Characterization of NDM-5- and CTX-M-55-coproducing Escherichia coli GSH8M-2 isolated from the effluent of a wastewater treatment plant in Tokyo Bay

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Tsuyoshi Sekizuka
Yuba Inamine
Takaya Segawa
Makoto Kuroda

Laboratory of Bacterial Genomics,
Pathogen Genomics Center, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan

Purpose: New Delhi metallo-β-lactamase (NDM)-5-producing Enterobacteriaceae have been detected in rivers, sewage, and effluents from wastewater treatment plants (WWTPs). Environmental contamination due to discharged effluents is of particular concern as NDM variants may be released into waterways, thereby posing a risk to humans. In this study, we collected effluent samples from a WWTP discharged into a canal in Tokyo Bay, Japan.

Methods: Testing included the complete genome sequencing of Escherichia coli GSH8M-2 isolated from the effluent as well as a gene network analysis.

Results: The complete genome sequencing of GSH8M-2 revealed that it was an NDM-5-producing E. coli strain sequence type ST542, which carries multiple antimicrobial resistance genes for β-lactams, quinolone, tetracycline, trimethoprim-sulfamethoxazole, florfenicol/chloramphenicol, kanamycin, and fosfomycin. The \textit{blaNDM-5} gene was found in the IncX3 replicon plasmid pGSH8M-2-4. Gene network analysis using 142 IncX3 plasmid sequences suggested that pGSH8M-2-4 is related to both clinical isolates of \textit{E. coli} and \textit{Klebsiella} species in Eastern Asia. GSH8M-2 also carries the \textit{blaCTX-M-55} gene in IncX1 plasmid pGSH8M-2-3.

Conclusion: This is the first report of environmental NDM-5-producing \textit{E. coli} isolated from a WWTP in Japan. NDM-5 detection is markedly increasing in veterinary and clinical settings, suggesting that dual β-lactamases, such as NDM-5 and CTX-M-55, might be acquired through multiple steps in environment settings. Environmental contamination through WWTP effluents that contain producers of NDM variants could be an emerging potential health hazard. Thus, regular monitoring of WWTP effluents is important for the detection of antimicrobial-resistant bacteria that may be released into the waterways and nearby communities.

Keywords: sewage, carbapenemase, NDM-5, IncX3, CTX-M-55, IncX1

Introduction

Antimicrobial resistance (AMR) is of global concern as the widespread detection of carbapenemase-producing Enterobacteriaceae (CPE) is an emerging issue with potentially serious public health implications.\(^1\) New Delhi metallo-β-lactamase (NDM) confers resistance to β-lactams with the exception of aztreonam, and the rapid global spread of NDM-1-producing strains has raised serious concerns that common infections with these organisms may soon be untreatable.\(^2\) Variants of NDM carbapenemase have been assigned to \textit{blaNDM-27} under curation by the National Database of Antibiotic Resistant Organisms (NDARO, https://www.ncbi.nlm.nih.gov).
AMR genes were identified in 2011 in the United Kingdom and was isolated from a multidrug-resistant *Escherichia coli* ST648 clinical isolate from a patient with a recent history of hospitalization in India.\(^3\)

NDM-5 exhibits expanded-spectrum cephalosporins and carbapenems degradation with two amino acid substitutions (Val88Leu and Met154Leu) in the NDM-1 amino acid sequence.\(^3\) NDM-5 has been characterized as exhibiting higher hydrolytic activity than other variants (NDM-1, NDM-4, NDM-6, and NDM-7).\(^4\)

NDM-5-producing Enterobacteriaceae have been identified from many different sources in multiple countries. Thus far, it has been reported that *bla*\(_{\text{NDM-5}}\) appears to be predominant among all potential carbapenemase genes (including other NDM variants and KPC-2) in China.\(^5\)

Moreover, diverse dissemination of the *bla*\(_{\text{NDM-5}}\) gene has been detected among non-clonal *E. coli* hosts (ST12, ST167, ST354, ST361, ST410, ST617, ST746, ST6335, and ST6395) via the IncX3 plasmid in China.\(^6,7\) To elucidate the dissemination of CPE including NDM variants, AMR monitoring of wastewater treatment plant (WWTP) effluent contributes to the detection of ongoing AMR bacterial dissemination in environment, and might provide early warning of potential dissemination in clinical settings and communities.

Here, we isolated an NDM-5-producing *E. coli* strain GSH8M-2 from an effluent sampled at a WWTP in Tokyo Bay, Japan. Then, we determined the complete genome sequence of GSH8M-2, and characterized the genetic features of *bla*\(_{\text{NDM-5}}\) and *bla*\(_{\text{CTX-M-55}}\)-positive plasmids.

**Materials and methods**

**Bacterial strains and identification**

The upper effluent flow from an urban WWTP was collected on August 6, 2018, in the canal (N35.631783, E139.7448151) at Tokyo Bay, Japan. The facilities of the WWTP process an influent sample in this study was collected within five consecutive days without recent rainfall to exclude the effect of the weather. Then, bacteria within the effluent sample were isolated as described previously for CPE.\(^8\) Briefly, 500 mL of effluent was filtered, and the membrane incubated in 20 mL of LB-broth supplemented with 1 mg/L meropenem at 37°C for 14 hrs, after which the culture was spread onto CHROMagar extended-spectrum β-lactamase (ESBL) plates (CHROMagar, Paris, France).

**Antimicrobial susceptibility and CPE screening tests**

Antimicrobial susceptibility was investigated by E test (bioMérieux, Marcy-l’Étoile, France) according to the European Committee on Antimicrobial Susceptibility Testing guideline v9.0. Carbapenemase production was assessed by performing a Carba NP test, as described previously.\(^9\)

**Whole-genome sequence analysis**

Determination of the complete genome sequence was performed as described, previously.\(^10\) Briefly, the complete genome sequence was determined using an Illumina NextSeq 500 (2×150-mer, 350× median coverage) (Illumina, San Diego, CA, USA), followed by a PacBio Sequel sequencer for long-read sequencing (PacBio, Menlo Park, CA, USA). A de novo assembly was performed.\(^10\) Annotation was conducted in DFAST.\(^11\) Multilocus Sequence Typing was characterized at the pubMLST site (https://pubmlst.org/escherichia/). Circular representations of complete genome sequences were visualized using the GView server.\(^12\) AMR genes were identified by homology searching against the ResFinder database.\(^13\) Plasmid replicon typing was performed using the curated PlasmidFinder database at the CGE website (https://cge.cbs.dtu.dk/services/PlasmidFinder/).\(^14\)

**Comparative plasmid genome network analysis**

Complete plasmid sequences were retrieved from NCBI “nt” and “RefSeq” database using a keyword search for the words “complete sequence” and “plasmid”, followed by gene prediction using the Prodigal program for normalization of gene finding. A plasmid network analysis was performed as previously described.\(^15\) In brief, amino acid sequences for the complete plasmids were clustered using the UCLUST program (version 6.0.307)\(^16\) with the following parameters: cluster_smallmem; id 1.0 (100% identity in amino acid sequence); target_cov 0.9, query_cov 0.9 (at least 90% coverage); minsl 0.9, minqt 0.9, maxqt 1.1 (less than 10% difference in length). Each plasmid, ranging from 30 to 60 kb in size, was connected to a network under the sharing threshold with at least 40 homologous
genes, followed by community assignment using the multilevel community method in the R igraph library. In order to visualize the level of similarity for individual plasmid sequences in a dataset, non-metric multidimensional scaling analysis was performed with the distance matrix calculated with the Simpson’s index using the metaMDS program from Vegan R package. The Cytoscape version 3.2.0 was used to draw the plasmid network graph.17

Results

NDM-5-producing E. coli strain GSH8M-2

The isolated E. coli effluent strain GSH8M-2 was Carba NP test positive. MIC testing suggested that GSH8M-2 was highly resistant to the β-lactams, fluoroquinolone, trimethoprim-sulfamethoxazole, and chloramphenicol, kanamycin, and fosfomycin. However, it was still susceptible to colistin and aminoglycosides (amikacin, gentamicin, streptomycin, and tobramycin) except kanamycin (Table 1).

Genome features of E. coli strain GSH8M-2

The complete genome sequence was determined, as described in the materials and methods. GSH8M-2 carries multiple antimicrobial resistance genes (ARGs) for β-lactams (blaNDM-5, blaCTX-M-55, blatETEC, and blatEM-138), quinolone (qnrS1 and qnrS2), tetracycline (tet (A)), trimethoprim-sulfamethoxazole (dfrA14 and dfrA17), florfenicol/chloramphenicol (floR), kanamycin (aph(3’)-Ia), and fosfomycin (fosA3).

Importantly, IncX3 plasmid pGSH8M-2–4 (46.1 kb) carries blaNDM-5 (Figure 1A), exhibiting an almost identical gene organization to pNDM-MGR194, which was first identified in IncX3 plasmids carrying blaNDM-5 in Klebsiella pneumoniae MGR-194 clinical isolates in India18 (Figure 1B). These two blaNDM-5-positive IncX3 plasmids showed partially distinct gene organization around the blaNDM-1 to IncX3 plasmid in p112298-NDM, which was first identified in 2013 in a Citrobacter freundii strain 112298 in China.19

IncX1 plasmid pGSH8M-2–3 (49.3 kb) carries multiple ARGs including blaCTX-M-55, aph(3’)-Ia, floR, fosA3, and qnrS2 (Figure 1C). Blast homology search revealed that three publicly complete sequences (two plasmids and one chromosome DNA of E. coli strains isolated from a dog and pig) showed homology with >80% region coverage, for all three plasmids sharing the aph(3’)-Ia, floR, and qnrS2 for three primarily veterinary use antimicrobials (aminoglycosides, florfenicol, and quinolone, respectively) (Figure 1D). However, pGSH8M-2–3 carries additional blaCTX-M-55 and fosA3 with IS26 mediated acquisition (Figure 1D).

Plasmid gene network analysis of IncX3 plasmids

To further elucidate the dissemination of blaNDM-5-positive IncX3 plasmids, gene clustering, and network analysis was performed using the IncX3 plasmids relevant to pGSH8M-2–4, resulting in 141 plasmid entries obtained from a public database (Table S1). Those 142 plasmid sequences were classified into four communities (Figure 2). The pGSH8M-2–4 strain originated from a community (C1) that shares blaNDM-5 with E. coli and Klebsiella clinical isolates in Eastern Asia. Of note, the community network analysis strongly suggested that blaNDM-5-positive IncX3 plasmids are of clonal lineage, but horizontally disseminated over multiple bacteria hosts (Escherichia, Klebsiella, Enterobacter, Citrobacter, and Klyvera). On the other hand, other β-lactamases [blaNDM-1, blaKPC, blaOXA-181], and ESBL gene-positive IncX3 plasmids can be clustered

| Antimicrobial agents | Minimum inhibitory concentration (mg/L) | SIR (EUCAST ver 9.0) |
|----------------------|----------------------------------------|---------------------|
| Ampicillin-sulbactam  | >256                                   | R                   |
| Cefepime             | 32                                     | R                   |
| Cefotaxime           | >32                                    | R                   |
| Ceftazidime          | >256                                   | R                   |
| Imipenem             | >32                                    | R                   |
| Meropenem            | >8                                     | R                   |
| Aztreonam            | 16                                     | R                   |
| Amikacin             | 2                                      | S                   |
| Gentamicin           | 1                                      | S                   |
| Tobramycin           | 1                                      | S                   |
| Kanamycin            | >256                                   | –                   |
| Streptomycin         | 8                                      | –                   |
| Tetracycline         | >256                                   | –                   |
| Ciprofloxacin        | 0.5                                    | I                   |
| Levofloxacin         | 4                                      | R                   |
| Azithromycin         | 8                                      | –                   |
| Trimethoprim-sulfamethoxazole | >32                              | R                   |
| Chloramphenicol      | >256                                   | R                   |
| Colistin             | 0.125                                  | S                   |
| Fosfomycin           | >1024                                   | R                   |

Note: –, not defined in EUCAST.
into different communities from each other (C2, C3, and C4 in Figure 2), because of horizontal acquisition with extra adjacent genes around the ARGs (Figure 1B). Thus, each β-lactamase-positive IncX3 plasmid appears to have disseminated in an independent manner among Enterobacteriaceae.

**Discussion**

In this study, we isolated the NDM-5-producing *E. coli* strain (GSH8M-2) from effluent of a WWTP in Tokyo Bay, Japan. This is the first report of an environmental NDM-5-producing *E. coli* isolate from a WWTP in Japan. Thus far, the detection of NDM variants in clinical settings is very rare and uncommon in Japan, a few reports suggested that NDM-5-producing *E. coli* and *K. pneumoniae* were identified in a Japanese individual traveling to Bangladesh in 2013 and also in a case in Indonesia in 2014. However, both of these were imported cases to Japan not a local occurrence. Thus, our findings in this study may indicate an emerging public health issue within the Japanese community.

In addition, previous environmental studies have suggested that NDM-5-producing Enterobacteriaceae have been detected in an urban river in Montpellier, France, hospital sewage water in India and Taiwan, and effluent from a WWTP in Switzerland. Although a few studies revealed dissemination of environmental NDM variants, WWTPs could be a primary source of most AMR bacteria in aquatic environments, because of the partial purification that occurs during the wastewater treatment processes. In particular, ongoing global dissemination could be archived by the rapid spread of the IncX3 replicon-based plasmid transmission among Enterobacteriaceae (Figure 2).

In addition to NDM-5, GSH8M-2 harbors CTX-M-55, which was first reported in 2006 in Thailand and is a variant of CTX-M-15 in the CTX-M-1 cluster, with only one amino acid substitution (Ala80Val). Over the past decade, CTX-M-55-producing *E. coli* isolated from animals and in clinical settings has been increasing. Moreover, the *bla*CTX-M-55-positive plasmid pGSH8M-2-3 is associated with other ARGs (*aph(3’)-Ia, flos, fosA3*, and *qnrS2*), the co-localization of the florfenicol resistance gene *flos* is of marked concern because *flos* is detected in increasing amounts in both veterinary and clinical isolates within China. Therefore, CTX-M-55-positive
E. coli could disseminate in other settings under highly selective pressure by co-selection of the veterinary antimicrobials (florfenicol) and clinical use (cephalosporin) and has become one of the most important emergent AMR bacteria in environment. These findings suggest that the dissemination of dual \( \beta \)-lactamase (carbapenemase and ESBL)-positive bacteria has become a global issue, and environmental contamination could act as a reservoir of NDM variants-producing AMR Enterobacteriaceae.

**Conclusions**

AMR monitoring of WWTP effluent may contribute to the detection of emerging AMR bacterial dissemination in clinical settings and communities. A comprehensive approach based on the complete determination of the genome sequence including the AMR plasmid will be required in order to fully understand AMR dissemination of these bacterial clones and relevant AMR plasmids within the environment.

**Data availability**

The complete, annotated genome sequence of *E. coli* GSH8M-2 was deposited in a public database as shown in Table 2. The short- and long-read sequences for DNA-Seq were deposited in the DNA Data Bank of Japan (BioProject PRJDB6962; BioSample SAMD00168435; DRA accession DRA008278).
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Author contributions

TS and MK contributed to the isolation of the NDM-5 producing strains. TS, YI, and MK performed the genome sequencing and the comparative genome analysis. TS and MK performed antimicrobial susceptibility testing. MK wrote the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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