#### ORIGINAL RESEARCH

# Characterization of NDM-5 Carbapenemase-Encoding Gene (*bla*<sub>NDM-5</sub>) – Positive Multidrug Resistant Commensal *Escherichia coli* from Diarrheal Patients

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**Purpose:** The multidrug resistance Enterobacteriaceae cause many serious infections resulting in prolonged hospitalization, increased treatment charges and mortality rate. In this study, we characterized  $bla_{\text{NDM-5}}$ -positive multidrug resistance commensal *Escherichia coli* (CE) isolated from diarrheal patients in Kolkata, India.

**Methods:** Three CE strains were isolated from diarrheal stools, which were negative for different pathogroups of diarrheagenic *E. coli* (DEC). The presence of carbapenemases encoding genes and other antimicrobial resistance genes (ARGs) was detected using PCR. The genetic arrangement adjoining  $bla_{NDM-5}$  was investigated by plasmid genome sequencing. The genetic relatedness of the strains was determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) methods.

**Results:** In addition to colistin, the  $bla_{\text{NDM-5}}$ -positive CE strains showed resistance to most of the antibiotics. Higher MICs were detected for ciprofloxacin (>32 mg/L) and imipenem (8 mg/L). Molecular typing revealed that three CE strains belonged to two different STs (ST 101 and ST 648) but they were 95% similar in the PFGE analysis. Screening for ARGs revealed that CE strains harbored *Int-1, bla*<sub>TEM</sub>, *bla*<sub>CTX-M3</sub>, *bla*<sub>OXA-1</sub>, *bla*<sub>OXA-7</sub>, *bla*<sub>OXA-9</sub>, *tetA*, *strA*, *aadA1*, *aadB*, *sul2*, *floR*, *mph(A)*, and *aac(6')-Ib-cr*. In conjugation experiment, transfer frequencies ranged from  $2.5 \times 10^{-3}$  to  $8.4 \times 10^{-5}$ . The *bla*<sub>NDM-5</sub> gene was located on a 94-kb pNDM-TC-CE-89 type plasmid, which is highly similar to the IncFII plasmid harboring an IS26-IS30-*bla*<sub>NDM-5</sub>-*ble*<sub>MBL</sub>-*trpF*-*dsbd*-IS91-*dhps* structure.

**Conclusion:** To the best of our knowledge, this is the first report on carbapenem resistance involving the  $bla_{\text{NDM-5}}$  gene in CE from diarrheal patients. The circulation of  $bla_{\text{NDM-5}}$  gene in CE is worrisome, since it has the potential to transfer  $bla_{\text{NDM-5}}$  gene to other enteric pathogens.

Keywords: antimicrobial resistance, carbapenem-resistance, commensal E. coli, plasmid; bla<sub>NDM-5</sub>

#### Introduction

The emergence and rapid spread of carbapenemases-producing Enterobacteriaceae is a serious public-health concern because carbapenems are the last resort antibiotics to treat extensively multidrug-resistant (MDR) bacterial infections.<sup>1</sup> Amongst the newly emerging carbapenemases-producers, NDM is very important due to its increased MDR phenotype and rapid global dissemination with frequent allelic variations.<sup>2</sup> Since its first discovery in 2008, more than 20 variants of NDM have been identified in different bacterial species spread across different countries.<sup>3–5</sup> In 2011, the NDM-5 was first identified from an MDR *E. coli* isolated in the United Kingdom from a patient who had been previously treated in India.<sup>6</sup> The NDM-5 varied from NDM-1 by only two amino acid replacements at positions 88 (Val  $\rightarrow$  Leu) and 54 (Met

© 2022 Chowdhury et al. This work is published and licensed by Dove Medical Press Limited. The full terms of this license are available at https://www.dovepress.com/ terms.php and incorporate the Creative Commons Attribution — Non Commercial (unported, v3.0) License (http://creativecommons.org/licenses/by-nc/3.0/). By accessing the work you hereby accept the Terms. Non-commercial uses of the work are permitted without any further permission for Dove Medical Press Limited, provided the work is properly attributed. For permission for commercial use of this work, please see paragraphs 4.2 and 5 of our Terms (https://www.dovepress.com/terms.php).  $\rightarrow$  Leu).<sup>6</sup> NDM-5-producing Enterobacteriaceae has been reported worldwide, including in Algeria,<sup>7</sup> Australia,<sup>8</sup> China,<sup>9</sup> Egypt,<sup>10</sup> India,<sup>11</sup> Italy,<sup>12</sup> Mali,<sup>13</sup> South Korea,<sup>14</sup> Spain,<sup>15</sup> and United States.<sup>16</sup>

The mechanism for dissemination and transmission of  $bla_{\text{NDM-5}}$  among Enterobacteriaceae is primarily associated with the transfer of plasmids or the transposon-related mobile genetic elements.<sup>17</sup> The  $bla_{\text{NDM-5}}$  has been reported worldwide with numerous incompatibility-type plasmid groups and the most prevalent being IncFIA and IncFK in India,<sup>18</sup> IncFII in the United States,<sup>16</sup> IncF and IncX3 in China,<sup>9</sup> IncFIA/B and IncX3 in Korea,<sup>14</sup> and IncFIB in Egypt.<sup>10</sup>

*E. coli* is the most common pathogen associated with community acquired infections. MDR in *E. coli* has become an increasing problem in human health management.<sup>19</sup> Acquisition of the gene encoding NDM by *E. coli* isolates is an important health issue due to its fast spread across the globe and also in other Gram-negative bacteria.<sup>20</sup> This emerging trend is directly associated with an increased use of carbapenems during the treatment of several infectious diseases.<sup>21</sup> In this study, we report on the incidence of the NDM-5-producing commensal *E. coli* isolated from diarrheal patients in Kolkata, India. To the best of our knowledge, this is the first report on CE strains producing NDM-5 carbapenemases. The present study characterized  $bla_{NDM-5}$  identified in CE isolated from diarrheal patients and its dissemination through conjugative plasmid into other enteric bacteria.

# **Materials and Methods**

#### **Clinical Specimens**

In this study, stool specimens collected from acute diarrheal patients hospitalized at the Infectious Diseases Hospital, Kolkata, India, were tested for the identification of enteric pathogens. Patients who excreted >3 loose or liquid stools per day with no, some or severe dehydration were considered to have diarrhea.<sup>22</sup> Fecal specimens were collected in McCartney bottles using sterile catheters or as rectal swab in Cary Blair transportation medium and processed in the laboratory within 2 hrs for common enteric pathogens.<sup>23</sup>

#### Identification and Isolation of Commensal E. coli

For identification and isolation of *E. coli*, fecal specimens were inoculated on MacConkey agar (Difco, USA) and incubated at 37°C for 16–18 hrs. Three typical colonies per sample from MacConkey agar plate (including both lactose fermenting and non-lactose fermenting colonies) were picked and sub-cultured on Luria Bertani agar (LBA, Difco, USA) plates. A small portion of cultures was taken from LBA and tested for indole-production by adding the Kovacs reagent. Colour changes to pink were recorded positive and additionally tested in triple sugar iron agar for typical *E. coli*. For further confirmation, all the isolates were individually tested on a Vitek-2 compact system (bioMérieux). Indole, biochemical and Vitek-2 compact-positive bacterial cultures from each of the three colonies plated on LBA plates were taken and mixed with 500  $\mu$ L of sterile water or phosphate-buffer saline in 1.5 mL microfuge tubes. The bacterial suspension was boiled in a water bath for 10 min and then kept on ice for snap chill for 5 min. Bacterial suspensions were centrifuged at 8000 rpm for 10 min and the supernatants were used as DNA templates in the multiplex PCR for the detection of virulence marker genes, such as CVD432 and *aaiC* (enteroaggregative *E. coli*), *eae* and *bfpA* (enteropathogenic *E. coli*), *elt* and *est* (enterotoxigenic *E. coli*).<sup>24</sup> Simplex PCR was also performed for the detection of Shiga toxin–producing *E. coli*, and/or enterohemorrhagic *E. coli*(*stx1*, *stx2*), enteroinvasive *E. coli* (EIEC) (*ipaH*) and cytolethal distending toxin (*cdt*) gene for *E. albertii*.<sup>25</sup> We defined commensal *E. coli* when the tested colonies were negative for all the above virulence marker genes.

# Antimicrobial Susceptibility Testing (AST)

AST was performed in accordance with the Clinical and Laboratory Standards Institute<sup>26</sup> by disc diffusion method using commercially available discs (Becton Dickinson Company, USA) namely, ampicillin (AMP), ceftriaxone (CRO), cefotaxime (CTX), ceftazidime (CAZ), chloramphenicol (CHL), nalidixic acid (NA), cipro-floxacin (CIP), ofloxacin (OFX), norfloxacin (NOR), meropenem (MEM), streptomycin (STR), erythromycin (E), azithromycin (AZM), gentamicin (GM), tetracycline (TET), doxycycline (D), trimethoprim/sulfamethoxazole

(SXT). In addition, MICs against ampicillin, azithromycin, ceftriaxone, cefotaxime, erythromycin, gentamicin, meropenem, and sulfamethoxazole/trimethoprim were determined using the E-test (AB Biodisk, Sweden), according to the manufacturer's instructions. Detection of the extended spectrum  $\beta$ -lactamase (ESBL) activity was made using the combination disc test (cefotaxime, 30 µg; ceftazidime, 30 µg; with and without clavulanic acid, 10 µg). Strains were considered as ESBL positive, if they exhibit >5 mm increase in the size of the zone of inhibition of the  $\beta$ -lactamase inhibitor combinations in comparison to a third-generation cephalosporin without the  $\beta$ -lactamase inhibitor. *E. coli* ATCC25922 was served as control in antimicrobial susceptibility testing.

## "O" Serogrouping of E. coli Isolates

The "O" serogrouping of *E. coli* was made using an *E. coli* "O" serotyping kit by slide agglutination test in accordance with the manufacturer's instructions (Denka-Seiken Co. Ltd., Japan). This kit consists of eight "O" polyvalent and 43 monospecific antisera.

# Molecular Typing

Pulsed-field gel electrophoresis (PFGE) was performed using a CHEF-Mapper (Bio-Rad, Hercules, USA) based on Pulse-Net standardized protocol.<sup>27</sup> PFGE was made by *Xba*I-digested genomic DNA of *E. coli* species. PFGE images were saved by using a Gel Doc XR system (Bio-Rad). The PFGE gel images were analysed using the BioNumerics software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) by normalizing and aligning the peaks of the *Salmonella enterica* serovar Braenderup H9812 size standard. The banding pattern similarity and the clustering correlation coefficients were calculated by comparison of the dice-coefficient and UPGMA, respectively. Multilocus sequence typing (MLST) was also carried out for molecular typing. Bacterial genomic DNA was extracted from the CE strains by DNA mini kit (Qiagen, Germany). Seven housekeeping genes (*adk, icd, fumC, gyrB, mdh, purA* and *recA*) were amplified by PCR,<sup>28</sup> and the amplicons were submitted for DNA sequencing. ST analysis was done according to the protocols specified at the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

## Antimicrobial Resistance Gene (ARGs) Screening

Different ARGs were screened by PCR, and the positive amplicons were confirmed by DNA sequencing. The PCR assays targeted AMR genes for carbapenemases ( $bla_{NDM}$ ,  $bla_{IMP}$ ,  $bla_{KPC}$ ,  $bla_{OXA-48}$ ,  $bla_{SIM}$ , and  $bla_{VIM}$ ), gentamicin (*aadB*), streptomycin (*aadA1* and *strA*), chloramphenicol (*catA1*, *floR*), extended-spectrum  $\beta$ -lactamase (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>PSE4</sub>, *bla*<sub>OXA-1</sub>, *bla*<sub>OXA-7</sub> and *bla*<sub>OXA-9</sub>), ciprofloxacin [*aac*(6')-*Ib-cr*], and macrolides {*mph*(A), *mph*(B), *erm*(A), *erm*(B)}. Simplex PCR was carried out as described previously.<sup>29</sup>

## Mutations in the Quinolone Resistance-Determining Regions (QRDRs)

The chromosomal genes gyrA and parC were amplified by PCR for the QRDRs and sequenced to analyse the potential mutations. We used published primers for the amplification of gyrA and parC genes.<sup>30</sup>

# Conjugation Assay

To test the transfer of the  $bla_{\text{NDM-5}}$ , conjugation assay was made using NDM-5 positive commensal *E. coli* (CE-89) as donor with two different recipients, namely *E. coli* J53 (sodium azide resistant) and *Salmonella enterica* serovar Bareilly. In brief, overnight seed cultures of the bacteria were inoculated in LB broth for late-exponential phase culture. Cell density of bacterial culture was adjusted to  $1.5 \times 10^8$  cells/mL. Both the cultures of donor and recipient were mixed (at the ratio of 1:2) in 5 mL of LB broth and kept at 37°C for overnight. Several selective media with antibiotics were used to recover  $bla_{\text{NDM-5}}$  positive transconjugants (TC). MacConkey agar containing both sodium azide (100 µg/mL) and meropenem (5 µg/mL) was used to select TC of *E. coli* J53 (TC-*E. coli* J53). Similarly, transconjugants of *Salmonella* (TC-*Salmonella*) were obtained by selecting on xylose lysine deoxycholate (XLD, Difco) containing meropenem (5 µg/mL). Putative transconjugants harboring  $bla_{\text{NDM-5}}$  were detected by PCR analysis followed by amplicon sequencing. The conjugation frequency was calculated as the number of transconjugants per recipient cell.

# **Plasmid Profiling**

Plasmid DNA was extracted from the CE strains and transconjugants using a modified Kado and Liu method.<sup>31</sup> The plasmid DNA of *E. coli* V517 and J53 pMG252 were used as molecular weight markers for determining the molecular weight of plasmid DNAs of CE strains and the transconjugants. Plasmid incompatibility of major groups was determined by using PCR-based replicon typing.<sup>32</sup>

#### Plasmid Sequence Analysis

Plasmid carrying  $bla_{NDM-5}$  was extracted from the TC-CE-89 using a QIAGEN plasmid midi kit according to the manufacturer's instructions (QIAGEN). The complete plasmid sequence was obtained using an Illumina HiSeq platform. The DNA library was prepared for the pair end sequencing. The derived reads were subsequently trimmed and assembled de novo using the CLC genomics work bench. The plasmid sequences were annotated by Prokaryotic Dynamic Programming Gene finding Algorithm (Prodigal tool v.2.6.3). The open reading frames were compared using BLAST against the non-redundant protein database. Different ARGs in the plasmid were identified by comparing the ORFs with the genes of the Comprehensive Antibiotic Resistance Database (CARD). For the ARGs, we selected the thresholds of identity >70% and subject coverage >90%. The circular map of the pNDM-TC-CE-89 plasmid was generated using the Snap Gene server.

#### Nucleotide Sequence

The complete nucleotide sequences of plasmid pNDM-TC-CE-89 were submitted to GenBank accession number MZ892875.

# Results

## Isolation, Identification and Serotyping of E. coli from Stool Specimens

Three carbapenem-resistant CE strains were isolated from three different hospitalized acute diarrheal patients in 2015. All three strains were confirmed as *E. coli* using biochemical and Vitek-2 compact system. As confirmed in the multiplex PCR assay, these CE strains did not harbour any virulence encoding genes specific to DEC. Serological results showed that the "O" antigen of the CE strains is different. One CE strain was untypable (ONT) and the other strains belonged to O167 and O86a (Table 1).

## Antimicrobial Susceptibility Testing and MIC

All the three CE strains shared the similar AMR profile. As shown in Table 1, CE strains are resistant to meropenem, ciprofloxacin, nalidixic acid, norfloxacin, ampicillin, ofloxacin, tetracycline, ceftriaxone, sulfamethoxazole/trimethoprim, streptomycin, ceftazidime, erythromycin, chloramphenicol, gentamicin, and cefotaxime but remained susceptible to doxycycline and colistin. CE displayed a higher MIC value for most of the antibiotics (Table 2). The modified Hodge test and imipenem-EDTA double-disc synergy test confirmed the production of metallo-B-lactamases (MBLs) by the CE strains. Carbapenemase gene screening revealed that all the CE strains harbored the  $bla_{NDM-5}$  gene that was further confirmed by amplicon sequencing. The sequence of the  $bla_{NDM-5}$ gene exhibited 100% homology with previously reported sequences.<sup>6</sup>

## PCR for Antimicrobial Resistance Genes and QRDRs

The results of ARGs detected in the CE are shown in Table 1. Class-1 integron was identified in the  $bla_{NDM-5}$  positive CE strains. In addition to  $bla_{NDM-5}$ , CE were positive for  $\beta$ - lactamase–encoding genes  $bla_{TEM}$ ,  $bla_{CTX-M3}$ ,  $bla_{OXA-1}$ ,  $bla_{OXA-7}$  and  $bla_{OXA-9}$ ; tetracycline resistance marker gene tet(A), streptomycin-encoding gene strA and aadA1; gentamicin encoding gene aadB; sulfonamides encoding gene sul2; chloramphenicol encoding gene floR, macrolide resistance phosphotransferase encoding gene mph(A); and ciprofloxacin modifying enzyme-encoding gene aac(6')-*Ib-cr* (amino glycoside actetyltransferase). Plasmid-mediated quinolone resistance (PMQR) qnrB gene was detected only in CE-89.

Strain ID	"O" Serotype	MLST Sequence Typing	Resistance Profile	Antibiotic Resistance Gene	Amino acid Substitutions in the QUINOLONE Resistance- Determining Region		
					GyrA	ParC	
CE-88	167	101	NA,CIP,NOR,OFX,TE, MEM,AM,CRO,SXT,S, CAZ,E,C,GM,CTX,AZM	Int-1, bla <sub>NDM</sub> , bla <sub>TEM</sub> , bla <sub>CTX-M3</sub> , bla <sub>OXA-1</sub> , bla <sub>OXA-7</sub> , bla <sub>OXA-9</sub> , tetA, strA, aadA I, aadB, sul2, floR, mph(A), aac(6')-lb-cr	Ser 83 Leu Asp 87 Asn	Ser 80 lle	
CE-89	86a	101	NA,CIP,NOR,OFX,TE, MEM,AM,CRO,SXT,S, CAZ,E,C,GM,CTX,AZM	Int-I, bla <sub>NDM</sub> , bla <sub>TEM</sub> , bla <sub>CTX-M3</sub> , bla <sub>OXA-I</sub> , bla <sub>OXA-7</sub> , bla <sub>OXA-9</sub> , tetA, strA, aadAI, qnrB, aadB, sul2, floR, mph(A), aac(6')-lb-cr	Ser 83 Leu Asp 87 Asn	Ser 80 lle	
CE-93	Untypable	648	NA,CIP,NOR,OFX,TE, MEM,AM,CRO,SXT,S, CAZ,E,C,GM,CTX,AZM	Int-I, bla <sub>NDM</sub> , bla <sub>TEM</sub> , bla <sub>CTX-M3</sub> , bla <sub>OXA-I</sub> , bla <sub>OXA-7</sub> , bla <sub>OXA-9</sub> , tetA, strA, aadA I, aadB, sul2, floR, mph(A), aac(6')-lb-cr	Ser 83 Leu Asp 87 Asn	Ser 80 lle	

#### Table I Characterization of AMR and Their Encoding Genes in NDM-5-Producing Commensal E. coli

**Abbreviations**: AM, ampicillin; AZM, azithromycin; C, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; E, erythromycin; GM, gentamicin; MEM, meropenem; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; TE, tetracycline.

Strain	Bacteria	Resistance Profile	*Frequency	MIC (μg/mL)						
ID			of Transfer	MEM	SXT	CRO	GM	AM	стх	AZM
CE-89	E. coli(Donor)	NA,CIP,NOR,OFX,TE,MEM,AM, CRO,SXT,S,CAZ,E,C,GM, CTX,AZM	-	32	>32	>256	>256	>256	>16	>256
J53	E. coli(Recipient)	AZD	-	0.064	0.047	0.75	0.25	١.5	0.064	0.75
TC- J53	E. coli	AM,MEM,CRO,CAZ,E,SXT,	8.4 × 10 <sup>-5</sup>	4	>32	>256	>256	>256	>16	>256
	(Transconjugant)	CTX,GM,AZM,AZD								
BCH	Salmonella	-	-	0.064	0.50	0.50	0.38	0.50	0.064	0.75
0704	Bareilly									
	(Recipient)									
TC-BCH	Salmonella	AM,MEM,CRO,CAZ,E,SXT,	$2.5 \times 10^{-3}$	4	>32	>256	>256	>256	>16	>256
0704	Bareilly	CTX,GM,AZM								
	(Transconjugant)									

 Table 2 Minimum Inhibitory Concentration (MIC) of Donor, Recipients, and Transconjugants

 $\textbf{Notes:} \ ^* Frequency \ of \ transfer \ was \ calculated \ as \ number \ of \ transconjugants \ per \ donor \ cell.$ 

Abbreviations: AM, ampicillin; AZM, azithromycin; AZD, azide; C, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; E, erythromycin; GM, gentamicin; MEM, meropenem; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; TE, tetracycline.

Additionally, three strains had amino acid substitutions in the QRDRs, ie, in GyrA (Ser 83 Leu and Asp 87 Asn) and ParC (Ser 80 Ile) (Table 1).

# MLST and PFGE

In the MLST sequence analysis, CE-88 and CE-89 were identified as ST101 Cplx (*adk* allele 43; *fumC* allele 41; *gyrB* allele 15; *icd* allele18; *mdh* allele 11; *purA* allele 7 and *recA* allele 6), whereas CE-93 was identified as ST648 Cplx (*adk* allele 92; *fumC* allele 4; *gyrB* allele 87; *icd* allele 96; *mdh* allele 70; *purA* allele 58 and *recA* allele 2, Table 1). PFGE revealed that isolates had ~90–95% similarity, suggesting that the three CE strains are genetically related clones (Figure 1).



Figure 1 Xba1 restriction patterns of genomic DNA of CE strains, Kolkata, India. Dendrogram was generated by using the unweighted pair group with the arithmetic mean method.

## Plasmid Characterisation and Conjugation

The  $bla_{\text{NDM-5}}$  harboring CE carried multiple plasmids ranging from 5 kb to 95 kb. In the transconjugants, only a single plasmid of size ~94 kb was detected. Plasmid harboring the  $bla_{\text{NDM-5}}$  was transferable from CE-89 to a laboratory strain *E. coli* J53 and a clinical isolate of *Salmonella enterica* serovar Bareilly. While using *E. coli* J53 as recipients, the conjugation frequency was higher (~10<sup>5</sup> transconjugants per donor cell) than *S*. Bareilly (~10<sup>3</sup> transconjugants per donor cell) (Table 2). TC-*E. coli* J53 and TC-*S*. Bareilly acquired resistance to ampicillin, meropenem, ceftriaxone, ceftazidime, erythromycin, sulfamethoxazole/trimethoprim, cefotaxime, gentamicin and azithromycin, indicating the possibility that the  $bla_{\text{NDM-5}}$  plasmid carried the corresponding resistance to these antimicrobials. However, the CT- *E. coli* J53 and TC-*S*. Bareilly were susceptible to quinolone, fluoroquinolones and tetracycline. The MIC values of antimicrobials of donor and recipient are shown in Table 2. For meropenem, the MIC value of transconjugants was 4 mg/L, but higher MIC values were observed for other antibiotics.

#### Plasmid Sequence Analysis

The plasmid typing has identified that the CE harbours IncFII replicon. Considering that the  $bla_{NDM-5}$  harbored transferable plasmids of the same size and incompatibility group, we have chosen pNDM-TC-CE-89 as a representative for whole-genome sequencing to identify the genetic background and the flanking regions of  $bla_{NDM-5}$ . This plasmid was identified to comprise several types of genes, including ARGs, mobile elements, and putative genes, genes encoding proteins that control plasmid replication, stability and transfer. Moreover, the sequence alignments based on BLAST revealed that pNDM-TC-CE-89 has 99% nucleotide identity with previously reported  $bla_{NDM-5}$  harboring plasmid of *E. coli* (99.9% sequence similarity, 100% query coverage; AP018144.1)<sup>33</sup> including the complete array of genes for replication, partitioning, type IV conjugative transfer system, maintenance and stabilization.

The nucleotide sequence length of pNDM-TC-CE-89 was 94,224-bp with an average GC content of 53%, which is slightly higher than that of *E. coli* (~50%). In addition, we observed that the plasmid has harbored different classes of ARGs, which include dihydrofolate reductase (*dhfR*), aminoglycoside 3"-nucleotidyltransferase (*aadA*), Class A  $\beta$ - lactamase (*bla*<sub>TEM</sub>), 23S rRNA dimethyl transferase (*ermB*) and macrolide 2'-phosphotransferase (*mphA*) family. The genes responsible for other antimicrobial resistance were not detected in this plasmid (Figure 2). The ARGs BLAST sequence alignments and the most similar nucleotide sequences to those of the previously reported plasmid have been shown in Table 3.

The  $bla_{\text{NDM-5}}$  within pNDM-TC-CE-89 was situated in a complex integron, connected by two IS26 sequences having an IS91 element and a class 1 integron with the *int11* gene truncated by one of the IS26 copies and the *aadA2-dfrA12* resistance gene cassettes. The flanking genetic structure of the  $bla_{\text{NDM-5}}$  of pNDM-TC-CE-89 plasmid composed of an IS30 and IS26 located upstream, and the genes  $ble_{\text{MBL}}$  (bleomycin resistance gene), *trpF* (phosphor ribosyl anthranilate isomerase), *dsbd* (Dsbd superfamily protein), IS91 and *dhps* (dihydropteroate synthase) in downstream (IS26-IS30-bla\_{\text{NDM-5}}-ble\_{\text{MBL}}-trpF-dsbd-IS91-dhps) (Figure 3).



**Figure 2** Schematic representation and comparison of the sequences of different antimicrobial resistance encoding genes and their association with mobile genetic elements of *E. coli* (accession no. AP018144.1), (*K*) *pneumoniae* (accession no. CP024840.1) and commensal *E. coli* (CE-89) strains harboring *bla*<sub>NDM-5</sub>. Arrow lengths are proportionate to the lengths of the genes or open reading frames. Genetic structure of CE NDM isolates was identified the link between resistance genes and mobile genetic element. *trpF*: phosphoribosylanthranilate isomerase gene; IS26: IS26 transposae; *Intl1*: class 1 integron integrase *Intl1*; *dfra12*: dihydrofolate reductase; *aad*A2: aminoglycoside resistance protein; SMR: quaternary ammonium compound efflux SMR transporter QacE delta 1; Dhps: dihydropteroate synthase. IS91: IS91 family transposae; Dsbd: Thiol: disulfide interchange protein DsbD; PRAI: phosphoribosylanthranilate isomerase; *ble*<sub>MBL</sub>: bleomycin resistance protein; *Sla*<sub>NDM-5</sub>: subclass B1 metallo-β-lactamase NDM-5; IS30: Transposae-like protein, IS30 family; *bla*<sub>TEM</sub>: β-lactamase; *tnpR*: Transposon Tn3 resolvase, *mphA*: macrolide 2'- phosphotransferase.

#### Discussion

The emergence of NDM-producing Enterobacteriaceae is an increasing threat to global health. The bla<sub>NDM</sub> harboring strains confer resistance to most of the  $\beta$ -lactams and also express resistance to cephalosporins, quinolones, and aminoglycosides with different ARGs, which allows pathogens to become MDR.<sup>34</sup> AMR is not restricted only to pathogenic bacteria, but the commensal gut bacteria also play a role in harboring and transmission of ARGs. E. coli is commonly found as a commensal gut microbiota and considered as a reservoir of acquired AMR determinants.<sup>35</sup> Since *bla*<sub>NDM-5</sub> was first identified in the UK, many bacterial species harboring this gene have emerged in several countries.<sup>36</sup> Although NDM-5-producing strains appear to be a common NDM variant, they remain less compared to NDM-1-variant.<sup>37</sup> In India, different sequence types (STs) of NDM-5 producing E. coli and other bacterial strains have been detected from clinical and other sources. The E. coli isolates harbour the *bla*<sub>NDM-5</sub> gene in either plasmids or the transposon-related mobile genetic elements. Different incompatibility groups of plasmids belonging to the IncF, IncFII IncX3, K and FrepB are reported to carry the bla<sub>NDM-5</sub> gene.<sup>11,38,39</sup> Furthermore, E. coli harbouring the bla<sub>NDM-5</sub> found on IncX3 plasmids isolated from patients who travelled from India to Australia and Denmark, suggesting the origin of NDM-5 with STs 648 and 1284, respectively.<sup>8,40</sup> In addition, the NDM-5-producing E. coli has been identified from fresh fish and mastitic milk samples in India.41,42 In this study, the three NDM-5-producing CE displayed close genetic similarity as evidenced from PFGE and plasmid typing and AMR profile.

CE strains harboring  $bla_{NDM-5}$  were typed as ST101 and ST648. ST101 of *E. coli* producing NDM-5 in China and Myanmar were documented as an epidemic clone of substantial public health concern carrying plasmids associated with ARGs.<sup>43</sup> *E. coli* ST648 was first reported in India and UK<sup>6</sup> and has also been sporadically detected worldwide.<sup>8–12</sup> The ST648 isolate detected in this study was highly resistant to several different classes of antimicrobial agents. A similar observation was made in earlier studies.<sup>8,43</sup>

Fluoroquinolone resistance in Enterobacteriaceae is primarily due to mutations in the QRDRs of DNA gyrase and topoisomerase IV. The two enzymes are encoded by the gyrA for DNA gyrase (topoisomerase II) and parC for

#### Table 3 Characteristics of Plasmid Encoded AMR and Other Genes in Different Bacterial Species

Gene Name	Size	GC	Similar Gene in Other	Similarity		Accession No.	
	(bр)	content (%)	Bacterial Species	ldentity (%)	QC (%)		
Dihydrofolate reductase (EC 1.5.1.3)	498	51.2	Klebsiella pneumoniae Acinetobacter baumanii E. coli	100	100	CP043863.1 CP027246.2 MN335921.1	
Integron integrase Intl I	888	61.1	E. coli Klebsiella pneumoniae Klebsiella variicola	100 99.77 97 99.77		MN218686.1 CP041948.1 CP026014.1	
Aminoglycoside 3"- nucleotidyltransferase (EC 2.7.7) => ANT(3")-la (AadA family)	780	51.7	Klebsiella pneumoniae E. coli Acinetobacter baumanii E. fergusonii	100	100	CP043863.1 MN335921.1 CP027246.2 CP040806.1	
Small multidrug resistance (SMR) efflux transporter)	348	50	Klebsiella pneumoniae Pseudomonas aeruginosa E. coli Acinetobacter towneri	100	100	CP043863.1 CP039991.1 MN335921.1 CP046045.1	
Dihydropteroate synthase type-2 (EC 2.5.1.15)	840	61.6	Klebsiella pneumoniae Pseudomonas aeruginosa Acinetobacter towneri E. coli Klebsiella variicola	100	100	CP043862.1 MN433457.1 CP046045.1 CP041338.1 CP026014.1	
Bleomycin resistance protein	366	60.9	Klebsiella pneumoniae E. coli Klebsiella variicola Acinetobacter towneri Pseudomonas aeruginosa	100	100	CP041949.1 CP041957.1 CP026014.1 CP046045.1 CP024630.1	
Subclass BI beta-lactamase (EC 3.5.2.6) => NDM family	813	61.5	Klebsiella pneumoniae E. coli Acinetobacter baumanii Pseudomonas sp. SKJ15	100	100	CP043863.1 MN197360.1 MN331780.1 MN331784.1	
23S rRNA (adenine(2058)-N(6))- dimethyltransferase (EC 2.1.1.184) => Erm(B))	738	33.1	Klebsiella pneumoniae E. coli	100	100	CP043863.1 MN218686.1	
Macrolide 2'-phosphotransferase => Mph(A) family	915	65.5	E. coli strain 5M Klebsiella pneumoniae	100	100	MN218686.1 CP043863.1	
Class A β-lactamase (EC 3.5.2.6) => TEM family	816	49.0	Klebsiella michiganensis Klebsiella pneumoniae strain HKU49 Acinetobacter baumanii strain MC75 E. fergusonii E. coli strain PE15	100	100	CP024641.1 MN543570.1 MK531541.1 CP040806.1 CP041631.1	



Figure 3 Salient features of *bla<sub>NDM-5</sub>* encoding plasmid pNDM-TC-CE-89 plasmid with other resistance genes, transposons and type IV conjugative transfer system. The circle in red colour indicates the flanking structure of *bla<sub>NDM-5</sub>*. Transfer machinery are showed in green colour. Cloned par loci genes are indicated in yellow colour and the transposons IS26 elements are marked in brown.

topoisomerase IV. In this study, all three isolates displayed mutations in the QRDR region of gyrA and parC, which has also been reported in many investigations.<sup>30</sup>

The IncFII plasmids are highly mobile and carry several  $bla_{\text{NDM}}$  variants in Enterobacteriaceae.<sup>44</sup> A recent study has shown the ability of  $bla_{\text{NDM}}$  bearing IncFII plasmid transfer to different members of Enterobacteriaceae along with the other ARGs.<sup>45</sup> In this study, the transfer of pNDM-CE89 carrying  $bla_{\text{NDM}-5}$  to other bacteria has been demonstrated along with the expression of MDR. Therefore, commensal *E. coli* having IncFII plasmids with  $bla_{\text{NDM}-5}$  are epidemiologically important, as there is a high possibility of its transfer to other pathogens, which will be a major concern in the clinical management of infections.

BLAST-based sequence alignments revealed that the plasmid pNDM-CE-89 had the most similar nucleotide sequences in other plasmids such as pYJ6-NDM5 (accession number AP023236.1) from an *E. coli* strain, pM941-NDM5 (accession number AP023454.1) from an *K. pneumoniae* strain, and pM2\_FII (GenBank accession AP018144.1) from an *E. coli* strain. The plasmid pNDM-CE-89 possesses complex structures as it contains the gene encoding MBLs *bla*<sub>NDM-5</sub>, a large group of genetic elements (integrons, transposons, and ISCRs), *IntI1* (class 1 integron integrase) and *mphA*, a family of macrolide phosphotransferase.

The location of  $bla_{NDM-5}$  in pNDM-CE-89 was identified between the two IS26 elements. The similar flanking genetic structure has previously been observed in other  $bla_{NDM-5}$  bearing plasmids.<sup>33</sup> The IS26 element has been reported in many members of Enterobacteriaceae and exists together with the  $\beta$ -lactamases region, which is a part of transposon-like structure in many plasmids.<sup>46</sup> In pNDM-CE-89, the association of  $bla_{NDM-5}$  with IS26 makes a condition favourable for its mobilization and function. The flanking region of NDM producers carried a highly conserved gene segment (IS26-IS30- $bla_{NDM5}$ - $ble_{MBL}$ -trpF) suggesting that these four genes are important for the transmission and expression of AMR.

In conclusion, the global dissemination of NDM-producing Gram-negative bacteria is of great concern. Our data suggest that commensal *E. coli* in the gut acts as a reservoir for NDM carrying plasmid and the other ARGs. More epidemiological and clinical studies are needed to elucidate the mechanisms of emergence, evolution and dissemination of NDM-5 in commensal *E. coli* and other gut microbes.

# **Ethical Approval**

This study was approved by the "Institutional Ethics Committee (IEC) of the National Institute of Cholera and Enteric Diseases in Kolkata, India (registration number: A-1/2015-IEC)". Written consent was obtained from each adult patient or the parent/guardian of the child patients enrolled in this study.

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## **Author Contributions**

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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# Disclosure

The authors report no conflicts of interest in relation to this work.

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