Title:

Detection of *Enterobacter cloacae* complex strain with a *bla*$_{NDM-1}$-harboring plasmid from an elderly resident at a long-term care facility in Okayama, Japan

Running title: *bla*$_{NDM-1}$-harboring *Enterobacterales* in Okayama

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Author Contribution

All authors met the ICMJE authorship criteria. KG performed the genetic analysis. KI was responsible for phenotypic testing of the isolate. HY collected the clinical data. KG and HH contributed to the drafting of the manuscript. OM and FO supervised the study. All authors interpreted the results, critiqued the manuscript, and approved the submitted manuscript.

Abstract

Amidst the global spread of antimicrobial resistance, New Delhi metallo-β-lactamase (NDM)-type carbapenemase-producing *Enterobacterales* (CPE) remain uncommon in Japan, and the detection of such highly drug-resistant organisms is limited to inbound cases. There is little evidence regarding the prevalence of NDM β-lactamase gene (*bla*$_{NDM}$)-harboring CPE in the domestic community, especially in the provincial cities of Japan. Herein, we report the isolation of a *bla*$_{NDM-1}$-harboring plasmid in *Enterobacter cloacae* complex strain isolated from an elderly woman without a history of traveling abroad who had resided in a long-term care facility in Okayama, Japan. The multidrug-resistant *bla*$_{NDM}$-harboring CPE isolate was detected in a stool sample of the patient during routine screening at admission. We performed whole-genome sequencing analysis of the isolate using MiSeq (Illumina) and MinION (Oxford Nanopore Technologies) platforms. The isolate was identified as sequence type 171, which has predominantly been reported in the United States and China. The *bla*$_{NDM-1}$ gene was encoded on the 46,161 bp IncX3 plasmid, with sequence similarity to plasmids of similar size isolated from individuals in China. Collectively, the genomic data suggest that an imported CPE isolate may have spread among healthy individuals in the regional area of Japan.

Keywords: antimicrobial resistance; carbapenemase-producing *Enterobacterales*; carbapenem-resistant *Enterobacterales*; New Delhi metallo-β-lactamase (NDM); *Enterobacter cloacae* complex

Text

Carbapenem-resistant *Enterobacterales* (CRE), especially carbapenemase-producing *Enterobacterales* (CPE), are the most serious emerging antimicrobial-resistant (AMR) pathogens globally. Carbapenemases vary based on the catalytic center of the enzyme, which include serine (*bla*$_{KPC}$ and *bla*$_{OXA48}$) or zinc ions (*bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$). A wide variety of CPE isolates are prevalent globally [1], but *bla*$_{IMP}$-harboring CPEs are exclusively dominant in Japan [2,3]. The detection of *bla*$_{NDM}$-positive organisms is quite rare domestically, although previous studies have suggested that non-medical intermediaries, such as international travelers [4] and imported foods [5], may be reservoirs of *bla*$_{NDM}$-harboring organisms. CPE may have spread in the community without being detected; however, as of 2021, Japanese researchers do not believe that New Delhi metallo-β-lactamase (NDM)-producing *Enterobacterales* exist latently in the domestic community. Here, we report the detection of a *bla*$_{NDM-1}$-positive *Enterobacter cloacae* complex strain in a fecal specimen from an elderly Japanese resident of a long-term care facility (LTCF) in Okayama, Japan, along with whole-genome analysis results.

The patient was a Japanese woman in her seventies. She had resided in an LTCF for 6 months and reported no history of overseas travel. Additionally, the patient had not received any antibiotic treatment over the last few months and had no contact with animals. The patient was admitted to an acute care hospital for symptomatic epilepsy, where every patient transferred from LTCF is supposed to be screened for AMR pathogens. On admission, a fecal sample was submitted for microbiological screening and CRE-suspected isolates were

detected on CHROMagar mSuper CARBA/ESBL agar (Kanto Chemical Co., Inc., Tokyo, Japan). The bacterial samples were transferred to our university hospital for further analysis.

Bacterial identification was performed using mass spectrometry (MALDI Biotyper; Bruker Daltonics Inc., Billerica, MA, USA), and the isolate was identified as *E. cloacae* complex strain. The organism was positive for both the sodium mercaptoacetic acid double-disk synergy test and the modified carbapenem inactivation method, suggesting the presence of a metallo-beta-lactamase. Antimicrobial susceptibility was examined by the microdilution method, using the Dry Plate "Eiken" reagent (Eiken Chemical Co., Ltd., Tokyo, Japan). The isolate showed multi-drug resistance, with a minimum inhibitory concentration of 8 μg/mL meropenem (**Table 1**). We used a multiplex PCR assay (Kaneka DNA Chromatography Carbapenemase Gene Detection Kit; Kaneka Corporation, Tokyo, Japan) and detected the bla_{NDM} in the isolate.

Whole-genome sequencing was subsequently performed using MiSeq (Illumina, San Diego, CA, USA) and MinION (Oxford Nanopore Technologies, Oxford, UK) platforms, as previously described [6]. The results of *de novo* assembly revealed a 4.7 Mb chromosome and five distinct plasmids in this isolate (**Table 2**). *In silico* DNA-DNA hybridization of chromosomes using GTDBtk v1.4.0 [7] suggested that the isolate was genetically identified as *E. hormaechei* subsp. *xiangfangensis*, an *E. cloacae* complex strain, with the highest average nucleotide identity value (99.7) compared to the National Center for Biotechnology Information (NCBI) RefSeq representative genome database. Multi-locus sequence typing using the chromosome sequence and *E. cloacae* PubMLST

(https://pubmlst.org/organisms/enterobacter-cloacae) classified it as sequence type 171, which has been reported to be predominant among carbapenem-resistant *Enterobacter* isolates in the United States and China [8,9]. Antimicrobial resistance genes were identified using ResFinder-3.2 [10]. Three *bla* genes were encoded on the chromosomes and plasmids. Among them, *bla*$_{\text{NDM-1}}$ was encoded on the IncX3-type, 46,161 bp conjugal plasmid, which was named pOU1-NDM1 (GenBank ON461804; plasmid 2 in Table 1). At least one hundred plasmid sequences that have 99.6–99.9% similarity with and more than 97% query cover with pOU1-NDM1 were found in the NCBI nucleotide database as those predominantly detected plasmid in China from 2017–2021. Phylogenetic analysis revealed that pYJ3-NDM1 (reported in Myanmar, 2020) [6] and pMH9B (reported in China, 2019) which is deposited in GenBank (MT012582.1) but unpublished were closely related to pOU1-NDM1. The genomic structures of these plasmids were quite similar, except for the IS*Kpn*26 inserted in the *bla*$_{\text{NDM-1}}$ flanking region in pOU1-NDM1 (**Fig. 1**). Interestingly, *bla*$_{\text{NDM-5}}$, instead of *bla*$_{\text{NDM-1}}$, was encoded by pMH9B. The host species for pYJ3~~0~~-NDM1 and pMH9B was *Escherichia coli*. The *bla*$_{\text{NDM-1}}$ positive strains were isolated from the stool alone, and not from other clinical samples.

Informed consent for the bacterial analysis and publication was obtained from the patient. A need for ethical approval was waived because the data comprised unidentifiable information alone.

This is the first ever case for the isolation of *bla*$_{\text{NDM-1}}$-harboring *Enterobacterales* in Okayama, a regional city in Japan. Importantly, the carrier patient was a resident of an LTCF

and had not traveled abroad. However, genetic analysis strongly suggested the *bla*$_{\text{NDM-1}}$-harboring *E. cloacae* complex strain was derived from an inbound route. In recent years, an increasing number of foreign workers, mainly from southeastern Asian countries, have been engaged in LTCFs as nursing care staff for the elderly in Japan. NDM-producing CRE strains are highly prevalent in such countries [1], even among healthy individuals [6]. Therefore, we suspected a staff-to-resident infection route. However, no foreign nationals were employed in the LTCF where the present patient resided. The presence of an index case other than the present case and a latent outbreak of the highly drug-resistant pathogen were also suspected, and active CRE surveillance of other residents and nursing care staff of the LTCF was later performed. However, no CRE carriers were found, and the patient was determined to be the only carrier of a *bla*$_{\text{NDM-1}}$-harboring CRE strain at the LTCF. Another possibility is that imported foods are transporters of AMR pathogens. Imported foods are potentially contaminated with CRE [11], and the patient may have been infected with a *bla*$_{\text{NDM-1}}$-harboring organism unknowingly through her daily diet. This is plausible; however, it has yet to be confirmed. Considering one-health circulation of AMR pathogen, an environmental-, or animal-derived strain should be listed as a source of *bla*$_{\text{NDM}}$-harboring *Enterobacterales* in humans. In fact, isolations of NDM-producing *Enterobacterales* have been reported in wastewater [12] and vegetables [13] in Japan. Whole genome analysis in Korea corroborated a high concordance rate with plasmids coding *bla*$_{\text{NDM}}$ detected in humans and animals [14].

So far, among various subtypes of *bla*$_{\text{NDM}}$, *bla*$_{\text{NDM-5}}$ has been predominantly isolated in Japanese clinical settings [4, 12, 15-18]. As far as we concern, *Klebsiella pneumoniae*

ST15, which was isolated from vegetable samples, was identified to have *bla*$_{NDM-1}$ on IncFII(K):IncR plasmid [13]. Other than this, there seems no detailed report on *bla*$_{NDM-1}$-bearing *Eneterobacterales* in the literature from Japan. However, our investigation uncovered the *bla*$_{NDM-1}$ was coded on IncX3 plasmid in the present case, inferring the presence of a variety of responsible plasmids spreading *bla*$_{NDM-1}$ in Japan.

To combat the domestic spread of CPE in Japan, it is essential to consider a one-health approach [19]. From a clinical perspective, the development of a platform for CPE monitoring is recommended in our region.

Conflict of interest

None to declare.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data Availability Statement

Data in detail will be available if requested to the corresponding author.

Declaration

This manuscript has not been published previously in any language, in whole or in part, and is not currently under consideration elsewhere. We have read and understood your journal's policies and believe that neither the manuscript nor the study violates any of them.

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Figure legends:

Figure 1. Comparison of plasmid sequences

The open reading frame filled with gray indicates *bla*$_{NDM}$. Plasmids with similar sequences (pYJ3-NDM1 and pMH9B, GenBank accession no. AP023227.1 and MT012582.1, respectively) are included for comparison.