

Plasmid analysis of clinically isolated *Enterobacter cloacae* in Showa University Hospital

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Abstract

We have reported the possibility of an outbreak of a plasmid-borne carbapenemase-producing *Enterobacteriaceae* at Showa University Hospital using conjugal transfer experiments; however, we could not perform plasmid profiling and fingerprinting to identify the plasmid responsible for the outbreak in clinical isolates. Therefore, to distinguish whether the appearance of metallo- β -lactamase IMP-11 (^{bla}IMP-11)-producing *Enterobacter cloacae* (*E. cloacae*) was due to the same plasmid, we established a plasmid testing system involving plasmid isolation, typing, profiling, and fingerprinting, as well as DNA sequencing analysis of genes surrounding the carbapenemase-encoding gene. Plasmid fingerprinting is an essential tool for identifying plasmids when next-generation sequencing methods cannot be employed. Of note, an important step in fingerprinting is plasmid isolation, which is difficult when large plasmids are involved. In addition, plasmid profiling using S1 nuclease pulse-field gel electrophoresis (PFGE) Southern blotting is an important tool for profiling the size and number of plasmids in bacteria. In this study, we successfully isolated an approximately 90-kb IncL/M plasmid by employing our plasmid analysis system. Importantly, as different ^{bla}IMP-11-producing *E. cloacae* isolates carried the same type of plasmid, with similar size and fingerprinting pattern, we suggest that the isolated IncL/M plasmid is the one present in this strain.

Key words : *Enterobacter cloacae*, *Enterobacteriaceae*, outbreak, IMP, plasmid fingerprinting

Introduction

After an outbreak of a plasmid-borne carbapenemase-producing *Enterobacteriaceae* in Osaka in June 2014¹, we considered the possibility of a horizontal gene transfer occurring via plasmid transfer among bacterial species. Major institutions, such as the National Institute of Infectious Diseases, Japan, started plasmid screening using next-generation sequencing (NGS). However, exhaustive plasmid screening was delayed due to the substantial cost of NGS technologies. Hence, we explored the possibility of horizontal

gene transfer using a conjugation-based approach². However, we could not determine whether the bacteria implicated in the outbreak shared a plasmid that was potentially responsible for the outbreak. In this study, we used a set of conventional methods of plasmid identification and unveiled the plasmid-encoding carbapenemase metallo- β -lactamase (MBL) IMP-11 (^{bla}IMP-11) in *Enterobacter cloacae* (*E. cloacae*) strains. Moreover, this set suggests that certain bacteria have been sharing the plasmid since the 2014–2015 outbreak of carbapenem-producing *Enterobacteriaceae*².

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Materials and methods

Strains

We routinely screened fecal samples from patients admitted the neonatal intensive care unit (NICU) of Showa University Hospital, Tokyo, as an ongoing active survey²⁻⁴. Two *E. cloacae* isolates 2017-444

and 2017-455 displayed resistance to ceftazidime (CAZ) and cefotaxime (CTX) and were isolated between September 18, 2017 and September 21, 2017.

Susceptibility to antibiotics

Bacterial susceptibility to antibiotics was determined by microdilution, according to the procedures implemented by the National Committee of the Clinical and Laboratory Standards Institute, and tested by Bio Medical Laboratories, Inc. (Tokyo, Japan).

Phenotypic testing for MBL

Screening tests for the identification of MBL types in the clinical isolates were performed using the double-disk synergy test (DDST) using the Kirby-Bauer disks (KBDs) (Eiken Kagaku, Tokyo, Japan). The DDST was performed using sodium mercaptoacetic acid (SMA), CAZ, CTX, or imipenem (IPM)-containing disks.

Identification of *bla*IMP using polymerase chain reaction (PCR) and DNA sequencing

PCR and DNA sequencing were performed following the methods described by Yamazaki *et al.*² Briefly, total bacterial DNA was extracted from colonies grown on Luria-Bertani (LB) medium agar plates. To detect carbapenemase genes, PCR was performed on strains that were either not susceptible to carbapenems (IPM and meropenem) or resistant to third-generation cephalosporins (CTX and CAZ)

and DDST-positive. Then, the samples were screened by PCR using five primer pairs to detect several β -lactamases. The β -lactamases screened, according to the Ambler classification, belong to class A (*bla*KPC), class B (*bla*VIM2, *bla*NDM1, and *bla*IMP), and class D (*bla*OXA48). The *IMP* gene subtypes that can be amplified with the primers used included subtypes 1, 3-7, 9-11, 15, 16, 25, 28, and 29. The PCR amplicons were separated by electrophoresis on 1% agarose gels, purified using a GenElute™ column (Sigma-Aldrich, St. Louis, MO, USA), and verified using sequence analysis.

Conjugational transfer experiments

Conjugational transfer experiments were performed as previously described². Rifampicin-resistant and cefotaxime-susceptible *Escherichia coli* TUM2235 strains provided by Toho University (Tokyo, Japan) were used as conjugation recipients. Two strains of *bla*IMP-producing *E. cloacae* isolated from patients were used as donors. Donor and recipient isolates were mixed at a donor-to-recipient ratio of 1 : 5 and incubated for 3 h in 2× yeast extract-tryptone (YT) medium at 37°C. Then, transconjugant colonies were selected on LB agar plates supplemented with rifampin (200 µg/ml) and ampicillin (100 µg/ml).

Plasmid extraction and DNA sequencing

Plasmid extraction and subsequent DNA sequencing were performed as previously described². Briefly,

Table 1. Sequence primers for the isolated plasmid from clinical isolates

Primer number	Primers	Position	Sequences
1	3F	3961-3980	5'-aaaagacggtaagggtcaag-3'
2	4F	4269-4288	5'-gggaacaggcggtaaaagg-3'
3	5F	4703-4722	5'-ctaaggccaatgtacaagg-3'
4	7F	3208-3228	5'-ccgaaaccttgcgctcgttc-3'
5	8R	2231-2250	5'-acgttcagtcgacccgtctt-3'
6	9R	1910-1929	5'-atggacaggctggcagcga-3'
7	11R	3624-3643	5'-tcctgcggcagtaatgtac-3'
8	12R	1379-1398	5'-tgcagcttctactctgca-3'
9	13F	5095-5117	5'-ccatctatctaccaacagatga-3'
10	17R	835-854	5'-taatgccagctactctgaa-3'
11	33R	368-387	5'-ggcgtttcccgggtgttct-3'
12	C5R6	3289-3308	5'-gcctcatccgttccacgg-3'
13	C5R7	2898-2917	5'-ccaaggatcggccttgatg-3'
14	IM11F	3633-3652	5'-ctgccgaggagcgtcttg-3'
15	IM11R	3965-3984	5'-ttagctgcacctaccgtc-3'

Primer position indicates the position of the reference *bla*IMP-11 integron, LC179842.

large plasmids harboring *bla*_{IMP} genes were purified from the transconjugants derived from clinical isolates using a Plasmid Midiprep Kit (Qiagen, Hilden, Germany) and subjected to DNA sequencing using the primers listed in Table 1.

Plasmid fingerprinting

Isolated plasmids were digested by restricting enzyme EcoRI and separated by electrophoresis on 0.8% agarose gels.

Plasmid profiling

For plasmid profiling, S1 nuclease pulse-field gel electrophoresis (PFGE) and Southern blotting were performed with slight modifications to the protocol described by McEllistrem *et al.*⁵. Briefly, *E. cloacae* cultures were grown overnight on 2× YT plates and adjusted to an optical density of 0.6–0.9 at 450 nm. Equal amounts of bacterial suspension and 2% PFGE-quality agarose (BioRad, Hercules, CA, USA) were mixed and pipetted into 100 µl plug molds. After being solidified on ice for 10 min, the plugs were lysed and digested in a single step. Each plug was incubated in 2 ml proteinase K (ProK) buffer containing 1% sarkosyl and 100-mM ethylene-diamine-tetra-acetic-acid (pH 8.0) for 4 h at 50°C. Then, the plugs were refreshed with new ProK buffer and incubated at 50°C for 4 h. After 30 min preincubation of a 2–5 mm section of each plug in S1 nuclease buffer (Promega, Madison, WI, USA), the plasmid DNA was nicked by incubating it with 125-U S1 nuclease at 37°C for 25 min. The digested DNA was separated by electrophoresis using a CHEF-DR III drive module (BioRad, Hercules, CA, USA). Then, electrophoretically separated fragments were transferred to a nylon membrane and visualized using a *bla*_{IMP}-specific probe² labeled with digoxigenin using a PCR DIG Probe Synthesis Kit (Sigma-Aldrich, St. Louis, MO, USA).

PCR-based replicon typing

Plasmids were classified into incompatibility groups using the PCR-based replicon-typing method⁶.

Multi locus sequence typing (MLST)

Seven housekeeping genes (*dnaA*, *fusA*, *gyrB*, *leus*, *pyrG*, *rplB*, and *rpoB*) were amplified from clinical isolates by PCR. Allele number and sequence type (ST) were assigned using the MLST website (<http://bigsgdb.pasteur.fr/klebsiella/>).

Ethics Approval and Consent to Participate

This was a retrospective study, and consent was obtained using the opt-out method. The study was approved by the Ethics Committee of Showa University Graduate School of Health Sciences (Kanagawa, Japan; approval number 371).

Results

We applied DDST assays to detect MBL activity in carbapenem-resistant *E. cloacae* strains isolated from patients. Then, PCR was performed to identify MBL genes in SMA-positive strains using five primer

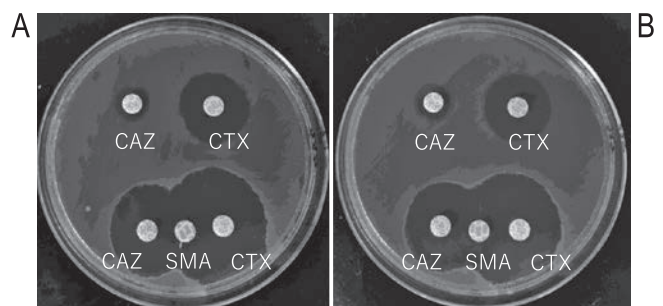


Fig. 1. Double-disk synergy test (DDST) assays of two transconjugants of *Escherichia coli* (*E. coli*) using cefotaxime (CTX), ceftazidime (CAZ), or sodium mercaptoacetic acid (SMA) disks.

Panel A: Transconjugant derived from 2017-444.
Panel B: Transconjugant derived from 2017-455.
Reproducibility was checked more than three times.

Table 2. Biological phenotypes and antibiotic susceptibilities of two isolated strains of *Enterobacter cloacae*

	Sample number	DDST	bla _{IMP}	MLST	MIC (µg/ml)			
					Cefotaxime	Ceftazidime	Imipenem	Meropenem
Clinical isolate	2017-444	+	+	ST190	>2	>8	≤1	2
	2017-455	+	+	ST190	>2	>8	2	>2
Transconjugant	2017-444	+	+	NT	NT	NT	NT	NT
	2017-455	+	+	NT	NT	NT	NT	NT

MLST, multi-locus sequence type; DDST, double-disk synergy test; MIC, minimum inhibitory concentration; NT, not tested; *bla*_{IMP-11}, metallo-β-lactamase IMP-11.

sets, including the *bla*IMP universal primer set²⁻⁴. The *bla*IMP-positive strains were further investigated using conjugation experiments, followed by DDST assays, which suggested that MBL activity is transferable from *E. cloacae* to recipient *E. coli* (Fig. 1 and Table 2). Then, plasmid isolation was performed using the transconjugants obtained by conjugation experiments. After isolating conjugative plasmids, plasmid sequence analysis by primer walking was performed (Fig. 2B and Table 1). *bla*IMP-11-positive *E. cloacae* 2017-444 and 2017-455 strains were identified; their antimicrobial minimum inhibitory concentrations and clinical characteristics are shown in Table 2 and Figure 1. These strains were selected for further study if both harbored the same plasmid. Fingerprinting of the plasmids from the two strains exhibited the same banding patterns (Fig. 3), and these plasmids belonged to the IncL/M type based on replicon-typing PCR (Table 3). Moreover, in addition to the identified transconjugants, we verified whether *E. cloacae* isolates derived from original patient fecal samples carried a single or multiple plasmids. Plasmid profiling revealed that two *E. cloacae* strains,

2017-444 and 2017-455, carried a single plasmid (~90 kb) harboring *bla*IMP (Fig. 4).

Discussion

The clinical isolates 2017-444 and 2017-455 shared a conjugative plasmid containing the *bla*IMP-11 integron. The DNA sequences of the *bla*IMP-11 integron from the two clinical isolates were identical to that of LC179842 during the 2014-2015 outbreak at Showa University Hospital, as previously described² (Fig. 2). Analysis of the two clinical isolates using plasmid profiling, plasmid fingerprinting, and PCR-based replicon typing revealed the same plasmid sizes, electrophoresis band patterns, and types. Altogether, these data suggested that these two strains carried the same IncL/M plasmid (90 kb). Moreover, integrons can be mobilized in association with functional transposons and/or conjugative plasmids^{7,8}. Therefore, it is possible that the plasmids derived from the two clinical isolates described in this study were circulating during the 2014-2015 outbreak at Showa University Hospital. To completely identify both

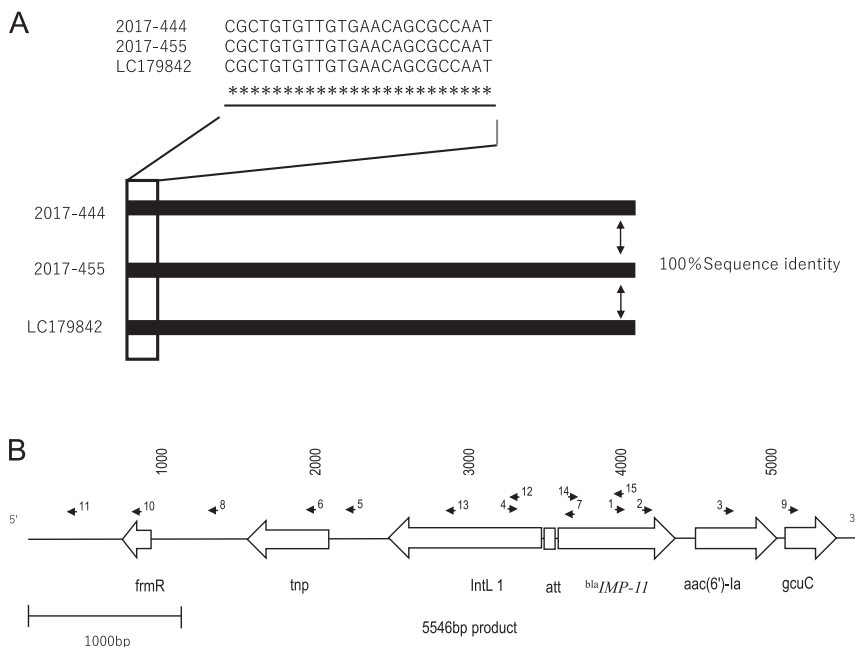


Fig. 2. (A) DNA sequences of integron region in plasmid DNA isolated from two transconjugants were identical to the reference integron LC179842.

Multiple alignments of representative partial sequence for *bla*IMP-11 integron corresponding to its 1-24 bps were generated from different clinical isolates (2017-444 and 2017-455) and reference sequence LC179842 using CLUSTAL 2.1. An asterisk indicates a position that has the same base. A solid black box shows the whole *bla*IMP-11 integron sequence. A blank black box indicates the position of the representative partial *bla*IMP-11 integron sequence. An arrowhead indicates identical integron sequences.

(B) Structure of *bla*IMP-11 class I integron LC179842.

The class I integron structure contained *frmR*, transposase (*tnp*), integrase L1 (*IntL 1*), *att*, metallo- β -lactamase IMP-11 (*bla*IMP-11), aminoglycoside 6'-N-acetyltransferase type Ia (*aac(6)-Ia*), and *gcuC*. An arrowhead indicates the primer position and direction. The number on the arrowhead indicates the primer number in Table 1.

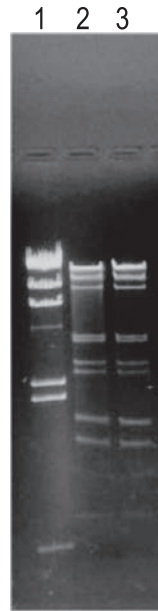


Fig. 3. Plasmid fingerprinting of two isolated strains of *Enterobacter cloacae*.

Lane 1: λ /HindIII marker. Lane 2: strain 2017-444. Lane 3: strain 2017-455. Reproducibility was checked more than three times.

Table 3. PCR-based replicon typing (PBRT) of two isolated strains of *Enterobacter cloacae*

Inc group	Clinical isolates	
	2017-444	2017-455
HI1	—	—
HI2	—	—
I1	—	—
X	—	—
L/M	+	+
N	—	—
FIA	—	—
FIB	—	—
W	—	—
Y	—	—
P	—	—
FIC	—	—
A/C	—	—
T	—	—
FIIAs	—	—
F	—	—
K	—	—
B/O	—	—

Table 3 shows whether the amplicon by each Inc group primer set⁶ was generated. Inc, incompatibility.

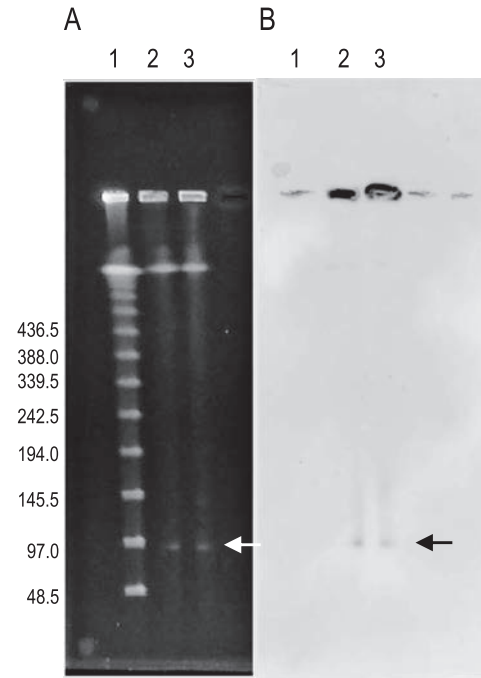


Fig. 4. (A) Pulse-field gel electrophoresis (PFGE) of S1 nuclease-digested total DNA from two isolated strains of *Enterobacter cloacae*.

Lane 1: phage λ DNA ladder (concatemers of 48.5-kb fragments). Lane 2: strain 2017-444. Lane 3: strain 2017-455.

(B) Hybridization with a specific probe for *bla*_{IMP} in two isolated strains of *Enterobacter cloacae*. Lane 1: phage λ DNA ladder (concatemers of 48.5-kb fragments). Lane 2: strain 2017-444. Lane 3: strain 2017-455. Reproducibility was checked more than three times.

plasmids, NGS would be required. However, several hospitals and institutions have not yet embraced NGS technology and are using an earlier method, that is, plasmid profiling using S1 nuclease-PFGE, Southern blotting, and plasmid fingerprinting^{9,10}. Therefore, compared with previous studies^{9,10}, the plasmid testing system we described in this study included almost all assays available for this purpose. For DNA isolation, we used the method reported by Kado *et al.*¹¹ or mini columns other than the Qiagen Plasmid Midiprep Kit. However, the DNA sequence reads from plasmids purified using these two methods were shorter than those obtained using the Qiagen Plasmid Midiprep Kit (data not shown). The protocol described in the Material and Methods section for the preparation of DNA plugs (small agarose blocks containing bacterial suspension) could be rendered more cost-effectively, as such plugs were successfully prepared more rapidly in the absence of the enzyme ProK than using the traditional protocol¹². MLST analysis revealed that the two strains belong

to the same ST190 strain (Table 2). Therefore, we could not demonstrate horizontal plasmid-borne gene transfer beyond the bacterial species isolated at Showa University Hospital. Conjugative transfer of bacterial plasmids is the most efficient method for horizontal gene propagation^{13,14}. The presence of a plasmid carrying *bla*IMP-6¹ has been suggested as a cause of the plasmid outbreak in Osaka. The *bla*IMP-1 and *bla*IMP-11 genes, which are also encoded in plasmids, were present in the positive clinically isolated samples in this study and have been previously detected on many occasions at the NICU of Showa University Hospital^{2,4}. In the future, we will perform routine plasmid analysis using our established analysis pipeline as much as possible.

Acknowledgments

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Conflicts of interest disclosure

The authors have no potential conflicts of interest to disclose.

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