ORIGINAL RESEARCH

Detection of Two Copies of a $bla_{\text{NDM-1}}$-Encoding Plasmid in *Escherichia coli* Isolates from a Pediatric Patient with Diarrhea

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Purpose: To elucidate the contribution of a transferable plasmid harboring the $bla_{\text{NDM-1}}$ gene in an *Escherichia coli* clinical isolate to the spread of resistance determinants.

Methods: Nine extended-spectrum β-lactamase-producing *E. coli* were collected from diarrhea samples from a pediatric patient and genetic linkage was investigated through enterobacteriaceae repetitive intragenic consensus polymerase chain reaction (PCR). Bacterial species were identified by 16s rRNA sequencing, susceptibility testing with the use of a BD Phoenix™-100 Automated Microbiology System, and assessment of virulence genes by PCR. The transferability of $bla_{\text{NDM-1}}$ in *E. coli* strain TCM3e1 was confirmed by conjugation experiments. Complete sequencing of *E. coli* strain TCM3e1 was determined with the PacBio and Illumina NovaSeq platforms and the characteristics were analyzed with bioinformatics software.

Results: The results showed that all nine *E. coli* strains were the same clone. *E. coli* strain TCM3e1 was resistant to 12 antimicrobial agents and carried the virulence gene EAST-1. Conjugation transfer analysis showed that $bla_{\text{NDM-1}}$ was carried on a self-transmissible plasmid. Two copies of the $bla_{\text{NDM-1}}$ gene were present on an IncC plasmid and some resistance genes with two or three copies were located downstream of the $bla_{\text{NDM-1}}$ gene and formed a tandem repeat fragment ($bla_{\text{DNM-1}}$-*bleo-sul1- aadA17-dfrA12*).

Conclusion: A transmissible plasmid harboring two copies of the $bla_{\text{NDM-1}}$ gene, including clonal dispersions of the $bla_{\text{NDM-1}}$ gene, was identified in clinical isolates. These findings emphasized the necessity of surveillance of the plasmid-borne $bla_{\text{NDM-1}}$ to prevent dissemination.

Keywords: diarrheagenic *Escherichia coli*, multi-drug-resistant, New Delhi metallo-β-lactamase, $bla_{\text{DNM-1}}$ gene

Introduction

Diarrhea is a severe public health problem and a leading cause of morbidity and mortality in infants and young children.[1,2] Severe diarrhea is among the most common reasons for hospital admissions of children in low- and middle-income countries in Africa, Asia, and Latin America. Over the past decade, there has been a substantial reduction in deaths due to diarrhea, yet diarrheal diseases account for nearly 10% of the 7.6 million deaths of children younger than 5 years annually.[3] Diarrhea is a multifactorial illness associated with wide spectrum of pathogens, including viruses, bacteria, and parasites. Among the bacterial pathogens, diarrheagenic *Escherichia coli* is an important cause of endemic and epidemic diarrhea worldwide,[4] as strains have evolved by horizontal gene transfer of a particular set of characteristics that have successfully persisted in the host.[5,6] Importantly, diarrheal patients are more prone to potentially fatal infections of carbapenem-resistant *E. coli*.

Carbapenems are important therapeutic agents for the treatment of infections of extended-spectrum β-lactamase (ESBL)-producing isolates.[7] Bacteria that possess the New Delhi metallo-β-lactamase (NDM) enzyme, the main carbapenemase detected in children,[8] are resistant to almost all β-lactams antibiotics.[9] NDM-1, which first emerged in 2009,[10] has become more of a global threat than other metallo-β-lactamases. Mobile-resistance elements, such as plasmids, are the main vehicles carrying the bla_{NDM-1} gene and regarded as major mechanisms driving the dramatically increased prevalence of carbapenemase-resistant *Enterobacteriaceae* isolates.[11,12]

In this study, we reported the discovery of an *E. coli* strain isolated from a diarrhea sample from a pediatric patient carrying a transferable IncC-type plasmid of pNDM-TCM3e1 with two copies of bla_{NDM-1} and $bla_{CTX-M-61}$.

Methods

Strain Information and Case History

A 4-month-old boy presented to the Affiliated Hospital of Changchun University of Traditional Chinese Medicine with abdominal pain, diarrhea, coughing, and wheezing accompanied by fever (the highest temperature reaching 38.5°C) for 3 days in June 2019. He received nonstandard antibiotic and antiviral treatment, which included injection of cefixime and the antiviral drug vidarabine monophosphate in another hospital 2 days before admission. On admission, his temperature was 38°C with a white blood cell count of $21.74×10^9$ /L, platelet count of $735×10^9$ /L, 0.34% neutrophils, and 0.52% lymphocytes. The *E. coli* isolates from fecal samples were extremely resistant to multiple antimicrobial agents. Subsequently, the boy received treatment consisting of an antibiotic (ceftazidime), antiviral (potassium sodium dehydroandrographolide succinate), and traditional Chinese medicine, including an ointment at the acupoint, manipulation, and chiropractic therapy for children for one week. On follow-up, the patient had quickly recovered.

Bacterial Isolates and Identification

Nine *E. coli* strains were isolated from a fecal specimen collected from the patient with the use of MacConkey agar with no antibiotic or containing 4 mg/L of imipenem or 8 mg/L of cefotaxime. Bacterial species identification was performed by 16s rRNA sequencing and further confirmed with a BD Phoenix™-100 Automated Microbiology System (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The genetic linkages of the nine *E. coli* strains were investigated using the ERIC-PCR technique.[13]

Antimicrobial Susceptibility Testing and bla_{NDM} Location

Antimicrobial susceptibility testing of 12 antimicrobial agents was performed using E-test strips (bioMérieux Sweden AB, Askim, Sweden) in accordance with the 2019 Performance Standards for Antimicrobial Susceptibility Testing of the Clinical and Laboratory Standards Institute (CLSI; https://clsi.org/). Susceptibility to imipenem was determined by the micro-broth dilution and the breakpoints of the other tested antibiotics were interpreted according to the CLSI-2019 guidelines. *E. coli* ATCC 25922 served as a quality control strain for susceptibility testing. The presence of key carbapenemase-encoding genes (bla_{NDM}, bla_{IMP}, bla_{VIM}) and ESBL genes (bla_{SHV}, bla_{TEM}, bla_{CTX-M}) were established by PCR and confirmed by DNA sequencing.

Previous reports have confirmed that most antibiotic resistance markers (bla_{NDM-1}, bla_{TEM}, bla_{CTX-M}) are located on plasmids.[14–16] The transferability of bla_{NDM-1} in *E. coli* strain TCM3e1 was determined by filter-mating using azide-resistant *E. coli* strain J53 as the recipient. Briefly, the donor and recipient strains were grown to the log-phase in Luria-Bertani (LB) broth and then mixed at a ratio of 1:3 and applied to a 0.22-μm filter. After culturing at 37°C for 16 h, the transconjugants putatively possessing the bla_{NDM-1}-bearing plasmid were screened on LB agar supplemented with imipenem (32 mg/L) and sodium azide (100 mg/L). PCR amplification and sequencing of the transconjugants were subsequently performed to confirm whether the plasmid harboring the antibiotic resistance marker was successfully transferred to the recipient. To determine whether the bla_{NDM-1} gene was expressed in *E. coli* strains TCM3e1 and *J53* AziR and the transconjugant strain J53-pTCM3e1, testing with imipenem was performed using E-test strips to evaluate the resistance to lactam antibiotics.

Screening of Virulence Genes

The presence of virulence genes and virulence-associated genes, including eae, F4, F5, F6, F18, F41, LT, STa, STb, EAST1, Stx1, Stx2, and Stx2e, in *E. coli* strain TCM3e1 was investigated by PCR with the use of ExTaq DNA polymerase (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) and gene-specific primers as described previously.[17–19]

Genome Sequencing and Component Prediction

Genomic DNA of *E. coli* strain TCM3e1 was extracted using the sodium dodecyl sulfate method.[20] The harvested DNA was separated by agarose gel electrophoresis, quantified with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and sequenced using the PacBio Sequel platform (Pacific Biosciences, Menlo Park, CA, USA) and NovaSeq 6000 System (Illumina, Inc., San Diego, CA, USA), which was performed by Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

A draft genome was assembled using the single molecule real-time (SMRT) Link v.5.0.1 program (Pacific Biosciences) and then corrected with Illumina data. Next, based on the overlap between the head and tail, the chromosomal sequence formed a circle. Then, the initial site was corrected in reference to the DNA database with the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, the chromosome and plasmid sequences were screened against the plasmid database using BLAST. The *E. coli* coding genes were predicted using the GeneMarkS program (http://opal.biology.gatech.edu/GeneMark/) and interspersed repetitive sequences were predicted using the RepeatMasker program (https://www.repeatmasker.org/). The Tandem Repeats Finder database (https://tandem.bu.edu/trf/trf.html) was searched to identify tandem repeats. The tRNAscan-SE web server (http://lowelab.ucsc.edu/tRNAscan-SE/) was used to predict genes coding for tRNA, while the rRNAmmer server (http://www.cbs.dtu.dk/services/RNAmmer/) was used to identify genes coding for rRNA. Small nuclear RNA was predicted with the use of BLAST against the Rfam database. The IslandPath-DIOMB program was used to predict the genomics islands, and the TransposonPSI tool (http://transposonpsi.sourceforge.net/) was used to identify transposons based on the homologous BLAST method. The PHAge Search Tool was used to identify prophages (http://phast.wishartlab.com/) and clustered regularly interspaced short palindromic repeat sequences (CRISPR) were identified with the CRISPRfinder program (https://crispr.i2bc.paris-saclay.fr/Server/). The online server Multi-Locus Sequence Typing (MLST) v 2.0 (http://www.cbs.dtu.dk/services/MLST-1.3/) was used to determine the sequence type of *E. coli* strain TCM3e1 with the assembled contigs. The serotype was predicted from the whole-genome sequences of *E. coli* isolates using the SeroTypeFinder database (https://cge.cbs.dtu.dk/services/SerotypeFinder/).

Gene Function Analysis

Seven databases were used to predict gene functions in this study, which included Gene Ontology (http://geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), Clusters of Orthologous Groups (http://www.ncbi.nlm.nih.gov/COG/), Non-Redundant Protein Database (https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/), Transporter Classification Database (http://tcdb.org/), and Swiss-Prot (http://www.ebi.ac.uk/uniprot/). A whole genome search was performed against the above seven databases using BLAST. Virulence genes were identified using VirulenceFinder v2.0 software of the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/VirulenceFinder/). Analysis of antimicrobial resistance genes was performed using the Antibiotic Resistance Genes Database (https://ardb.cbcb.umd.edu/) and Comprehensive Antibiotic Research Database (https://card.mcmaster.ca/).

Plasmid Analysis

Plasmid DNA was extracted using a HiSpeed Plasmid Midi kit (Qiagen, Valencia, CA, USA) and sequenced using the NovaSeq 6000 System with 350-bp paired-end reads, which was performed by Beijing Novogene Bioinformatics Technology Co., Ltd. Identification of plasmid incompatibility types was performed with the use of complete plasmid sequences via the PlasmidFinder v2.0 tool (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The plasmid sequences were annotated using the Rapid Annotations using Subsystems Technology server (https://rast.nmpdr.org/) and edited manually. Sequence alignment of bla$_{NDM-1}$-carrying plasmids was performed using BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) or the conserved domain database Domain

(https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and visualized with the application Easyfig v 2.2.3.[21] Highly homologous complete plasmid sequences available in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) were aligned using the BLAST Ring Image Generator (BRIG) tool. Circular maps of chromosomes and plasmids were also generated using the BRIG tool.[22]

Results

Antimicrobial Susceptibility Analysis

The results of the BD Phoenix™-100 Automated Microbiology System and 16s rRNA sequencing confirmed that nine ESBL-producing *E. coli* strains shared the same biochemical phenotype and were resistant to 15 classes of antibiotics, thus, considered as multi-drug resistant. The ERIC-PCR results revealed that all nine *E. coli* strains had the same ERIC pattern (ERIC-types), as shown in Figure S1. Therefore, these strains were uniformly named *E. coli* strain TCM3e1 in this article. The antimicrobial susceptibility test results determined that *E. coli* strain TCM3e1 was resistant to multiple antimicrobial agents, including cephalosporins, carbapenems, penicillins, sulfonamides and tetracyclines, and susceptible to quinolones, chloramphenicols, aminoglycosides, and polymyxin according to the CLSI breakpoints (M100-S29) (Table 1).

Detection of Antibiotic Resistance Genes and Conjugation Experiment

PCR amplification and sequencing confirmed that *E. coli* strain TCM3e1 harbored the bla_{NDM-1} and bla_{TEM} genes. Furthermore, the results of the conjugation assays showed that bla_{NDM-1} was successfully transferred from *E. coli* strain TCM3e1 to laboratory *E. coli* strain J53 at a frequency of 10^{-6}, suggesting that bla_{NDM-1} was located on a self-transmissible plasmid, while transfer of bla_{TEM-1} was unsuccessful (Figure S2). The transconjugant (designated J53-pTCM3e1) was resistant to both of the tested β-lactams (imipenem and meropenem). Interestingly, strain J53-pTCM3e1 exhibited a resistance profile similar to that of the parental isolate *E. coli* strain TCM3e1, with the exception of tetracycline (Table 1).

Genomic Sequence Analysis

Assembly of whole-genome sequencing data revealed that *E. coli* strain TCM3e1 consisted of a 4,994,807-bp circular chromosome and two circular plasmids (Table 2). The chromosome had a GC content of 50.51% and coded for 22 rRNA operons, 88 tRNAs, 61 nRNAs, and 5052 predicted protein coding sequences (CDSs) (Table 2 and Figure 1). MLST analysis showed that *E. coli* strain TCM3e1 belonged to sequence type 1722 (ST1722) (adk-fumC-gyrB-icd-mdh-purA-

Table 1 Antimicrobial Susceptibility Testing of *E. coli* Strain TCM3e1 Isolates and Associated Transconjugants

Antibiotics	MIC (mg/mL)/Antimicrobial Susceptibility		
	TCM3e1	**J53-pTCM3e1**	**J53**
Ampicillin	≥256/R	>16/R	= 4/S
Cefazolin	>16/R	>16/R	≤4/S
Ceftazidime	≥256/R	>16/R	≤1/S
Cefotaxime	≥256/R	>32/R	≤1/S
Cefepime	≥96/R	>16/R	≤2/S
Imipenem	8/R	8/R	≤1/S
Meropenem	8/R	8/R	≤1/S
Piperacillin	>64/R	>64/R	≤4/S
Amoxicillin-clavulanate	16/R	>16/8/R	≤4/2/S
Ampicillin/sulbactam	>16/8/R	>16/8/R	≤4/2/S
Piperacillin-tazobactam	>64/4/R	>64/4/R	≤4/4/S
Trimethoprim-sulfamethoxazole	>2/38R	>2/38R	≤0.5/9.5/S
Tetracycline	≥256/R	≤2/S	≤2/S

Note: These data are derived from the 2019 CLSI guidelines.
Abbreviations: S, sensitive; R, resistant; I, intermediately resistant.

Table 2 Genome and Plasmids of *E. coli* Strain TCM3e1

Genomic Structure	Size	GC Content	CDS No.	Accession No.	Resistance Genes Present	Replicon Type
TCM3e1 chromosome	4,994,807 bp	50.51%	5030	CP066806	bla_{CMY-62}, *arnA*, *PmrB*, *PmrC*, *PmrF*, *fyuA*, *kdpE*, APH (3")-Ib, *sul2*, APH (6)-Id, *PmrE*, bla_{TEM-1}, $bla_{CTX-M-105}$	IncQ
pTCM3e1-1	131,861 bp	47.21%	182	CP066807	bla_{TEM-1}	IncFIB
pTCM3e1-2	191,962 bp	51.72%	254	CP066808	bla_{NDM-1}, *bleo*, *sul1*, *aadA17*, *dfrA12*, $bla_{CTX-M-61}$	IncC

recA). Further analysis determined that strain TCM3e1 had three replicon types, IncFIB, IncC, and IncQ, which is uncommon for plasmids associated with bla_{NDM-1} (Table 2).

Plasmid pTCM3e1-1 contained 182 CDSs and sequence analysis revealed query coverages of 47.02–66.97% and sequence identities of 96.59– 98.59% with five plasmids listed in Table 3 at the nucleotide level. The *E. coli* species harbored all five of the plasmids (Figure S4). Similarly, complete sequence analysis revealed that the 191,962-bp plasmid pTCM3e1-2 contained 254 CDSs. Plasmid pTCM3e1-2 had query coverages of 65.98–87.47% and sequence similarities of 99.22–99.99% with another five plasmids in Table 3. Surprisingly, none of these plasmids, as opposed to plasmid
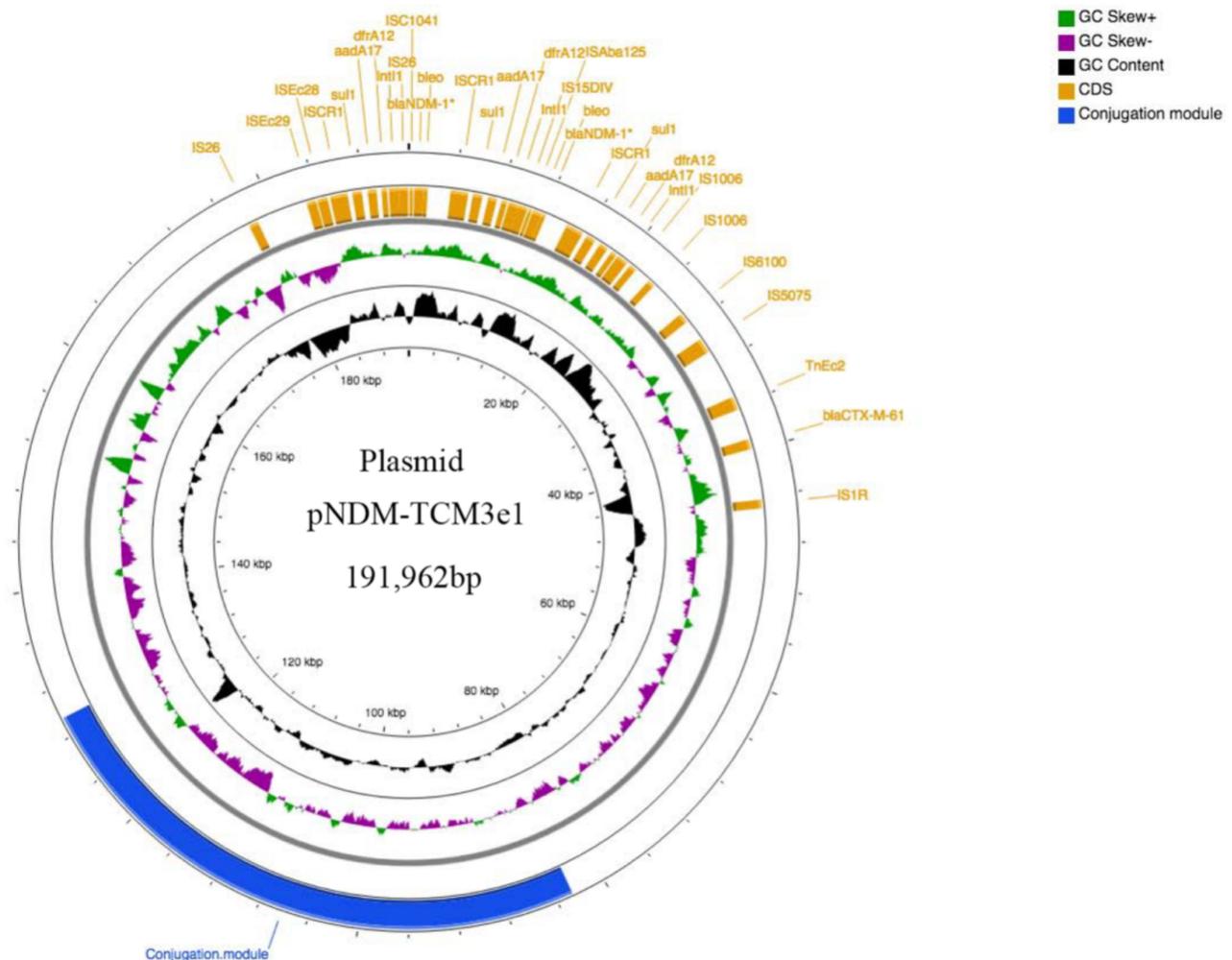

Figure 1 Genetic structure of plasmid pNDM-TCM3e1. This circular graph was created to illustrate the backbone and the location of the genetic load region of pNDM-TCM3e1. Genes are denoted by yellow rectangles. The GC skew is depicted as the inner circle in black and the GC content as the outer circle in green and purple. The blue region represents conjugation.

Table 3 Match in GenBank of the *E. coli* Strain TCM3e1 Plasmid

	GenBank_ID	Length (bp)	Covery (%)	Identity (%)	Species Origin
Plasmid pTCM3e1-1	NZ_CP015160.1	212,180	54.28	98.97	*Escherichia coli*
	NZ_LT985252.1	179,021	66.97	98.59	*Escherichia coli*
	NZ_CP031295.1	245,305	47.05	98.83	*Escherichia coli*
	NZ_CP024238.1	274,465	47.02	96.59	*Escherichia coli*
	NZ_AP018785.1	169,561	52.37	98.93	*Escherichia coli*
Plasmid pTCM3e1-2	NZ_LR697099.1	273,158	87.47	99.22	*Klebsiella pneumoniae*
	NZ_CP028197.1	444,417	65.98	99.99	*Salmonella enterica subsp.*
	NZ_CP023274.1	191,769	80.16	99.22	*Proteus mirabilis*
	NZ_LT904892.1	192,619	73.77	100	*Salmonella enterica subsp.*
	NZ_CP008824.1	319,976	69.67	99.99	*Enterobacter cloacae*

pTCM3e1-2, were derived from *E. coli*, but rather other species, including *Klebsiella pneumoniae, Proteus mirabilis*, and *Salmonella enterica* (Figure S5).

Resistance Gene Analysis

Multiple resistance genes responsible for resistance to β-lactams, aminoglycosides, polymyxin, fluoroquinolones, sulfonamide, and tetracycline were identified in *E. coli* strain TCM3e1. Specifically, 13 antimicrobial resistance genes mediating resistance to β-lactams (bla_{TEM-1}, $bla_{CTX-M-105}$ and bla_{CMY-62}), aminoglycosides [$kdpE$, $aph (3'')-Ib$ and $aph (6)-Id$], polymyxin ($arnA, PmrB, PmrC$ and $PmrF$), fluoroquinolones (mfd), sulfonamide ($sul2$), and tetracycline ($fyuA$) were coded by the genomic chromosome, while nine (bla_{NDM-1}, $sul1$, $aadA17$, $bla_{CTX-M-61}$, $dfrA12$, $mphA$, Mrx, $NmcR$, and $mphG$) were coded by plasmid pTCM3e1-2, and only one β-lactams resistance gene (bla_{TEM-1}) by plasmid pTCM3e1-1 (Table 2). Therefore, the two plasmids were designated as pTEM-TCM3e1 and pNDM-TCM3e1, respectively. Surprisingly, the whole-genome sequencing results revealed that plasmid pNDM-TCM3e1 carried two copies of the bla_{NDM-1} gene (Figure 1). Additionally, there were two or three copies of the sulfonamide resistance gene $sul1$, the aminoglycoside resistance gene $aadA17$, the diaminopyrimidine resistance genes $dfrA12$, and the bleomycin resistance protein located downstream of the bla_{DNM-1} gene, which formed a tandem repeat fragment (bla_{DNM-1}-$bleo$-$sul1$- $aadA17$- $dfrA12$), as shown in Figure 1.

Virulence of *E. coli* Strain TCM3e1 Isolates

The presence of enteroaggregative *E*. coli heat-stable enterotoxin 1 (EAST-1) in *E. coli* strain TCM3e1 was confirmed by PCR and sequencing (Figure S3). Furthermore, the virulence and virulence-related genes were identified with the use of the VirulenceFinder database. The results indicated that *E. coli* strain TCM3e1 harbored the EAST-1 heat-stable toxin gene $astA$, which was consistent with the PCR results. In addition, strain TCM3e1 harbored genes encoding regulators of the enteroaggregative immunoglobulin repeat protein (air), afimbrial adhesion (afaD), siderophore receptor ($fyuA$), heat-resistant agglutinin (bra), outer membrane protein complement resistance (traT), outer membrane hemin receptor (chuA), and long polar fimbriae (lpfA). The plasmid pTEM-TCM3e1 did not code for the afaD and traT genes. Strain TCM3e1 belonged to the serotypes O11:H25, as determined with the SeroTypeFinder database.

Discussion

Globally, diarrheal diseases remain the third leading cause of morbidity and mortality among children under 5-years-old, after pneumonia and preterm birth complications.[23] Every day, approximately 2195 children die due to diarrheal diseases, which accounts for one in nine child deaths worldwide.[24] According to the "Chinese Clinical Practice Guidelines for Acute Infectious Diarrhea in Children," the incidence of acute infectious diarrhea in Chinese children under 5-years-old is 2.50–3.38 per person-year. While many etiological agents can cause diarrhea, diarrheagenic *E. coli* is a major contributor. Importantly, >40% of diarrheal episodes among children are caused by diarrheagenic *E. coli* pathotypes.[25]

Nine *E. coli* strains were isolated from clinical fecal specimens of the patient and were identified with the BD Phoenix™-100 Automated Microbiology System. The results showed that all of the *E. coli* isolates had the same

biochemical and drug-resistant phenotype, the same ERIC patterns, and carried the same virulence gene. Significantly, all nine *E. coli* strains harbored the bla_{NDM-1} and bla_{TEM-1} genes, as confirmed by PCR amplification. The cloned strains were named *E. coli* TCM3e1 for use in future studies.

The O11:H25 serotype of *E. coli* strain TCM3e1 was identified with the use of the SeroTypeFinder database. The results showed that O11:H25 was a non-classical serotype and was difficult to classify as a particular pathotype of diarrheal *E. coli*. Nevertheless, EAST-1, which was detected in *E. coli* strain TCM3e1 by PCR and sequencing, is the first toxin characterized for the enteroaggregative *E. coli* (EAEC) pathotype.[26] EAEC has been systematically identified as an emerging enteropathogen that is strongly associated with acute and persistent diarrhea among children younger than 5 years living in developing countries.[27] Considering the significant health burden of diarrheagenic *E. coli* in children, an in-depth study of the dominant strain *E. coli* TCM3e1 was conducted.

The BD Phoenix[TM]-100 Automated Microbiology System determined that strain TCM3e1 produced ESBL and was resistant to 15 classes of antibiotics, and thus was considered as multi-drug resistant. Importantly, the *E. coli* strain TCM3e1 was resistant to all of the tested β-lactam antibiotics (Table 1). Additionally, PCR amplification and genome sequencing confirmed that *E. coli* strain TCM3e1 harbored the bla_{NDM-1} and bla_{TEM-1} genes.

NDM-1 is a broad-spectrum β-lactamase that is capable of hydrolyzing almost all β-lactam antimicrobials. NDM-1-producing Enterobacteriaceae isolates have swept across the globe since first reported in 2009[10] and the prevalence has dramatically increased recently not only in the United States and European Union, but also Asian countries.[12] Many provinces of China, including Beijing,[11] Zhejiang,[15] and Henan,[28] have reported the emergence of Enterobacteriaceae positive for the bla_{NDM-1} gene. Enterobacteriaceae isolates producing NDM-1-type carbapenemase are often called "super bugs" due to extreme drug resistance.[28] Studies have confirmed that this super resistance gene contains genetic determinants that widely exist on mobile genetic elements, such as plasmids, and thus are easily transferred to other organisms, which is also considered to be the main reason for the rapid dissemination of bla_{NDM-1} into various Enterobacteriaceae.[29] Conjugation experiments with azide-resistant *E. coli* strain J53 were conducted with the use of broth and filters. The result showed that strain TCM3e1 successfully transferred a plasmid carrying bla_{NDM-1} into recipient *E. coli* strain J53 at a frequency of 10^{-6} (transconjugant/recipient) by mating, suggesting that bla_{NDM-1} was carried on a self-transmissible plasmid. Compared to *E. coli* strain J53, the transconjugants exhibited significantly increased resistance to carbapenems (Table 1). In addition to bla_{NDM-1}, strain TCM3e1 had other resistance genes, including the ESBL genes $bla_{CTX-M-61}$ and bla_{TEM-1}, but neither was capable of transfer by conjugation.

To determine the molecular characterization of carbapenemase-resistant NDM-1-producing *E. coli* strains and the genetic location of bla_{NDM-1}, strain TCM3e1 was subjected to sequencing using the Illumina HiSeq X10 platform. The assembly of whole-genome sequencing data revealed that the bla_{NDM} gene was detected in a 191,962-bp circular plasmid (designated pNDM-TCM3e1) containing an IncC replicon. Studies have confirmed that the IncC plasmid in an important vector for the transmission of drug-resistance genes. In Guangdong, Fujian, and other provinces of China, there have been reports of IncC-type plasmids from *E. coli, Salmonella, Klebsiella pneumoniae*, and other bacterial isolates in humans and various animals, including pigs, dogs, cattle, chickens, fish, and shrimp. This type of plasmid is the main type of conjugation plasmid carrying the bla_{NDM} and bla_{CMT} genes in China.[30–32] Surprisingly, strain TCM3e1 harbored two copies of bla_{NDM-1}, both of which were present on pNDM-TCM3e1. The two bla_{NDM} genes were identical in sequence, but present at different locations on pNDM-TCM3e1. Importantly, there were two or three copies of the class 1 integrin integrase IntI1 with the *aadA17–dfrA12* cassette array, *sul1* (encoding sulfonamide resistance protein), and bleomycin resistance protein that were all located downstream of the bla_{DNM-1} gene and formed a tandem repeat fragment (bla_{DNM-1}-*bleo-sul1-aadA17-dfrA12*), suggesting that this duplicated region may be transferred horizontally among different strains as a whole (Figure 1). Most previous studies have reported only one copy of the bla_{DNM-1} gene coded by the chromosome or plasmid, as the coexistence of two bla_{DNM-1} genes has only been described in the chromosomes of an ST167 *E. coli* strain Y5 in China (GenBank accession no. CP013483)[12] and *Pseudomonas aeruginosa* strain MMA83 (GenBank accession no. HF546976) in Serbia.[33] To our knowledge, this is the first report of two bla_{NDM-1} gene copies coded by a single plasmid. The genetic environment of the bla_{NDM-1} gene in the plasmid of strain TCM3e1 showed certain similarities to, but was general different from, the nucleotide sequences of the two strains analyzed. As observed in other bla_{NDM-1} gene environments, ISAba125 insertion sequences were identified immediately upstream of the bla_{NDM-1} genes (Figure 2), which provides the −35 promoter sequence for the bla_{NDM-1} gene.[34] The
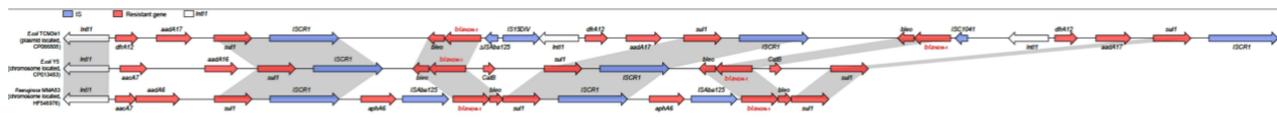
Figure 2 Alignment of sequences surrounding the region of bla_{NDM-1}. Genes are denoted by arrowheads and colored based on gene function classification. Red letters are for bla_{NDM-1}. Resistant genes are indicated with red arrowheads. IS elements are indicated in blue. White arrows indicate integron IntI1. The tandem repeat fragment surrounding the bla_{NDM-1} gene is highlighted with a light grey frame.

Abbreviations: bla_{NDM-1}, New Delhi metallo-β-lactamase gene; $bleo$, bleomycin resistance gene; $sul1$, sulfonamide resistance gene; $aadA17$, aminoglycoside 3-N-acetyltransferase ANT-(3"); $dfrA1$, dihydrofolate reductase; IS, insertion sequence; $intI1$, integron gene.

genetic organization downstream of the bla_{NDM-1} genes in strain TCM3e1 is the same as *P. aeruginosa* strain MMA83, encompassing a bleomycin resistance gene followed by the $sul1$ gene. In addition, other antibiotic-resistance genes, such as the aminoglycoside resistance gene $aadA17$ and the diaminopyrimidine resistance gene $dfrA12$, appeared after the $sul1$ and the integrin-1 ($intI1$) genes, which were downstream of the $dfrA1$ gene. It has been reported that the IS$CR1$ mobile element was usually associated with $intI1$, at a point where cassette integration usually occurred.[35] IS$CR1$, which was first reported by Wang et al, is usually neighbored by a wide array of antibiotic resistance genes and exhibits potential for mobilizing adjacent antibiotic-resistance genes.[36,37] Considering the important role of the IS$CR1$ element in the transposition of antibiotic resistance genes, further analysis of the insertion sequence revealed three tandem copies of the IS$CR1$ element surrounding bla_{NDM-1}, suggesting that bla_{NDM-1} in *E. coli* strain TCM3e1 was carried in a similar IS$CR1$ element in *E. coli* strain Y5 and *P. aeruginosa* strain MMA83, although the sequences within the element were significantly different (Figure 2). Additionally, *E. coli* strain TCM3e1a harbored a composite Tn structure consisting of two insertion sequences (IS$CR1$ and IS$Aba125$). Given that both IS$CR1$ and IS$Aba125$ are capable of transfer and the important role played by the IS$CR1$ element in the genetic transmission of bla_{NDM-1}, future studies are warranted to explore the effect of this insertion region on the transfer efficiency of the pNDM-TCM3e1 plasmid.

Importantly, the copy number of the bla_{NDM-1} gene in this strain had no effect on carbapenemase resistance. The minimum inhibitory concentration of imipenem against *E. coli* strain TCM3e1 carrying two copies of the bla_{NDM-1} gene was 8 μg/mL, which was equal to or lower than that of strains with a single copy of bla_{NDM-1}.[38] Our presumption is that the increased copy number of bla_{NDM-1} gene in pNDM-TCM3e1 of strain TCM3e1 did not convey higher resistance to carbapenemase as compared with that of a plasmid with a single copy as the result of one or even both of the following reasons (i) two copies of the bla_{NDM-1} gene were transferred as a block from an ancestor strain and as a nonfunctional multiplication mechanism, and (ii) as a hydrolase, NDM-1 is able to hydrolyze all β-lactam antimicrobials, with the exception of aztreonam, as its activity is not only affected by the expression of the bla_{NDM-1} genes, but also by substrate or/and enzyme inhibitors, such as metal chelators and Ryukyu compounds. Hence, future studies are needed to elucidate the effects of carbapenemase-resistance and the exact mechanism for the repeated fragment.

Conclusion

This is the first report of an *E. coli* clinical isolate carrying two copies of the bla_{NDM-1} gene on a IncC transferable plasmid, although the coexistence of two bla_{NDM-1} genes on the chromosome was reported and a plasmid coding for one bla_{NDM-1} gene is already widespread. This study provided new data on the complexity and diversity of genetic features associated with the bla_{NDM-1} gene, which are of great importance for further analyses of the dissemination routes and mechanisms, including clonal dispersions of the bla_{NDM-1} gene within clinical isolates.

Accession Number(s)

The complete sequences of the chromosome and two plasmids of *E. coli* strain TCM3e1 have been deposited in the GenBank database under accession numbers CP066806, CP066807, and CP066808, respectively.

Ethics Statement

The study protocols were approved by the Ethics Committee of Changchun Children's Hospital (Changchun Traditional Chinese Medicine University, Changchun, China) (Review No.: CCZYFYLL9) and signed informed consent was

obtained from the guardians of the patient, which included consent to publish the case details and any accompanying images.

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 declare that none have any financial or non-financial conflicts of interest associated with this study.

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