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NDM-8 Metallo-β-Lactamase in a Multidrug-Resistant *Escherichia coli* Strain Isolated in Nepal

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A novel metallo-β-lactamase, NDM-8, was identified in a multidrug-resistant *Escherichia coli* isolate, IOMTU11 (NCGM37), obtained from the respiratory tract of a patient in Nepal. The amino acid sequence of NDM-8 has substitutions at positions 130 (Asp to Gly) and 154 (Met to Leu) compared with NDM-1. NDM-8 showed enzymatic activities against β-lactams similar to those of NDM-1.

Metallo-β-lactamases (MBLs) produced by Gram-negative bacteria confer resistance to all β-lactams except monobactams (1). New Delhi metallo-β-lactamase-1 (NDM-1), a recently discovered MBL, was initially isolated from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 in Sweden (2). Since then, NDM-1-producing members of the *Enterobacteriaceae* have been isolated in various parts of the world, including Australia, Bangladesh, Belgium, Canada, France, India, Japan, Kenya, the Netherlands, New Zealand, Pakistan, Singapore, Taiwan, and the United States (3, 4). In addition, isolates producing six NDM variants have been reported, including NDM-2-producing *Acinetobacter baumannii* strains from Egypt (5, 6), Israel (5), Germany (7), and the United Arab Emirates (8); an NDM-3-producing *E. coli* strain from Australia (accession no. JQ734687); an NDM-4-producing *E. coli* strain from India (9); an NDM-5-producing *E. coli* strain from the United Kingdom (10); an NDM-6-producing *E. coli* strain from New Zealand (11); and an NDM-7-producing *E. coli* strain from Canada (accession no. JX262694).

E. coli IOMTU11 (NCGM37) and *Pseudomonas aeruginosa* IOMTU9 (NCGM1841) were isolated from pus from a surgical site and from sputum of patients, respectively, in 2012 at Tribhuvan University Teaching Hospital in Kathmandu, Nepal. The isolates were phenotypically identified, and species identification was confirmed by 16S rRNA sequencing (12). MICs were determined using the microdilution method recommended by the Clinical and Laboratory Standards Institute (13). *E. coli* IOMTU11 was resistant to all antibiotics tested excepted fosfomycin (MIC, 4 μg/ml). The MICs of β-lactams are shown in Table 1, and those of other antibiotics were as follows: arbekacin, >1,024 μg/ml; amikacin, >1,024 μg/ml; colistin, 0.25 μg/ml; gentamicin, >1,024 μg/ml; and tigecycline, 0.5 μg/ml. MBL production was examined with an MBL Etest (Sysmex; bioMérieux Co., Marcy l'Etoile, France), with MICs of 256 μg/ml of imipenem and 2 μg/ml of imipenem-EDTA. PCR analysis for MBL genes (14, 15, 16) and 16S rRNA methylase genes (17) was performed. The isolates were positive for bla_{NDM} and *rmtB*. Sequence analysis showed that the *bla*NDM was a novel variant, and it was designated bla_{NDM-8}. Multilocus sequence typing (MLST) of IOMTU11 showed that it was ST101 (*Escherichia coli* MLST database [http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html]). *P. aeruginosa* IOMTU9 had bla_{NDM-1}, which was used as a reference gene.

The sequence of the bla_{NDM-8} gene showed mutations corresponding to two amino acid substitutions compared with bla_{NDM-1} (accession number JF798502). Analysis of the predicted amino acid sequence revealed two substitutions (D130G and M154L) compared with NDM-1, one substitution (D130G) compared with NDM-4, and one substitution (L88V) compared with NDM-5.

The bla_{NDM-8} and bla_{NDM-1} genes were cloned into the corresponding sites of pHSG398 (TaKaRa Bio, Shiga, Japan) with the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCA ATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGC AGCTTGTCGGCCAT-3′). *E. coli* DH5α was transformed with pHSG398-NDM-8 or pHSG398-NDM-1 to determine the MICs of β-lactams.

The open reading frames of NDM-1 and NDM-8 without signal peptide regions were cloned into the expression vector pQE2 (Qiagen, Tokyo, Japan) with the primer set SacI-NDM-F (5′-CCCC TCGAGCAGCAAATGGAAACTGGCGACCAACGGT-3′) and SalI-NDM-R (5′-CCCGAGCTCTCAGCGCAGCTTGTCGGCC ATGCGGGCC-3′). The plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA). The recombinant NDM proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instruction (Qiagen). His tags were removed by digestion with DAPase (Qiagen), and untagged proteins were purified by an additional passage over Ni-NTA agarose. The purities of NDM-1 and NDM-8 were over 90%, as estimated by SDS-PAGE. During the purification procedure, the presence of β-lactamase activity was monitored with nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM phosphate buffer (pH 7.0) at 25°C with 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 by use of the Lineweaver-Burk plot. Wavelengths and extinc-

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TABLE 1 MICs of various β-lactams for *E. coli* strain IMOTU11 and *E. coli* strains transformed with NDM-1 or NDM-8

Antibiotic	MIC (μg/ml)			
	IOMTU11	pHSG398/NDM-8	pHSG398/NDM-1	pHSG398
Ampicillin	>1,024	256	256	4
Ampicillin-sulbactam	>1,024	128	128	2
Aztreonam	>1,024	0.03	0.03	0.03
Cefepime	1,024	0.5	0.5	<0.25
Cefmetazole	ND[a]	4	2	1
Cefoselis	ND	8	4	<0.25
Cefotaxime	>1,024	8	8	<0.25
Cefoxitin	>1,024	64	64	8
Cefozopran	ND	8	8	<0.25
Cefpirome	ND	2	1	<0.25
Cefsulodin	ND	>512	>512	256
Ceftazidime	>1,024	256	256	<0.25
Ceftriaxone	ND	16	32	<0.25
Cefuroxime	ND	512	512	4
Cephradine	>1,024	512	256	16
Doripenem	ND	0.125	0.06	0.03
Imipenem	256	0.5	0.25	0.06
Meropenem	256	0.25	0.5	0.03
Moxalactam	ND	16	8	<0.25
Panipenem	ND	0.5	0.25	0.06
Penicillin G	>1,024	256	256	32
Piperacillin	>1,024	16	16	2
Piperacillin-tazobactam	>1,024	8	8	1
Ticarcillin	>1,024	>512	>512	2
Ticarcillin-clavulanic acid	512	512	512	4

[a] ND, not determined.

tion coefficients for β-lactam substrates have been reported elsewhere (18, 19, 20).

Expression of the bla_{NDM-8} and bla_{NDM-1} genes in *E. coli* DH5α conferred resistance or reduced susceptibility to all cephalosporins, moxalactam, and carbapenems (Table 1). The MICs of cefmetazole, cefoselis, cefpirome, doripenem, imipenem, panipenem, and moxalactam were one dilution higher for the *E. coli* strain expressing NDM-8 than for that expressing NDM-1. In contrast, those of ceftriaxone and meropenem were one dilution lower for the NDM-8-expressing strain than for the NDM-1-expressing strain.

As shown in Table 2, recombinant NDM-8 and NDM-1 hydrolyzed all β-lactams tested except aztreonam. The profile of enzymatic activities of NDM-8 against β-lactams was similar to that of NDM-1, although NDM-8 had slightly lower k_{cat}/K_m ratios for penicillin G, ampicillin, cephradine, cefotaxime, and meropenem than NDM-1.

Two amino acid substitutions at positions 88 and 130 slightly affected the enzymatic activities of NDM-8 compared to those of NDM-1 (Table 2). Among all eight NDM variants, amino acid substitutions were found at 6 positions (i.e., positions 28, 88, 95, 130, 154, and 233). It is not yet known which position(s) plays a critical role in the enzymatic activities. The crystal structure of NDM-1 revealed that the active site of NDM-1 is located at the bottom of a shallow groove enclosed by 2 important loops, L3 and L10 (21, 22, 23, 24). Residues 88 and 130, however, were not lo-

TABLE 2 Kinetic parameters of NDM-8 and NDM-1[a]

β-Lactam	NDM-8			NDM-1		
	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Penicillin G	74 ± 10	91 ± 3	1.20	29 ± 2	79 ± 1	2.70
Ampicillin	193 ± 6	158 ± 5	0.82	122 ± 12	137 ± 5	1.10
Cephradine	52 ± 7	52 ± 4	1.00	37 ± 4	63 ± 1	1.70
Cefoxitin	34 ± 1	3 ± 0.1	0.10	25 ± 6	4 ± 0.3	0.05
Cefotaxime	30 ± 6	38 ± 3	1.30	28 ± 4	45 ± 1	1.70
Ceftazidime	63 ± 3	12 ± 0.2	0.20	74 ± 9	32 ± 2	0.45
Cefepime	153 ± 13	25 ± 1	0.17	152 ± 31	33 ± 5	0.22
Aztreonam	NH[c]	NH	NH	NH	NH	NH
Imipenem	167 ± 8	46 ± 2	0.28	194 ± 38	60 ± 7	0.31
Meropenem	127 ± 20	169 ± 12	1.30	54 ± 10	66 ± 3	1.20

[a] The proteins were initially modified by a His tag, which was removed after purification.
[b] Values are means from three 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.

cated in these loops. These residues may indirectly affect the formation of the active site. NDM-1 may not bind to the carbapenems as tightly as IMP-1 or VIM-2, and it turns over the carbapenems at a rate similar to that of VIM-2 (2). NDM-4 possessed increased hydrolytic activity for carbapenems and several cephalosporins compared to NDM-1 (9). NDM-4 with an amino acid substitution at position 130 (Met to Leu) showed increased hydrolytic activity for carbapenems and several cephalosporins compared to NDM-1 (9). NDM-5 with substitutions at positions 88 (Val to Leu) and 154 (Met to Leu) reduced the susceptibility of *E. coli* transformants to cephalosporins and carbapenems (9). The drug susceptibilities of *E. coli* transformants with bla_{NDM}-2, bla_{NDM-3}, bla_{NDM-6}, and bla_{NDM-7} have not yet been reported. NDM must have only recently started to evolve, and therefore careful monitoring of NDM-producing pathogens is required.

bla_{NDM-8} was found in a plasmid of >100 kb (data not shown). The plasmid was sequenced by using the GS Junior system (Roche Diagnostics K.K, Tokyo, Japan). The sequence surrounding bla_{NDM-8} was *tra-*bla_{NDM-8}*-ble-trpF-tat*, and the genetic environment of bla_{NDM-8} had more than 99.9% identity at the nucleotide sequence from position 4564 to 8780 bp of *K. pneumoniae* strain GN529 (accession no. HQ416416), which was isolated in Ontario, Canada.

This is the first report describing NDM-1- and NDM-8-producing Gram-negative pathogens in Nepal.

Nucleotide sequence accession number. bla_{NDM-8} has been deposited in GenBank with the accession number AB744718.

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