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NDM-12, a Novel New Delhi Metallo-β-Lactamase Variant from a Carbapenem-Resistant *Escherichia coli* Clinical Isolate in Nepal

Tatsuya Tada,[a] Basudha Shrestha,[c] Tohru Miyoshi-Akiyama,[a] Kayo Shimada,[a] Hiroshi Ohara,[b] Teruo Kirikae,[a] Bharat M. Pokhrel[c]

Department of Infectious Diseases, Research Institute,[a] and Department of International Medical-Cooperation,[b] National Center for Global Health and Medicine, Shinjuku, Tokyo, Japan; Department of Microbiology, Institute of Medicine, Tribhuvan University, Maharajgunj, Kathmandu, Nepal[c]

A novel New Delhi metallo-β-lactamase variant, NDM-12, was identified in a carbapenem-resistant *Escherichia coli* clinical isolate obtained from a urine sample from a patient in Nepal. NDM-12 differed from NDM-1 by two amino acid substitutions (M154L and G222D). The enzymatic activities of NDM-12 against β-lactams were similar to those of NDM-1, although NDM-12 showed lower k_{cat}/K_m ratios for all β-lactams tested except doripenem. The bla_{NDM-12} gene was located in a plasmid of 160 kb.

Metallo-β-lactamases (MBLs) usually confer reduced susceptibility to carbapenems, cephalosporins, and penicillins but not monobactams (1). Acquired MBLs are produced by Gram-negative bacteria, including *Acinetobacter* spp., *Pseudomonas aeruginosa*, and several *Enterobacteriaceae* (1). MBLs are categorized by their amino acid sequences into various types (2–4), including AIM (5), DIM (6), FIM (7), GIM (8), IMPs (9), KHM (10), NDMs (11), SMB (12), SIM (13), SPM (14), TMBs (15), and VIMs (16). The most prevalent types of MBLs are IMP-, VIM-, and NDM-type enzymes (1, 2, 17). NDM-1 was initially isolated from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 in Sweden (11). Subsequently, at least 11 NDM variants (www.lahey.org/studies) have been reported in several countries (4, 18–29).

This study was ethically reviewed and approved by the Institutional Review Board of the Institute of Medicine at Tribhuvan University (reference 6-11-E) and the Biosafety Committee at the National Center for Global Health and Medicine (approval no. 26-D-088 and 26-D-089).

E. coli IOMTU388.1 was isolated from a urine sample obtained from a patient in 2013 in a university hospital in Nepal. The isolate was phenotypically identified, and the species identification was confirmed by 16S rRNA sequencing (30). *E. coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and for expression of bla_{NDM-1} and bla_{NDM-12}, respectively.

MICs were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (31). The MICs of β-lactams for *E. coli* IOMTU388.1 are shown in Table 1, and the MICs of other antibiotics were as follows: amikacin, >1,024 μg/ml; arbekacin, >1,024 μg/ml; ciprofloxacin, 128 μg/ml; colistin, ≤0.125 μg/ml; fosfomycin, 8 μg/ml; gentamicin, >1,024 μg/ml; kanamycin, >1,024 μg/ml; levofloxacin, 32 μg/ml; minocycline, 8 μg/ml; tigecycline, ≤0.125 μg/ml; and tobramycin, >1,024 μg/ml. PCR analysis was performed to detect the MBL genes bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM} (32, 33). The isolate was PCR positive for bla_{NDM} but negative for the other MBL genes tested. The DNA sequence of the PCR product revealed that the isolate had bla_{NDM-12}. Multilocus sequence typing (MLST) of IOMTU388.1 typed it as ST635 (*E. coli* MLST Database; http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html). bla_{NDM-1} obtained from *P. aeruginosa* IOMTU9 (29) was used as a reference gene.

The bla_{NDM-12} sequence had 2 amino acid substitutions (M154L and G222D) compared with bla_{NDM-1} (accession no. JF798502) and one substitution (G222D) compared with NDM-4 (accession no. JQ348841).

The bla_{NDM-1} and bla_{NDM-12} genes were cloned into the corresponding sites of pHSG398 (TaKaRa, Shiga, Japan) using the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′). *E. coli* DH5α was transformed with pHSG398-NDM-1 or pHSG398-NDM-12.

The open reading frames of NDM-1 and NDM-12 without signal peptide regions were cloned into the pET28a expression vector (Novagen, Inc., Madison, WI) using the primer set BamHI-TEV-NDM-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCCAGCAAATGGAAACTGGCGAC-3′) and XhoI-NDM-R (5′-ATCTCGAGTCAGCGCAGCTTGTCGGCCATG-3′). The resulting plasmids were transformed into *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA). Both recombinant NDM-1 and NDM-12 were purified simultaneously using Ni-nitrilotriacetic acid (NTA) agarose according to the manufacturer's instruction (Qiagen, Hilden, Germany). His tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA) and untagged proteins were purified by an additional passage over the Ni-NTA agarose. The purities of NDM-1 and NDM-12, which were estimated by SDS-PAGE, were greater than 90%. During the purification procedure, the presence of β-lactamase activity was monitored using nitrocefin (Oxoid, Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl and 5 μM $Zn(NO_3)_2$ at 37°C, using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis with a Lineweaver-Burk plot. Wavelengths and extinction coefficients for

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Address correspondence to Teruo Kirikae, tkirikae@ri.imcj.go.jp.
T.T. and B.S. contributed equally to this article.
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TABLE 1 MICs of various β-lactams for *E. coli* IOMTU388.1 and *E. coli* DH5α transformed with plasmids encoding NDM-12 or NDM-1

Antibiotic(s)	MIC (μg/ml) for strain:			
	IOMTU388.1	DH5α(pHSG398/NDM-1)	DH5α(pHSG398/NDM-12)	DH5α(pHSG398)
Ampicillin	>1,024	256	512	4
Ampicillin-sulbactam	>1,024	128	128	2
Aztreonam	64	≤0.063	≤0.063	≤0.063
Cefepime	512	0.5	1	≤0.063
Cefoselis	1,024	16	8	1
Cefotaxime	>1,024	8	16	≤0.063
Cefoxitin	>1,024	64	16	≤0.063
Cefpirome	512	2	2	≤0.063
Ceftazidime	>1,024	256	256	≤0.063
Ceftriaxone	>1,024	16	16	≤0.063
Cefradine	>1,024	512	256	16
Doripenem	32	0.063	0.063	≤0.063
Imipenem	16	0.5	0.25	≤0.063
Meropenem	64	0.25	0.125	≤0.063
Moxalactam	>1,024	16	4	0.125
Penicillin G	>1,024	256	256	32

β-lactam substrates have been reported previously (34–36). The K_m and k_{cat} values (means ± standard deviations) were obtained from three individual experiments. The enzymatic activities of NDM-1 were measured in parallel with those of NDM-12.

The plasmid harboring bla_{NDM-12} was extracted (37) and sequenced using MiSeq (Illumina, San Diego, CA). The size of the plasmid harboring bla_{NDM-12} was determined using pulsed-field gel electrophoresis (PFGE) and Southern hybridization. A probe for bla_{NDM-12} from IOMTU388.1 was amplified by PCR using the primer sets for EcoRI-NDM-F and PstI-NDM-R. Signal detection was carried out using the digoxigenin (DIG) High Prime DNA labeling and detection starter kit II (Roche Applied Science, Indianapolis, IN).

Mating-out assays between the parental strain IOMTU388.1 and the chloramphenicol-resistant *E. coli* strain BL21 were performed in LB broth using a 1:4 donor/recipient ratio for 3 h at 37°C. Transconjugants were selected on Muller-Hinton agar plates containing ceftazidime (100 μg/ml) and chloramphenicol (30 μg/ml). Selected transconjugants harboring bla_{NDM-12} were confirmed by PCR with the primer set EcoRI-NDM-F and PstI-NDM-R.

E. coli DH5α harboring bla_{NDM-1} or bla_{NDM-12} showed reduced susceptibility to moxalactam and all penicillins, cephalosporins, and carbapenems tested compared with DH5α harboring a vector control (Table 1). The MICs of the β-lactams cefoxitin and moxalactam for DH5α harboring bla_{NDM-12} were 4-fold less than those for DH5α harboring bla_{NDM-1} (Table 1).

As shown in Table 2, recombinant NDM-1 and NDM-12 hydrolyzed all β-lactams tested except for aztreonam. The profiles of enzymatic activities of NDM-12 against β-lactams tested were similar to those of NDM-1, although NDM-12 had lower k_{cat}/K_m ratios for all β-lactams tested except for doripenem. The lower k_{cat}/K_m ratios were likely to be caused by the lower k_{cat} values of NDM-12 compared with those of NDM-1, as the values of NDM-12 were 11.4 to 73.6% of those of NDM-1 (Table 2). The profiles of enzymatic activities of NDM-1 except for cefoxitin were similar to those of NDM-1 that we reported previously (29). The k_{cat}/K_m ratio for cefoxitin in Table 2 was 10-fold higher than that

TABLE 2 Kinetic parameters of the NDM-1 and NDM-12 enzymes[a]

β-Lactam	NDM-1[b]			NDM-12[b]		
	K_m (μM)	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Ampicillin	231 ± 33	249 ± 22	1.1	126 ± 4	136 ± 2	1.1
Aztreonam	NH[c]	NH	NH	NH	NH	NH
Cefepime	162 ± 7	31 ± 1	0.19	103 ± 6	11.1 ± 0.2	0.11
Cefotaxime	102 ± 16	137 ± 7	1.1	45 ± 4	38 ± 1	0.84
Cefoxitin	13 ± 1	6.7 ± 0.1	0.50	26 ± 2	0.66 ± 0.01	0.02
Ceftazidime	202 ± 7	56 ± 1	0.28	53 ± 4	5.7 ± 0.1	0.11
Cefradine	27 ± 3	72 ± 1	2.7	57 ± 4	16 ± 1	0.28
Doripenem	201 ± 27	114 ± 9	0.57	88 ± 2	53 ± 1	0.60
Imipenem	249 ± 43	44 ± 2	0.34	125 ± 22	22 ± 2	0.18
Meropenem	81 ± 10	139 ± 10	1.7	91 ± 8	53 ± 2	0.58
Moxalactam	4.5 ± 2.3	7.6 ± 0.3	2.0	67 ± 5	6.0 ± 0.2	0.09
Penicillin G	67 ± 6	104 ± 1	1.6	64 ± 8	42 ± 2	0.66

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] The K_m and k_{cat} values shown represent the means from 3 independent experiments ± standard deviations.
[c] NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

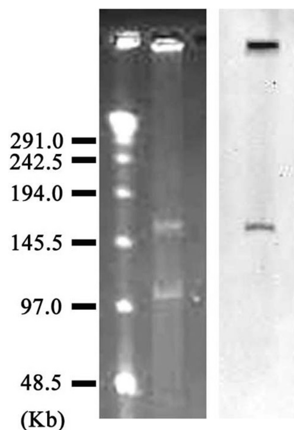

FIG 1 Localization of bla_{NDM-12} on a plasmid from *E. coli* strain IOMTU388.1 separated by PFGE. Left lane, MidRange PFG marker (New England BioLabs, Tokyo, Japan); middle lane, plasmids from *E. coli* strain IOMTU388.1; right lane, hybridization of the plasmid with a probe specific for bla_{NDM-12}.

in our previous study (see Table 2 in reference 29). The difference between the ratios may be explained by the use of different buffer solutions in the kinetics assays (Tris buffer and phosphate buffer, respectively). It was reported that phosphate ions affected the enzymatic activities of metallo-β-lactamase IMP-1 (38). Phosphate ions may affect the enzymatic activities of NDM-1 against cefoxitin.

The MBL gene bla_{NDM-12} in *E. coli* IOMTU388.1 was detected in a plasmid, pIOMTU388-NDM (accession no. AB926431), with a size of 160 kb (Fig. 1). The sequence surrounding bla_{NDM-12} was *bla_{NDM-12}-ble_{MBL}-trpF-dsbC-tnpA-sulI-qacEΔI*. This plasmid showed more than 99.9% identity at the nucleotide sequence level to the sequence located from bp 70978 to 77904 in the pGUE-NDM plasmid (accession no. JQ364967) from *E. coli* strain GUE, which was isolated in India (39), and also showed 99.9% identity at the nucleotide sequence level to the sequence located from bp 372 to 7298 in the pEC77-NDM plasmid (accession no.AB898038) from *E. coli* strain NCGM77, which was isolated in Japan (40). The plasmid harboring bla_{NDM-12} belonged to the IncF incompatibility group and was conjugated from IOMTU388.1 to *E. coli* BL21 at a conjugative frequency of 1.63×10^{-3}.

The 2 substitutions M154L and G222D in NDM-12 (compared with NDM-1) affected the activity of the enzyme (Table 2). Nordmann et al. (24) reported that a mutant containing M154L (NDM-4) possessed increased hydrolytic activity toward carbapenems and several cephalosporins compared to NDM-1. Unexpectedly, NDM-12, which contains the M154L substitution, did not show an increase in hydrolytic activities. The substitution at position 222 found in NDM-12 has been not reported in other variants, to our knowledge. Although we did not directly compare the enzymatic activity of NDM-12 with those of NDM-4, the substitution of G222D in NDM-12 may be associated with a decrease in hydrolytic activities toward these antibiotics (Table 2). Position 222 is located in loop L10 of NDM-1, which forms the active site of NDM-1 with L3 at the bottom of a shallow groove (41–44). Among all known 11 NDM-1 variants, amino acid substitutions were found at 13 amino acid positions, including positions 28, 32, 36, 69, 74, 88, 95, 130, 152, 154, 200, and 233. Positions 28, 32, and 36 were in the signal peptide region. Positions 95, 130, and 154 have been reported to affect β-lactam-hydrolyzing activities, although whether the activities are affected by the other 6 substitutions has not been reported. Residue 95 is located in α1 on the protein surface, and the amino acid substitution at position 95 affected the k_{cat} values of NDM-3 (40). The substitution at position 130 (Met to Leu) showed increased hydrolytic activity toward carbapenems and several cephalosporins compared to NDM-1 (24, 29).

This is the first report describing NDM-12-producing *E. coli* in Nepal. NDMs seem to evolve rapidly; therefore, careful monitoring of NDM-producing pathogens is required.

Nucleotide sequence accession number. The plasmid sequence including bla_{NDM-12} has been deposited in GenBank under accession no. AB926431.

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