A transferrable IncL/M plasmid harboring a gene encoding IMP-1 metallo-β-lactamase in clinical isolates of Enterobacteriaceae

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Abstract

Background: The worldwide spread of carbapenemase-producing Enterobacteriaceae (CPE) has reduced the clinical utility of carbapenems. Plasmids often play an important role in the spread of genes encoding drug-resistance factors, especially in the horizontal transfer of these genes among species of Enterobacteriaceae. This study describes a patient infected with three species of CPE carrying an identical transferrable IncL/M plasmid.

Methods: Clinical isolates of CPE were collected at St. Luke’s International Hospital, Tokyo, Japan, from 2015 to 2019. Three species of CPE isolates, Enterobacter cloacae, Klebsiella aerogenes and Serratia marcescens, were isolated from a patient who developed severe gallstone pancreatitis associated with bloodstream infection, with all three isolates producing IMP-1 metallo-β-lactamase. The complete sequences of the plasmids of the three isolates were determined by both MiSeq and MinION. The medical chart of this patient was retrospectively reviewed conducted to obtain relevant clinical information.

Results: The three CPE species carried an IncL/M plasmid, pSL264, which was 81,133 bp in size and harbored blaIMP-1. The genetic environment surrounding blaIMP-1 consisted of int1-blaIMP-1-aac(6’)-Ib-qacEdelta1-su11-ist8-IS21. Conjugation experiments showed that S. marcescens could transmit the plasmid to E. cloacae and K. aerogenes. In contrast, pSL264 could not transfer from E. cloacae or K. aerogenes to S. marcescens.

Conclusion: The IncL/M plasmid pSL264 harboring blaIMP-1 was able to transfer among different species of Enterobacteriaceae in a patient receiving long-term antimicrobial treatment. The worldwide emergence and spread of IncL/M plasmids harboring carbapenemase-encoding genes among species of Enterobacteriaceae is becoming a serious public health hazard.

Keywords: Carbapenemase producing Enterobacteriaceae, IncL/M, IMP-1 metallo-β-lactamase

Background

Carbapenems are often the treatment of last resort for patients with serious infections caused by gram-negative bacteria. The worldwide emergence of carbapenemase-producing Enterobacteriaceae (CPE) has limited the clinical utility of this class of antimicrobial agents [1]. The most common carbapenemases among CPE are Klebsiella pneumoniae carbapenemases (Amber class A), IMP-type metallo-β-lactamases (MBLs), VIM-type MBLs, NDM-type MBLs (class B), and OXA-48-like (class D) enzymes [2]. MBLs produced by gram-negative bacteria confer resistance to all β-lactams, except for aztreonam, and are characterized by their efficient hydrolysis of carbapenems [2, 3]. IMP-1 was first discovered in Japan in 1991 [4] and IMP-producing Pseudomonas aeruginosa and

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Enterobacteriaceae are frequently detected in patients in the Asia–Pacific region [2, 4–7].

Plasmids often play an important role in the spread of genes encoding drug resistance factors, with these plasmids often involved in the horizontal transfer of drug resistance genes among Enterobacteriaceae [8]. For example, the plasmid IncX4, which harbors mcr-1, a gene associated with colistin resistance, was shown to easily transfer between Escherichia coli and K. pneumoniae [9]. In addition, IncX3 plasmids carrying blaNDM are effectively transferred among Enterobacteriaceae species and contribute to the worldwide dissemination of NDM producers [10, 11]. IncFII(K) plasmids carrying blaIMPs and several Inc type plasmids, including IncL/M, IncN2, IncHI1B-IncFIB (Mar) and IncX3-IncC-like plasmids, carrying blavIMs, have contributed to the global spread of blaIMPs and blavIMs among different species of Enterobacteriaceae [12].

Few reports have described the in vitro and in vivo horizontal transfer of identical carbapenemase-encoding plasmids [13–16]. This study describes a patient infected with three species of Enterobacteriaceae, all of which carried a transferrable IncL/M plasmid producing IMP-1.

Methods
Bacterial strains and antimicrobial susceptibility
All clinical isolates of Enterobacteriaceae not susceptible to carbapenems were prospectively collected and stored at the microbiological laboratory of St. Luke's International Hospital, Tokyo, Japan, from April 2015 to May 2019. Initial identification and susceptibility tests were performed using Microscan WalkAway 96 Plus (Beckman-Coulter) and Microscan Nog Series NC-EN2J (Beckman-Coulter). Susceptibility results were interpreted in accordance with the Performance Standards for Antimicrobial Susceptibility Testing M100-25 of the Clinical Laboratory Standards Institute (CLSI) [17]. These isolates were screened for production of carbapenemase by a modified carbapenem inactivation method. Thereafter, MBLs were detected using sodium mercaptoacetic acid. During the screening of CPE, one patient was found to harbor three species of CPE, Enterobacter cloacae, Serratia marcescens and Klebsiella aerogenes.

Identification and whole genome sequencing of CPE harboring blaIMP-1
The CPE isolates were screened for the presence of blaIMP-1-like by PCR using the primers IMP-F (5’-ATG AGCAAGTTATCTGATTTCTTTA-3’) and IMP-R (5’-TTAGTGCTTGGTTTGTGAATTT-3’). All PCR products were sequenced using ABI PRISM 3500XL DNA Analyzer (Applied Biosystems, Foster, CA). The whole genome of each isolate was extracted using DNeasy Blood and Tissue kits (Qiagen, Tokyo, Japan) for MiSeq (Illumina, San Diego, CA) and QIA-GEN Genomic-tip 100/G and Genomic DNA Buffer Set (Qiagen) for MiniION (Oxford Nanopore Technologies, Oxford, UK). Whole genomes and plasmids of the three isolates were sequenced by MiSeq platform using 600 cycle Reagent Kit v.3 and MinION platform using R9.4 flow cell (FLO-MIN106), according to the manufacturers’ instructions. The sequence reads generated by MiSeq were quality trimmed and filtered using CLC Genomics Workbench v11 (CLC bio, Aarhus, Denmark). MiniION data were base called by Guppy v3.6.1 (Oxford Nanopore), trimmed by NanoFilt v2.2 (https://github.com/wdecoster/nanofilt), and adaptors trimmed by Porechop v0.2.3 (https://github.com/rrwick/Porechop). The long reads generated by MinION and the short reads generated by Miseq were assembled and polished using Unicycler v0.4.7 [18]. The sequence of drug resistance genes and plasmid typing were determined using Resfinder 4.1 and PlasmidFinder 2.1, respectively, from the Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/).

Structure of the plasmids harboring blaIMP-1
The sequences of the plasmids in the three isolates were compared using GENETYX-MAC ver. 19.0.1 (GENETYX Co.), and the detailed genetic structure of the plasmid in E. cloacae was determined by the Rapid Annotation using Subsystem Technology version 2.0 (https://rast.nmpdr.org) [19]. Whole genome sequences of the plasmids were analyzed by BLAST® website (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed September 21, 2020) and in silico

| Table 1 | Characteristics of IMP-1 metallo-β-lactamase producing Enterobacteriaceae |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Isolates | ST | Tissue source | MIC (µg/mL) | AMK | AZT | CAZ | CIP | CST | MPM | TIG |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| E. cloacae SL264 | 17S | Pancreatic cyst | 0.5 | 64 | 256 | 1 | ≤0.016 | 2 | 1 |
| S. marcescens SL267 | N/A | Pancreatic cyst | 2 | ≤0.25 | 64 | ≤0.25 | >32 | 4 | ≤0.25 |
| K. aerogenes SL269 | N/A | Blood | 2 | 32 | 256 | 2 | 0.063 | 4 | 2 |

ST, sequence type; N/A, not applicable; AMK, amikacin; AZT, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; MPM, meropenem and TIG, tigecycline
Conjugation of the plasmid among the isolates

The type strains of *E. cloacae* (NBRC13535), *K. aerogenes* (NBRC13534) and *S. marcescens* (NBRC102204) were plated on Mueller–Hinton broth (MHB) containing 50 µg/ml rifampicin. The grown cells were selected as recipient strains. The donor strains were cultivated in MHB containing ceftazidime and the recipient strains in MHB overnight. The cells were harvested by centrifugation (5000 × g for 3 min), washed three times with PBS and resuspended in PBS to an OD = 3.5. Donor and recipient cells, mixed at an optimal ratio of 1:3 [20], were incubated for 3 h at 37 °C and plated on selective media, consisting of MHB containing 50 µg/ml rifampicin or 50 µg/ml ceftazidime and 50 µg/ml rifampicin, for 18 h. The conjugation frequencies of donor cells were calculated based on the number of colonies on each selective medium.

Multilocus sequence typing and antimicrobial resistance genes

Multilocus sequence typing (MLST) was performed using MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST-2.0/) [21], and antimicrobial resistant genes of *E. cloacae* SL264 were determined using ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) [22].

Clinical information

The medical chart of the patient was retrospectively reviewed conducted to obtain relevant clinical information.

Nucleotide sequence accession number

The chromosome and two plasmids (pSL264 and pSL264-2) sequences of *E. cloacae* SL264 have been deposited at GenBank under accession number AP024913, AP024914 and AP024915, respectively. The chromosome and the plasmid (pSL267) sequences of *S. marcescens* SL267 were AP024916 and AP024917, respectively. The chromosome

![Fig. 1 Structure of the plasmid, pSL264, harboring blaIMP-1.jpg](image-url)
Fig. 2 Comparative analysis of plasmids highly similar to pSL264. pSL264 was 98.51% identical to the plasmid pEB-1 in *E. cloacae* (KX230795), 98.51% identical to pACM1 in *Klebsiella oxytoca* (KJ541681), 98.51% identical to p51929_MCR_VIM in *Citrobacter freundii* CP059429), and 98.52% identical to pKp616_2 in *K. pneumoniae* (CP026497).

Fig. 3 Comparative analysis of the genomes of pSL264 and pTMTA63632. The sequence qacEdelta1-sul1-istB-IS21 of pSL264 was 99.25% identical to that of the plasmid TMTA63632 harboring blaIMP-68 (Accession Number AP019667) in a *K. pneumoniae* isolate obtained from a patient in Japan.

Fig. 4 The scheme of transferability of pSL264 among *E. cloacae*, *K. aerogenes* and *S. marcescens*. 
and two plasmids (pSL269 and pSL269-2) sequences of K. aerogenes SL269 have been deposited at GenBank under accession number AP024918, AP024919 and AP024920, respectively.

Results
Description of the patient
The patient was an 86-year-old man with a history of pancreatic cancer who developed severe gallstone pancreatitis and complicated pancreatic cyst infection. Of note, he was empirically treated with meropenem and a surveillance culture of his pancreatic fluid yielded an IMP-1-producing strain of E. cloacae, designated SL264, which was considered colonization (Table 1). Two months after the initial event, an IMP-1-producing strain of S. marcescens, designated SL267, was isolated from the pancreatic drainage tube (Table 1). Five days later, however, the patient developed a high fever. Culture of a blood sample resulted in the isolation of an IMP-1-producing strain of K. aerogenes, designated SL269 (Table 1). The patient was successfully treated with prolonged infusion of meropenem, colistin and tigecycline for 14 days.

Structure of the plasmid harboring bla\textsubscript{IMP-1}

The plasmid identified in E. cloacae SL264 was designated pSL264; it was found to be 81,133 bp in length and have a genetic structure of int\textsubscript{I}-bla\textsubscript{IMP-1}-aad\textsubscript{6′}-aac\textsubscript{3}-1-aph\textsubscript{3′}-qacE\textsubscript{delta1}-sul1-istB-IS21 (Fig. 1). The $\text{bla}_{\text{IMP-1}}$ gene was on a class 1 integron with a unique structure. The sequence of qacEdelta1-sul1-istB-IS21 was 99.25% identical to that of the plasmid TMTA63632 harboring $\text{bla}_{\text{IMP-68}}$ (accession number AP019667) in a K. pneumoniae isolate obtained from a patient in Japan (Figs. 2 and 3) [23]. The plasmid incompatibility complex of pSL264 was IncL/M. Comparative analysis showed that the plasmids in E. cloacae SL 264 (pSL264), S. marcescens SL267 (pSL267) and K. aerogenes SL269 (pSL269) were completely identical. This plasmid, designated pSL264, was similar to other, previously identified plasmids, including pEB-1 in E. cloacae with 98.51% identity (KX230795) [24], pACM1 in Klebsiella oxytoca with 98.51% identity (KJ541681) [25], p51929_MCR_VIM in Citrobacter freundii with 98.51% identity (CP059429) [26], and pKp616_2 in K. pneumoniae with 98.52% identity (CP026497) [27]. However, the genetic environment surrounding $\text{bla}_{\text{IMP-1}}$ in pSL264 was found to be unique.

Transferability of plasmid pSL264 among the three species of Enterobacteriaceae

Table 2 Transferability of pSL264 among the three species of Enterobacteriaceae

| From            | To            | Experiment No. |
|-----------------|---------------|----------------|
| E. cloacae      | S. marcescens | 0              |
| E. cloacae      | K. aerogenes  | 0              |
| S. marcescens   | E. cloacae    | 7.6 x 10^{-7}  |
| S. marcescens   | K. aerogenes  | 0              |
| K. aerogenes    | E. cloacae    | 3.8 x 10^{-8}  |
| K. aerogenes    | S. marcescens | 0              |

Table 2 shows that conjugation experiments indicated that pSL264 did not equally transfer among these species. Rather, it transferred from S. marcescens to E. cloacae and K. aerogenes, from E. cloacae to K. aerogenes and from K. aerogenes to E. cloacae. In contrast, pSL264 could not transfer from E. cloacae or K. aerogenes to S. marcescens (Fig. 4 and Table 2).

Discussion
This study indicated that the plasmid, pSL264, transferred among three species of bacteria, E. cloacae, K. aerogenes and S. marcescens, in a single patient. However, our conjugation experiments indicated that pSL264 did not equally transfer among these species. Rather, it transferred from S. marcescens to E. cloacae and K. aerogenes, but not from E. cloacae or K. aerogenes to S. marcescens, suggesting that, in this patient, pSL264 was initially present in S. marcescens and subsequently spread to the other species. Although transferability rate in vitro was not that high, treatment with carbapenem for more than 28 days may have triggered the transfer of resistance.

This study had several limitations. First, this was a single center study. The transferability of pSL264 may not be general, and external validation may be required. Second, although in vitro conjugation experiments suggested that S. marcescens initially harbored pSL264, E. cloacae was isolated from this patient earlier than the other two species.

Incl/M plasmids harboring $\text{bla}_{\text{IMP}}$ and $\text{bla}_{\text{NDM}}$ in Enterobacteriaceae may be emerging and spreading, especially in Asian countries. An Incl/M plasmid was first reported in a multidrug-resistant strain of Morganella morgani isolated in South Africa in 1972 [28]. These plasmids are now commonly identified among environmental and clinical isolates [29, 30]. Because these plasmids are carriers of genes encoding carbapenemases, mostly OXA-48 carbapenemases, they can be regarded as a public health threat [23, 28, 30–44].

To our knowledge, this is the first report of an Incl/M plasmid harboring $\text{bla}_{\text{IMP-1}}$ in three species of Enterobacteriaceae isolated from an individual patient. This plasmid may have spread clonally in the hospital environment, suggesting the need to assess the presence of Enterobacteriaceae harboring $\text{bla}_{\text{IMP-1}}$ in medical settings in Japan.
Conclusion
This study found that pSL264 harboring bla IMP-1 could easily transfer among species of Enterobacteriaceae in a patient during long-term antimicrobial treatment. Molecular and genomic analyses of plasmids may reveal the horizontal transmission of plasmids in CPE.

Acknowledgements
Not applicable.

Authors’ contributions
The manuscript was seen and approved by all the authors and is not under consideration elsewhere. All the authors contributed to the work in this study. NM collected clinical data and wrote the initial draft of the manuscript; TT performed the drug susceptibility profiles and data analysis; TK supervised this study and edited the manuscript; and YU helped perform genomic analyses and supervised this study. All authors read and approved the final manuscript.

Funding
This study was supported by grants from the Research Program on Emerging and Re-emerging Infectious Diseases from Japan Agency for Medical Research and Development (Grant Number: 21fk0108604h00701). The funding organization had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials
The chromosome and two plasmid sequences of E. cloacae SL264 have been deposited at GenBank under accession number AP024913, AP024914 and AP024915, respectively. The chromosome and the plasmid sequences of S. marcescens SL267 were AP024916 and AP024916, respectively. The chromosome and two plasmid sequences of K. aerogenes SL269 have been deposited at GenBank under Accession Number AP024918, AP024919 and AP024920, respectively.

Declarations
Ethics approval and consent to participant
This study was approved by the Institutional Review Board of St. Luke’s International University in Tokyo, Japan (Number: 20-R151) and by the Biosafety Committee, Juntendo University (Approval Number BSL2/29-1). Individual informed consent was waived by the ethics committee listed above because this study used currently existing sample collected during the course of routine medical care and did not pose any additional risks to the patients. Informed consent about study participation was officially announced by mail and poster. All patient data were anonymized prior to the analysis.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 10 December 2020 Accepted: 4 October 2021 Published online: 13 October 2021
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