietz et al., 1997). Mutations in *ampD*, have reportedly been the most frequent mechanism that underlie hyperproduction of AmpC and β-lactam resistance in the *Enterobacteriaceae* family (Hanson and Sanders, 1999; Kaneko et al., 2005).

a*mpD* plays an important role in regulating the expression of chromosomal cephalosporinase - *ampC,* overexpression of which ultimately leads to high-level resistance to cephalosporins. Thus, it is important to study *ampD* and/or *ampD* homologs in various bacterial species expressing chromosomal cehalosporinases. The discovery of three *ampD* homologs in *Pseudomonas aeruginosa* (Juan et al., 2006) and AmpD-like lipoproteins, AmiD, in *Escherichia coli* (Uehara and Park, 2007) suggested a complex mechanism which regulates the expression of *ampC*. Though, a member in the family *Enterobacteriaceae*, regulation of *ampC* is not well understood in *Y. enterocolitica.* A recent study reported three homologs of *ampD* and a three-step regulation mechanism of expression of *ampC* in a strain of *Y. enterocolitica* subsp. *palearctica* 105.5 R(r) of bioserotype 3/O: 9 (Liu et al., 2016). Earlier studies reported that expression and inducibility of AmpC-like β-lactamases in *Y. enterocolitica* differed significantly among different biotypes (Stock et al., 1999, 2000; Bonke et al., 2011). Induction of expression of *ampC* by imipenem varied within strains of biotype 2 and 5 (Stock et al., 1999), while strains of biotype 1A were reportedly more inducible than strains of biotype 1B (Stock et al., 2000). The objective of this study was to investigate the distribution and characteristics of *ampD* homologs in different biotypes (1A, 1B, 2, 3 and 4) to understand the differential regulation of *ampC* expression in different biotypes of *Y. enterocolitica*.

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2. Materials & methods

2.1. Bacterial strains

Y. enterocolitica strains of biotypes 1A, 1B, 2 and 4 were maintained on trypticase soy agar at 4 °C. Strain 8081 of bio-serotype 1B/O: 8, was a clinical isolate of American origin. Strains IP27938 of bio-serotype 1A/O: 6, 30; W22703 of bio-serotype 2/O: 9 and IP134 of bio-serotype 4/O: 3 were clinical isolates of European origin.

2.2. Isolation of genomic DNA, PCR amplification and sequencing of ampD

Genomic DNA was isolated using DNeasy Tissue kit (Qiagen, Hilden, Germany). Primers ampDF 5′GCCAGAAGGTGAAGCTCCTT 3′ and ampDR 5′CTCTGGTTAATACTGCATGA 3′ were designed to amplify the complete coding sequence (CCDS) of *ampD* using NCBI-PrimerBlast. The components of PCR reaction mixture and reaction conditions have been described previously (Singhal et al., 2014), except that the annealing temperature used in the present study was 56 °C. PCR amplicons were sequenced at a commercial facility (Invitrogen BioServices India Pvt. Ltd., Bangalore, India) and confirmed by homology search using NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Homology search using whole genome tBLAST

CCDS of *ampD* were translated using expasy (http://web.expasy.org/translate/). The information about AmpD homologs of the biotype 3 was retrieved from a published study (Liu et al., 2016). For identifying AmpD homologs present in the biotypes 1A, IB, 2, 3 and 4, AmpD sequences were searched using tBLAST, against complete genomes of several strains of *Y. enterocolitica* (of each biotype) available at NCBI. The detailed information about these strains has been summarized in Supplementary Table 1.

2.4. Phylogenetic analysis, InterProScan and multiple sequence alignment (MSA)

The phylogeny of AmpD was inferred using Neighbor-Joining method with Mega 7.0.26 program (http://www.megasoftware.net/). Bootstrap values were estimated from 1000 replicates. Functional classification of the AmpD homologs into protein families was done using InterProScan (Jones et al., 2014). MSA of the ampD homologs identified in biotypes 1A, 1B, 2, 3 and 4 was carried out using the program Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/).

2.5. 3-D structure predictions: modeling and validation

Since the protein structure of AmpD of *Y. enterocoloitica* was not known, the 3D structures of AmpD homologs of different biotypes were predicted using the web interface *Iterative Threading Assembly Refinement (I-TASSER) (*https://zhanglab.ccmb.med.umich.edu/I-TASSER*/). For those AmpD homologs whose protein sequences were similar, only one representative protein was modeled. Finally,* the 3D structures which were modeled included: first homolog of AmpD present in biotypes 1A, 1B, 2, 3 and 4, second homolog of AmpD of biotypes 1A, 1B, 2, 4, second homolog of AmpD of biotype 3, third AmpD homolog of biotypes 2 and 4 and third AmpD homolog of biotype 3. Of the five models predicted by I-TASSER the best model was selected on the basis of confidence score (C-score). *The selected models were validated using PROCHECK* (Laskowski et al., 1993)*, ERRAT* (Colovos and Yeates, 1993) *and Verify 3D* (Lüthy et al., 1992)*; superimposed and visualized using PyMol (*https://pymol.org/2/*).*

3. Results

3.1. PCR amplification of CCDS of ampD and sequence confirmation

The primer pair, ampDF and ampDR resulted in the expected amplicon of 550 bp in strains of all biotypes. BLAST analysis of the sequenced amplicons confirmed that these encoded for AmpD of *Y. enterocolitica*.

3.2. Homology search using whole genome tBLAST, MSA of the AmpD homologs and InterProScan analysis

tBLAST search revealed that two homologs of AmpD were present in biotypes 1A and 1B, while three homologs each were present in biotypes 2 and 4 (Supplementary Table 1). An earlier study (Liu et al., 2016) reported three homologs of AmpD in biotype 3, AmpD1 (CCDS 576 bp), AmpD2 (CCDS 852 bp), and AmpD3 (CCDS 777 bp) with accession numbers, WP_005156822.1, WP_005164953.1 and WP_013649890.1 respectively. In the present study, AmpD homologs were designated as per the recommendations made by Karah et al. (2017) and Hall and Schwarz (2016). For biotype 3, the designations for the first, second and third homologs of AmpD were kept same as reported by Liu et al. (2016). Other AmpD homologs were numbered as per their chronological order in MSA (Fig. 1a). Accordingly, the first AmpD homolog of biotype 1B was named as AmpD4 (CCDS 465 bp) and of biotype 4 as AmpD5 (CCDS 465 bp). Since the difference in protein sequence of the first AmpD homolog of biotype 1B and biotype 1A was < 2%, these were considered as one protein and named AmpD4, as suggested (Hall and Schwarz, 2015). The second AmpD homologs detected in biotype 4 and biotype 1B were named as AmpD6 (CCDS 348 bp) and AmpD7 (CCDS 339 bp) respectively. Since the difference in protein sequences of the second homolog of AmpD of biotype 1B and biotype 1A was < 2% it was named as AmpD7. Similarly, due to < 2% difference in protein sequences, the first and second homologs of AmpD of biotype 4 and biotype 2 were named as AmpD5 and AmpD6, respectively. The third homolog of AmpD of biotype 4 (identical with third AmpD homolog of biotype 2) was named as AmpD8 (CCDS 297 bp). The designations of AmpD homologs discerned in various biotypes of *Y. enterocolitica* have been summarized in Table 1.

In the first AmpD homolog of all biotypes, the amidase catalytic sites, substrate binding sites and Zn binding residues were identical, and a domainn of Pfam family, Amidase_2 was present. The amidase catalytic sites, substrate binding sites and Zn binding residues though similar in the second AmpD homolog of biotypes 1A, 1B, 2, 3 and 4, there were additional catalytic, substrate and Zn binding sites in biotype 3. In all the biotypes, a common *N*-acetylmuramoyl-L-alanine amidase domain was present in the second AmpD homolog. However, in biotype 3, an additional peptidoglycan binding-like domain and a signal peptide of 21 amino acids were present at the N-terminal of the second AmpD homolog. Analysis of the third AmpD homolog revealed that though a common *N*-acetylmuramoyl-L-alanine amidase domain was present in biotypes 2, 3 and 4, the third AmpD homolog of biotype 3 had additional amidase catalytic, substrate binding and Zn binding sites.

3.3. 3D Modeling, evaluation and superimposition

The top three templates (PDB ID: 4BOL_A, 4BXJ_A, 2Y28_A); (PDB ID: 2BH7_A, 4BOL_A, 3D2Y_A); (PDB ID: 4BXD_A, 4BXJ_A, 3D2Y_A) with sequence identity of > 90%, 85% & 95% were used as a primary templates to model the structures of first, second and third AmpD homologs respectively. The predicted C-score for the final protein models of first, second and third AmpD homologs were −3.27, −1.51, and 1.75 respectively. The PROCHECK results indicated that in the protein models of first, second and third AmpD homologs 96%, 95% and 92% residues respectively were present in the allowed regions of

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AmpD1   MQLENNWQLDNGWISGVKRVVSPHCDQRPEGEAPSLLVIHNISLPPGEFGGPYIDQLFTGTLNPDEHPYFTDIVHLRVSAHCLIRRDG-
AmpD2   ------MRKLLSTGLLLLLAGCSSSHRDNPHQLIDRGEYKVDTSTPSIAQNERVRFLVLHYTATDDAESLRLLTQGDVSAHYLVKTYPE
AmpD3   ------------------------------------MYMIDYNNFRAIKSFNSRVRFLVLHYTAENFENSINSLTGNNVSVHYLVPDLDD
AmpD4   ------------------------------------VIHNISLPPGEFGGPYIDQLFTGTLNPDEHPYFADIVHLRVSAHCLIRRDG-
AmpD5   ------------------------------------VIHNISLPPGEFGGPYIDQLFTGTLNPDEHPYFTDIVHLRVSAHCLIRRDG-
AmpD6   ------------------------------------------------------------------------------SAHYLVKTYPE
AmpD7   ------------------------------------------------------------------------------VSAHYLVKTHPE
AmpD8   ------------------------------------------------------------------------------------------

AmpD1   ----------EIIQYVPFDKRAWHAGVSVFAERERCNDFSIGIELEG---TDVL------PFTAAQYRSLSEVSTLLFAHYP-ITAEQ
AmpD2   SLNGKP-----VVLQLVPESQRAWHAGVSYWQGRNNLNDTSIGIEIVNRGFTERMLRREWYAYNESQIELIERLTKDIVERYN-IDPAN
AmpD3   DSYKKAGFNNIRIFNLVDENARAWHAGVSSWAGRANINDTSIGIEIVNLA-TEHSGVFDFPPYPENQIAAVKQLAANILQRYPDISPTH
AmpD4   ----------EIIRYVPFDKRAWHAGVSVFSGRERCNDFSIGIELEG---TDVL------PFTAAQYRSLSEVSALLFAHYP-ITAEQ
AmpD5   ----------EIIQYVPFDKRAWHAGVSVFAERERCNDFSIGIELEG---TDVL------PFTAAQYRSLSEVSTLLFAHYP-ITAEQ
AmpD6   SLNGKP-----VVLQLVPESQRAWHAGVSYWQGRNNLNDTSIGIEIVNRGFTERMLRREWYAYNESQIELIERLTKDIVERYN-IDPAN
AmpD7   SLNGKP-----VVLQLVPESQRAWHAGVSYWQGRNSLNDTSIGIEIVNRGFTERMLRREWYPYNESQIELIERLTKDIVERYN-IEPAN
AmpD8   ----------RIFNLVDENARAWHAGVSSWAGRANINDTSIGIEIVNLA-TEHSGVFDFPPYPENQIAAVKQLAANILQRYPDISPTH
                  ::. *  . ******** : .*   ** *****:     *         .:   * :. .     .   *  * . :

AmpD1   VVGHSDIAPGRKTDPGPAFDWVLYQQNLAKSSLPS----------------------------------------------------------
AmpD2   VVAHSDIAPLRKSDPGPLFPWKRLAEQGIGAWPDDATVNKYINGRNMSGMASVTIIQQALARYGYQIPQSGELDNET..(n=40).....
AmpD3   VVAHSDIAPTRKSDPGPKFPWLELYNNGIGAWYDSETKEKFVQQFTQQGLPTKADLLSYFKTYGYDISIASS-DDGY..(n=40).....
AmpD4   VVGHSDIAPGRKTDPGPAFNWALYQQSLAKSSLPS----------------------------------------------------------
AmpD5   VVGHSDIAPGRKTDPGPAFNWALYQQSLAKSSLPS----------------------------------------------------------
AmpD6   VVAHSDIAPLRKSDPGPLFPW----------------------------------------------------------------------
AmpD7   VVAHSDIAPLRKSDPGP----------------------------------------------------------------------
AmpD8   VVAHSDIAPTRKSDPGPKFPWL----------------------------------------------------------------------
        **.****** **:****
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(a)

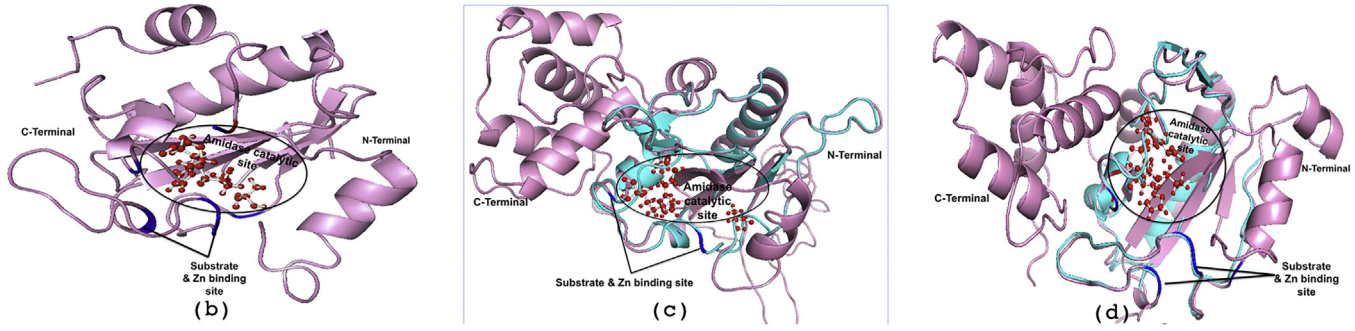

Fig. 1. (a) MSA of AmpD homologs detected in biotypes 1A, 1B, 2, 3 and 4 of *Y. enterocolitica*. Amidase catalytic sites are highlighted in red colour, * denotes identical amino acids,: denotes similar amino acids, n indicates number of amino acids (b) representative 3D protein model of first AmpD homolog of *Y. enterocolitica* biotypes 1A, 1B, 2–4 (c) superimposed 3D protein models of second AmpD homolog of biotype 3 (pink colour) and of biotypes 1A, 1B, 2 and 4 (cyan colour) (d) superimposed 3D protein models of third AmpD homolog of biotype 3 (pink colour) and of biotype 2/4 (cyan colour). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the Ramachandran map. The average ERRAT score for the protein models of first, second and third AmpD homologs were 86.301, 87.963 and 98.551 respectively, indicating high accuracy of the predicted models. The compatibility scores of an atomic model (3D) with its own amino acid sequence (1D) as revealed by Verify-3D program for the modeled first, second and third AmpD homologs were 88.89%, 88.79% and 84.42% respectively, indicating accuracy of the predicted models. The results of PROCHECK, ERRAT & Verify-3D confirmed that the predicted 3-D models were reliable and within the acceptable range. The final 3D model representing the first AmpD homologs of biotypes 1A, 1B, 2, 3 and 4 is shown in the Fig. 1b. The superimposed structure representing 3D model of the second AmpD homolog of biotypes 1A,

Table 1
AmpD homologs discerned in various biotypes of *Y. enterocolitica.*

S. No.	Protein designation	Description of the protein	Biotype in which it was discerned	Reference
1	AmpD1	1st homolog	Biotype 3	Liu et al. (2016)
2	AmpD2	2nd homolog	Biotype 3	Liu et al. (2016)
3	AmpD3	3rd homolog	Biotype 3	Liu et al. (2016)
4	AmpD4	1st homolog	Biotypes 1A/ 1B	This study
5	AmpD5	1st homolog	Biotypes 2/4	This study
6	AmpD6	2nd homolog	Biotypes 2/4	This study
7	AmpD7	2nd homolog	Biotypes 1A/ 1B	This study
8	AmpD8	3rd homolog	Biotypes 2/4	This study

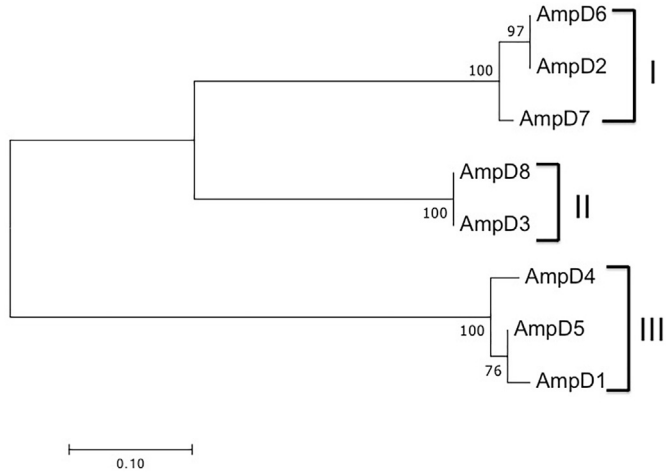

Fig. 2. Phylogeny of AmpD homologs present in different biotypes of *Y. enterocolitica.*

1B, 2 and 4 collectively, and second AmpD homolog of biotype 3 is shown in Fig. 1c. The superimposed structure representing 3D model of the third AmpD homologs present in biotype 2 /4 and of biotype 3 is shown in Fig. 1d.

3.4. Phylogenetic analysis

The AmpD homologs formed three separate clusters. The first cluster was comprised of the second homologs of AmpD (AmpD2, AmpD6 and AmpD7), second cluster comprised the third homologs of AmpD (AmpD3 and AmpD8), and the third cluster comprised the first homologs of AmpD (AmpD1, AmpD4 and AmpD5) (Fig. 2). Thus, the overall topology of the phylogenetic tree appeared to conform well to the separate evolution of the AmpD homologs in *Y. enterocolitica.*

4. Discussion

It was earlier believed that in *Enterobacteriaceae* family, the expression of *ampC* was regulated by a one-step inducible-derepressed mechanism, where AmpD inactivation leads to constitutive hyperproduction of AmpC. But, recent studies have revealed that, besides the main AmpD, additional AmpD homologs and regulatory proteins (like AmiD in *E. coli*) were present in several members of the family *Enterobacteriace* (Juan et al., 2006; Uehara and Park, 2007). In the present study we noted that besides the main AmpD, varying number of additional AmpD homologs were present in different biotypes of *Y. enterocolitica*. Two homologs of AmpD were detected in biotypes 1A and 1B, while three homologs each were detected in biotypes 2 and 4. Liu et al. (2016) reported that three AmpD homologs regulated the expression of *ampC* in *Y. enterocolotica* biotype 3. To the best of our knowledge, no earlier studies have reported varying number of AmpD homologs within a single bacterial species.

The main AmpD of biotypes 1A, 1B, 2, 3 and 4 (in the present study, named as AmpD4, AmpD5 and AmpD1) had similar catalytic, interacting and Zn binding sites. The secondary and 3D structures were also similar. The second AmpD homologs of biotypes 1A, 1B, 2 and 4 (in the present study named as AmpD7 and AmpD6) showed similar catalytic, interacting and Zn binding sites; however the second AmpD homolog of biotype 3 (named as AmpD2) had extra catalytic, amidase and Zn binding sites, an additional peptidoglycan binding-like domain, and a signal peptide at the N-terminal. Liu et al. (2016) experimentally showed that mutational inactivation of first or/and second AmpD homolog(s) (AmpD1 or AmpD2 alone or AmpD1 + AmpD2 together) failed to significantly derepress the expression of *ampC* in biotype 3. Since, the protein models of first and second AmpD homologs of biotypes 1A, 1B, 2 and 4 coincided with the first and second AmpD homologs (AmpD1 and AmpD2) of biotype 3, these might also be less effective in regulating the expression of *ampC*, as reported for in biotype 3 strains.

Liu et al. (2016) reported that in biotype 3, mutational inactivation of the third AmpD homolog (AmpD3 alone) or first and third AmpD homolog together (AmpD1 + AmpD3), led to a modest increase in expression of *ampC*. However, an over expression of *ampC* was observed when second and third homologs (AmpD2 + AmpD3) or first, second and third homologs (AmpD1 + AmpD2 + AmpD3) were inactivated together. This implied that the third homolog, (AmpD3) played the primary role and the role of first AmpD homolog (AmpD1) and/or second AmpD homolog (AmpD2) was only secondary (might be synergistic) in regulating the expression of *amp*C. Since, the major active sites in 3D models of the third AmpD homologs of biotypes 2 and 4 coincided with the 3D model of third AmpD homolog of biotype 3; it is reasonable to assume that the third AmpD homolog might also be the primary regulator of *amp*C expression in biotypes 2 and 4. The role of other AmpD homologs might be secondary or synergistic as suggested earlier. In the backdrop of this information it is reasonable to question as to how the expression of *ampC* was regulated in biotypes 1A and 1B which seemed to possess only the first and the second homologs of AmpD, which were less effective in regulating expression of *ampC*. It is therefore suggested that in absence of a third AmpD homolog, other proteins (like AmiD in *E.coli*) might be involved in regulating the expression of *ampC* in *Y. enterocolitica* biotypes 1A and 1B.

To conclude, the results of the current study indicate that multiple but varying numbers of homologs of AmpD are present in different biotypes of *Y. enterocolitica.* Thus, while a three-step regulation seems to be functional in biotypes 2, 3 and 4, a two-step regulation using AmiD-like proteins might be functional in biotypes 1A and 1B. The existence of *ampD* homolog(s)-mediated expression of *ampC* in other members of the family *Enterobacteriaceae may provide further credence to our findings.* Also, besides structural differences in the AmpD homologs it would be worthwhile to explore the mechanisms underlying the AmpD homolog mediated expression of cephalosporinases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.01.033.

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Author contributions statement

Conceived, designed and performed the experiments: NS, DP, NSS and MK. Data analysis: NS and MK. Written the manuscript: NS and JSV.

Conflict of interest statement

The authors declare no conflict of interests.

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