

Characterization of the IncFII-IncFIB(pB171) Plasmid Carrying *bla*_{NDM-5} in *Escherichia coli* ST405 Clinical Isolate in Japan

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Yoko Takayama^{1,2}
Tsuyoshi Sekizuka³
Hidehito Matsui⁴
Yuzuru Adachi⁴
Ryotaro Eda⁵
Shin Nihonyanagi²
Tatsuhiko Wada²
Mari Matsui⁶
Satowa Suzuki⁶
Masashi Takaso⁷
Hidero Kitasato⁵
Makoto Kuroda³
Hideaki Hanaki⁴

¹Department of Infection Control and Infectious Diseases, Research and Development Center for New Medical Frontiers, Kitasato University School of Medicine, Kanagawa, Japan; ²Department of Infection Control and Prevention, Kitasato University Hospital, Kanagawa, Japan; ³Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan; ⁴Infection Control Research Center, Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan; ⁵Department of Microbiology, School of Allied Health Sciences, Kitasato University, Kanagawa, Japan; ⁶Antimicrobial Resistance Research Center, National Institute of Infectious Diseases, Tokyo, Japan; ⁷Department of Orthopaedic Surgery, Kitasato University School of Medicine, Kanagawa, Japan

Correspondence: Yoko Takayama
1-15-1 Kitasato, Minami-Ku, Sagami-hara,
Kanagawa 252-0374, Japan
Tel/Fax +81-42-778-9119
Email yoko@med.kitasato-u.ac.jp

Purpose: New Delhi metallo-β-lactamase 5 (NDM-5) shows stronger resistance to carbapenems and broad-spectrum cephalosporins than NDM-1 because NDM-5 differs from NDM-1 by two amino acid substitutions. In this study, our aim was to characterize a NDM-5-producing *Escherichia coli* isolate KY1497 from a patient with urinary tract infection in Japan, who had no recent history of overseas travel.

Patients and Methods: NDM-5-producing *E. coli* isolate KY1497 was detected in the urine sample of a patient hospitalized in a tertiary hospital in Japan. The complete genome sequence of isolate KY1497 was determined by short- and long-read sequencing with hybrid assembly, followed by multilocus sequence typing (MLST), core-genome phylogeny analysis, plasmid analysis, and transconjugation experiments.

Results: KY1497 was classified as ST405 by MLST, and core-genome phylogeny exhibited the closest lineage to the clinical isolates in Nepal (IOMTU605) and Canada (FDAARGOS_448). KY1497 harbors *bla*_{NDM-5} in the IncFII-IncFIB(pB171) replicon plasmid (pKY1497_1, 123,767 base pairs). Plasmid analysis suggested that the cognate plasmids of pKY1497_1 have a minor plasmid background, rather than the globally disseminated IncX3 plasmid carrying *bla*_{NDM-5}. Transconjugation analysis revealed that pKY1497_1 is transmissible to the recipient *E. coli* J53 strain.

Conclusion: We characterized a novel Inc replicon plasmid (IncFII-IncFIB[pB171]) carrying *bla*_{NDM-5} and its host *E. coli* strain. NDMs are associated with a high risk of infection worldwide because of their antibiotic resistance and untreatable and hard-to-treat infections. Other patients in the hospital showed negative results for carbapenem-resistant *Enterobacteriaceae*. As NDM-producing strains are only sporadically detected in Japan, attention should be provided to the community prevalence of NDM-producing *E. coli* strains to prevent nosocomial infections.

Keywords: *bla*_{NDM-5}, *Escherichia coli*, sequence type 405, IncFII, IncFIB

Introduction

Bacterial resistance due to β-lactamase is increasingly associated with carbapenemases encoded by various plasmids. Among these newly emerging carbapenemases, New Delhi metallo-β-lactamase 1 (NDM-1) was first reported in 2009.¹ NDMs can hydrolyze all β-lactams, but not monobactams, and are associated with a high risk of causing a global health crisis.

NDM-5 is a variant that differs from other NDM enzymes because it contains two substitutions (Val88Leu and Met154Leu) and shows increased resistance to carbapenems and broad-spectrum cephalosporins.² In 2011, NDM-5 was first

identified in the UK in a strain of *Escherichia coli* isolated from a patient with a recent history of hospitalization in India.² *Escherichia coli* strains possessing NDM-5 were subsequently reported to be prevalent in Denmark, France, and Algeria.³ In Japan, detection of an NDM-5-producing clinical isolate of *E. coli* was first reported in 2014; this isolate belonged to sequence type (ST) 540, and the patient had traveled to Bangladesh.⁴ Herein, we report the first detection of an NDM-5-producing *E. coli* strain belonging to ST405 in Japan.

Materials and Methods

Bacterial Isolates

In October 2015, a 79-year-old man was admitted to Kitasato University Hospital with cervical spinal cord injury causing respiratory muscle paralysis and upper and lower limb weakness. The patient developed pneumonia on day 5 of hospitalization; therefore, empirical antimicrobial treatment with vancomycin (VCM) (1 g twice daily) and tazobactam/piperacillin (TAZ/PIPC) (3.5 g three times daily) was initiated. Notable pathogens including gram-positive pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* were not cultured from the blood, sputum, and pleural effusion specimens. Therefore, VCM treatment was discontinued after 6 days, while TAZ/PIPC treatment was continued to manage the patient's condition for 29 days. On hospital day 52, the patient developed a urinary tract infection, and a strain of carbapenem-resistant *E. coli* (KY1497 strain) was isolated from a urine specimen, although the other patients in the hospital showed negative results for carbapenem-resistant *Enterobacteriaceae*. The carbapenem-resistant *E. coli* was continuously isolated from the patient's stool and urine specimens as colonies. The cardiopulmonary function of the patient gradually weakened, respiratory failure progressed, and the patient died on day 149. He had been admitted directly to our hospital without a history of international travel.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolate was determined by microdilution according to the Clinical and Laboratory Standards Institute (CLSI) reference methods,⁵ except that European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints⁶ were used to evaluate tigecycline and polymyxin B. Two disks of ceftazidime and sodium mercaptoacetic acid (Eiken Chemical Co., Ltd.,

Tokyo, Japan) were used as indicators of metallo- β -lactamase production.

Antimicrobial Resistance Gene Screening and Molecular Typing

Polymerase chain reaction (PCR) was performed to detect the *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{OXA-48}, and CTX-M-1 group genes in the isolate.⁷⁻⁹ The bacterial PFGE plug was digested with S1 nuclease, followed by PFGE using a previously reported method with some modifications,¹⁰ and visible DNA bands, which were possible plasmids, were excised to extract DNA.

Whole-Genome Sequencing

DNA libraries were constructed using the Nextera XT sample prep kit according to the manufacturer's instructions (Illumina, Inc., San Diego, CA, USA), followed by next-generation sequencing (Miseq, Illumina, Inc.).¹¹ We performed long-read sequencing with PacBio RSII and obtained the resulting unitigs with HGAP v. 4.0 de novo assembler, followed by error-correction and complete genome sequence determination by Illumina short-read sequencing. Genome annotation was carried out using DFAST.¹² Strain genotyping was determined in silico by MLST (<http://cge.cbs.dtu.dk/services/MLST/>).

Plasmid Conjugation

Plasmid conjugation using the broth method¹³ was carried out between the *bla*_{NDM5}-positive isolate KY1497 and sodium azide-resistant *E. coli* J53 as the recipient strain. Transconjugants were selected on selection plates supplemented with a combination of ceftriaxone (8 mg/L) and sodium azide (100 mg/L), and the presence of the NDM-5 gene was confirmed by PCR.

Ethics Statement

This study was approved by the research ethics committee of Kitasato University Hospital (approval no. B17-123) and complied with the Declaration of Helsinki. Written informed consent was obtained from the patient for publication of this case report.

Results and Discussion

Comparative Analysis of the IncFII-IncFIB Plasmids Harboring Carbapenemases

The complete genome sequence of KY1497 suggested that it belongs to ST405 and O102: H6 (Figure 1A) and carries

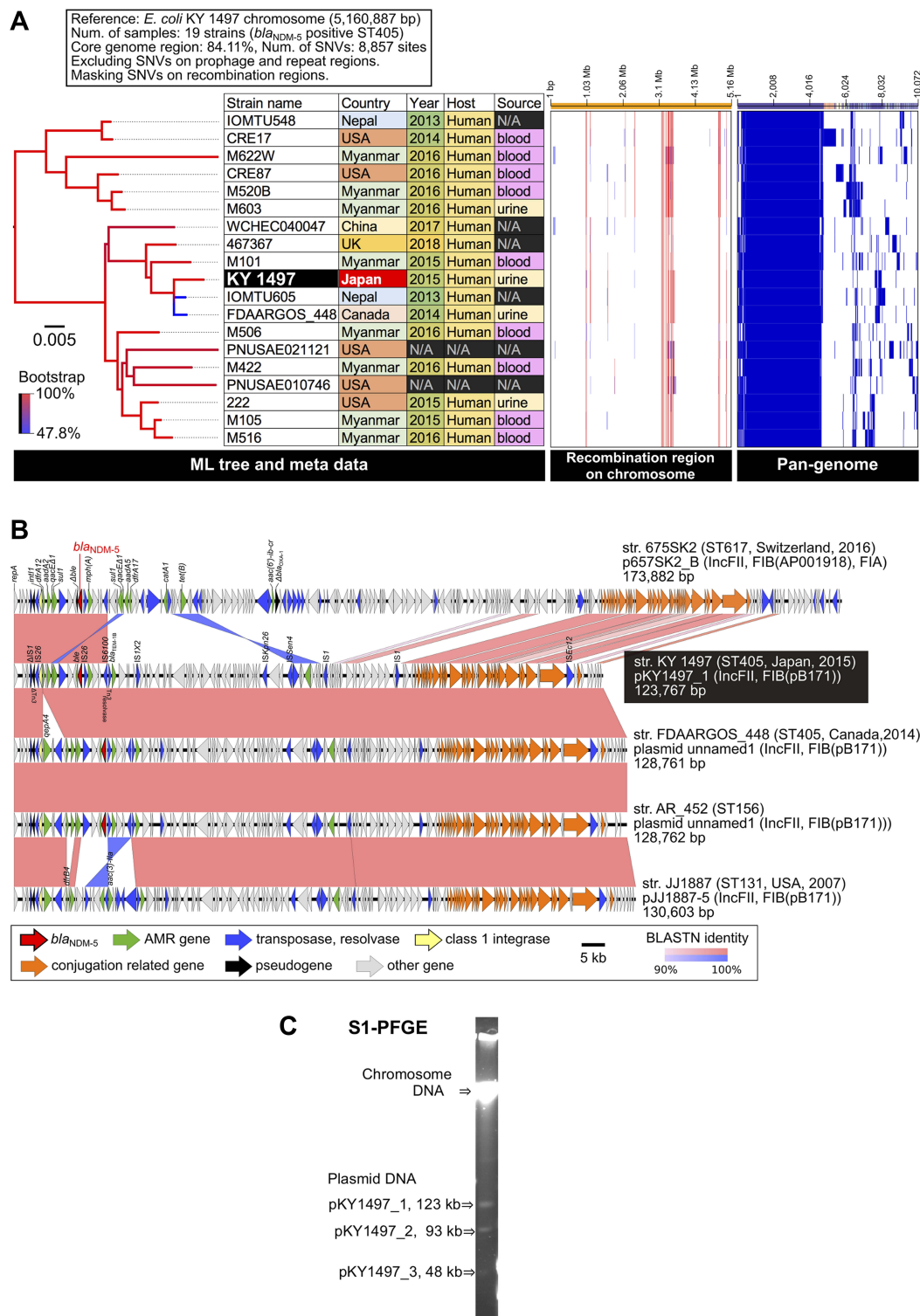


Figure 1 Comparative genomic analysis among 19 strains of *bla*_{NDM-5}-positive *E. coli* ST405. **(A)** Core-genome SNV analysis and pan-genome analysis. Detected SNVs in the repeat and prophage regions were excluded. Recombination regions of the chromosome were predicted using Gubbins v. 2.3.4, followed by masking SNVs in the recombination regions. A maximum likelihood phylogenetic tree was constructed from 8,857 SNV sites in the core genome region. Pan-genomic analysis was performed using Roary v. 3.12.0. The recombination region and pan-genomic data were visualized using Phandango. The red and light blue bars indicate recombination regions of the ancestral type and single isolate, respectively. The blue bars in pan-genomic data indicate the presence of a gene cluster. **(B)** Comparative representation of complete *bla*_{NDM-5}-positive plasmid sequences among five *E. coli* strains. The plasmid sequences were aligned using BLASTN, followed by visualization using Easyfig. Similarity of homologous and inversion blocks is indicated in red and blue, respectively. The backbone of the IncFII-IncFIB(pBI71) plasmids is highly conserved among the different ST types. **(C)** S1-PFGE analysis of *E. coli* KY1497.

three plasmids. *bla*_{NDM-5} is located on the 123.7-kb plasmid pKY1497_1, which is an IncFII-IncFIB(pB171) replicon type (Figure 1B). S1-PFGE revealed three plasmid bands with lengths corresponding to those of the complete genome sequences (Figure 1C). Core-genome phylogeny of *bla*_{NDM-5}-positive *E. coli* ST405 (19 strains in total) suggested that KY1497 had the most similar lineage to clinical isolate IOMTU605 in Nepal and FDAARGOS_448 in Canada, with 38 and 39 single nucleotide variants (SNVs), respectively (Figure 1A). These two strains carry the homologous *bla*_{NDM-5}-positive plasmid (Figure 1B). Pair-wise alignment of pKY1467_1 displayed homologous regions with other IncFII-IncFIB(pB171) plasmids except for the *qepA4* quinolone-resistance gene (Figure 1B). Regarding the IncFII-IncFIB(pB171) background, pKY1497_1 shares most of its plasmid backbone, whereas pJJ1887-5 in *E. coli* JJ1887 (ST131) carries other antimicrobial resistance genes rather than *bla*_{NDM-5}, indicating that pJJ1887-5 is one of the most common ancestral plasmids for *bla*_{NDM-5} acquisition.¹⁴ We detected *bla*_{NDM} variants on plasmids >100-kb in size, with IncF, IncA/C, and untypeable replicons. Previous reports have indicated that the IncX3-type plasmid plays a major role in the global dissemination of NDM-producing *Enterobacteriaceae*.^{11,15,16} In this study, molecular characterization of KY1497 revealed that it carried *bla*_{NDM-5} in a 123.7-kb plasmid harboring IncFII-IncFIB(pB171), suggesting that IncF has a similar role of dissemination as IncX3.

Strain Features

In the isolate, *bla*_{NDM} was the only carbapenem-resistance gene detected. The MLST analysis classified *E. coli* (KY1497 strain) as ST405, suggesting that *E. coli* ST405 strains have the potential to become a reservoir for the *bla*_{NDM-5} gene. NDM-5-producing *E. coli* belonging to ST405 was previously detected in Spain and Italy.¹⁷ *Escherichia coli* ST405 was found to carry *bla*_{CTX-M}, *bla*_{NDM}, and a repertoire of virulence genes comparable to those in O25b: H4ST131.¹⁸ According to a previous study, among NDM-producing *E. coli*, ST405 was the fourth most common reported ST and the most abundant ST in Nepal and Europe, showing the highest distribution in the UK and Italy.¹⁹ Sporadic occurrence of NDM-5 producers in Japan has been reported,^{3,4} however, NDM-5-producing *E. coli* belonging to ST405 has not been detected.

Transferability of *bla*_{NDM-5}

KY1497 was resistant to fluoroquinolones and all β -lactams, including broad-spectrum cephalosporins and carbapenems, whereas it remained susceptible to tigecycline. The antimicrobial susceptibilities of transconjugants derived from *E. coli* J53 were similar to those of the donor KY1497 strain, particularly for penicillin, cephalosporin, and tigecycline (Table 1). The KY1497 strain successfully transferred the resistance plasmid at a frequency of 8.3×10^{-6} , creating *E. coli* J53 KY1497T, suggesting the horizontal transfer of *bla*_{NDM-5} in the IncFII-IncFIB(pB171) plasmid. The IncF type plasmids were conjugatable, which may explain the rapid spread of the NDM-carrying isolates. Therefore, effective and feasible measures must be taken immediately to control the dissemination of these resistant plasmids.

How Does *bla*_{NDM-5} Spread?

Travelers contribute significantly to the global spread of microbes and resistance genes. KY1497 was isolated from a non-traveler, suggesting that it is caused by an autochthonous strain or transmission by undetected carriers. In this case, TAZ/PIPC was prescribed for 29 days before detecting KY1497. Long-term broad-spectrum antibiotics may enable the detection of carbapenem-resistant strains.^{20,21} As the patient stayed in a private room, environmental

Table 1 Antimicrobial Susceptibility Profile of *E. coli* Isolate with the *bla*_{NDM-5} Gene and Its Transconjugant

Antimicrobial Agent	MIC (mg/L)		
	KY1497	<i>E. coli</i> J53 KY1497 T (Transconjugant of <i>E. coli</i> J53)	<i>E. coli</i> J53
Ampicillin	>128	>128	8
Amoxicillin/clavulanate	>64/32	>64/32	8/4
Piperacillin	>128	>128	4
Tazobactam/piperacillin	>4/128	>4/128	4/2
Cefoxitin	>128	>128	4
Cefotaxime	>128	128	0.25
Ceftazidime	>64	>64	0.5
Cefepime	>128	16	≤0.06
Flomoxef	>128	16	0.12
Imipenem	16	4	0.12
Meropenem	64	4	≤0.06
Aztreonam	64	0.12	0.12
Levofloxacin	64	0.25	≤0.06
Gentamicin	2	0.5	1
Amikacin	4	2	4
Fosfomicin	4	2	0.5
Tigecycline	0.25	0.25	0.25

Abbreviation: MIC, minimum inhibitory concentration.

investigation was performed after his death. Swab samples from a shelf close to the bed, hand-wash sink, drain ditch, inside of bedpan, and toilet-cleaning apparatus were tested by cultivation as possible sources of NDM-5 transmission; however, NDM-5-producing bacteria were not isolated. There have been previous reports of community-acquired NDM-producing isolates, indicating the existence of an undetected reservoir and potential transmission among colonized carriers in hospitals.^{22,23}

One limitation of this study was that the undetected reservoirs formed by NDM-5-producing isolates were not investigated. Our results strongly emphasize that while strains producing NDM enzymes are rarely reported among hospitalized patients in Japan, attention should be paid to the community prevalence of such strains to monitor future trends and prevent further horizontal spread.

Conclusion

In this study, a self-transmissible IncFII-IncFIB plasmid carrying *bla*_{NDM-5} was detected in ST405 *E. coli*, which is a novel Inc replicon plasmid. We highlighted the dissemination potential of the IncFII-IncFIB plasmids harboring *bla*_{NDM-5}. Effective infection control steps should be taken to prevent nosocomial infections.

Abbreviations

MLST, multilocus sequence typing; NDM-5, New Delhi metallo- β -lactamase 5; PCR, polymerase chain reaction; ST, sequence type; TAZ/PIPC, tazobactam/piperacillin; VCM, vancomycin.

Data Sharing Statement

The complete, annotated genome sequence of *E. coli* KY1497 has been deposited in a public database (chromosome, AP019803; pKY1497_1, AP019804; pKY1497_2, AP019805; and pKY1497_3: AP019806). The short- and long-read sequences for DNA-Seq have been deposited in the DNA Data Bank of Japan (DRA accession DRA008639; BioProject PRJDB8512; BioSample SAMD00178070; DRR accession DRR184076-DRR184080).

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Disclosure

The authors report no conflicts of interest in this work.

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