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A Novel New Delhi Metallo-β-Lactamase Variant, NDM-14, Isolated in a Chinese Hospital Possesses Increased Enzymatic Activity against Carbapenems

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A novel New Delhi metallo-β-lactamase (NDM) variant, NDM-14, was identified in clinical isolate *Acinetobacter lwoffii* JN49-1, which was recovered from an intensive care unit patient at a local hospital in China. NDM-14, which differs from other existing enzymes by an amino acid substitution at position 130 (Asp130Gly), possesses enzymatic activity toward carbapenems that is greater than that of NDM-1. Kinetic data indicate that NDM-14 has a higher affinity for imipenem and meropenem.

The emergence and global spread of carbapenem-resistant *Enterobacteriaceae* is of great concern. A novel metallo-β-lactamase (MBL), New Delhi MBL-1 (NDM-1), has attracted wide attention in recent years because it confers resistance to all classes of β-lactam antibiotics except for the monobactam aztreonam (1). NDM-1 is identified mainly in *Escherichia coli*, *Acinetobacter* spp., and *Klebsiella pneumoniae*. Ongoing research suggests that the gene conferring antibiotic resistance on these bacteria, bla_{NDM-1}, is now widely spread throughout the world (2). Currently, there are 12 variants of NDM (NDM-1 to NDM-10, NDM-12, and NDM-13; NDM-11 is assigned without any information in GenBank) that differ by one, two, or five amino acid substitutions at 13 positions (see www.lahey.org/studies).

Acinetobacter lwoffii JN49-1 was isolated from the infected wound and feces of an intensive care unit (ICU) patient in Jinan, China. Identification of the isolate to the species level was carried out by using the Vitek 2 system (bioMérieux, France), 16S rRNA gene sequencing, and 16S-23S rRNA gene intergenic spacer sequencing (3). Antimicrobial susceptibility testing was performed by broth microdilution according to the Clinical and Laboratory Standards Institute (4). *A. lwoffii* JN49-1 was highly resistant to β-lactams, including imipenem and meropenem, and susceptible to tigecycline and colistin (Table 1). MBL detection with Etest MBL strips (bioMérieux, France) was positive. PCR screening for known β-lactamase genes and aminoglycoside resistance genes was also performed (5, 6). Interestingly, PCR product sequencing results revealed that JN49-1 carries the bla_{NDM} and $bla_{aac(6')-Ib}$ genes. Subsequent sequencing revealed that JN49-1 harbored a novel bla_{NDM} gene with a point mutation at position 389 (A→G). Analysis of the predicted amino acid sequence showed an amino acid substitution (Asp130Gly), and it was designated NDM-14. Moreover, another NDM-1-positive *A. lwoffii* strain, JN247, that had a resistance pattern similar to that of JN49-1 was recovered from a different ICU patient at the same hospital (Table 1).

The horizontal-transfer capability of the bla_{NDM} gene was assessed by broth and filter mating by using a standard *E. coli* J53 azide-resistant strain as the recipient. MacConkey agar containing 100 mg/liter sodium azide and 0.5 mg/liter meropenem was used to select for *E. coli* J53 transconjugants (7; see Materials and Methods in the supplemental material). Putative transconjugants were confirmed by bla_{NDM} detection by PCR assay as described above.

Southern blot analysis was performed to locate the bla_{NDM} genes by using specific bla_{NDM} digoxigenin-labeled probes (Roche) (8). *K. pneumoniae* ATCC BAA-2146 was used as a positive control, and *E. coli* J53 was used as a negative control. Plasmid DNA was extracted and sequenced by the Ion Torrent sequencing platform (9).

To compare the relative contributions of NDM-1 and NDM-14 to carbapenem resistance, the entire open reading frame (ORF) (primers NDM-F [5′-CGGGATCCATGGAATTGCCCAATATTATG-3′] and NDM-R [5′-CCCAAGCTTTCAGCGCAGCTTGTCGGCCAT-3′]) and the complete gene with its native promoter (primers NP-NDM-F [5′-CGGGATCCCACCTCATGTTTGAATTCGC-3′] and NP-NDM-R [5′-CCCAAGCTTCTCTGTCACATCGAAATCGC-3′]) were amplified and cloned into the corresponding sites of pHSG398 (TaKaRa Bio). *E. coli* DH5α cells were transformed with pHSG398-NDM-1, pHSG398-NDM-14, pHSG398-NP-NDM-1, and pHSG398-NP-NDM-14 to determine β-lactam MICs (10, 11).

The ORFs of NDM-1 and NDM-14 without signal peptide regions were cloned into expression vector pET28a with primers BamHI-TEV-NDM-F (5′-CGGGATCCGAAAACCTGTATTTCCAAGGCCAGCAAATGGAAACTGGCGAC-3′) and XhoI-NDM-R (5′-CCGCTCGAGTCAGCGCAGCTTGTCGGCCATG-3′) (11).

E. coli BL21(DE3) was used to express the recombinant NDM

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TABLE 1 Antibiotic susceptibility profiles of NDM-carrying clinical isolates, transconjugants, and transformants

Antibiotic	MIC (mg/liter) for NDM-carrying clinical isolate, transconjugant, and transformant:									
	JN247 (NDM-1)	JN49 (NDM-14)	JN49-J53	JN247-J53	J53	DH5α(pHSG398)	DH5α(pHSG398-NDM-1)	DH5α(pHSG398-NDM-14)	DH5α(pHSG398-NP-NDM-1)	DH5α(pHSG398-NP-NDM-14)
Ampicillin	>256	>256	>256	>256	4	2	>256	>256	>256	>256
Ceftazidime	>256	>256	>256	>256	0.25	0.25	32	32	>256	>256
Cefotaxime	>256	>256	>256	>256	2	1	128	128	>256	>256
Meropenem	≥32	≥32	4	2	0.023	0.023	0.094	0.094	4	16
Imipenem	≥32	≥32	2	1	0.19	0.19	0.25	0.38	6	16
Aztreonam	8	>256	0.062	0.062	0.062	0.062	0.062	0.062	0.062	0.062
Amikacin	2	16	0.062	0.062	0.031	0.031	0.062	0.062	0.062	0.062
Ciprofloxacin	4	2	0.031	0.031	0.031	0.031	0.031	0.031	0.031	0.031
Tigecycline	0.125	0.125	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Colistin	1	1	0.25	0.125	0.062	0.015	0.015	0.015	0.031	0.031

proteins, which were purified by using nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instructions (Qiagen). His tags were cleaved with the TurboTEV protease (Accelagen, San Diego, CA), and the tags and protease were removed by an additional passage over Ni-NTA agarose. The purity of the recombinant NDM proteins was estimated up to 90% by SDS-PAGE. The protein concentration was measured with the Pierce bicinchoninic acid protein assay kit (Thermo Scientific). The hydrolysis rates were monitored in 50 mM phosphate buffer (pH 7.0) at 37°C with a SpectraMax 190 microplate reader (Molecular Devices). K_m and k_{cat} values and k_{cat}/K_m ratios were determined by using a Lineweaver-Burk plot. Wavelengths and extinction coefficients for β-lactam substrates have been reported previously (12–14).

The bla_{NDM-1} (in *A. lwoffii* JN247) and bla_{NDM-14} (in *A. lwoffii* JN49-1) genes were successfully transferred into *E. coli* J53 by filter mating with a low transfer frequency of approximately 1.0×10^{-8}. No transfer was observed by broth mating. Transconjugation assays suggested that bla_{NDM} might be located on a plasmid, and the *E. coli* J53 transconjugants carrying bla_{NDM} from these two *E. coli* isolates were named JN49-J53 and JN247-J53. Both isolates exhibited resistance to ampicillin, ceftazidime, and cefotaxime and susceptibility to aztreonam. Two isolates showed different susceptibilities to meropenem and imipenem; JN49-J53 was resistant to meropenem and intermediately susceptible to imipenem, whereas JN247-J53 was intermediately susceptible to meropenem and susceptible to imipenem (Table 1).

A Southern blot assay showed that each isolate harbored multiple plasmids, yet only one plasmid in each isolate was positive for the bla_{NDM} probe (Fig. 1). Further, these data clearly indicated that bla_{NDM} was located on a plasmid of approximately 40 kb. The plasmid harboring bla_{NDM-14} from strain JN49-1 was named pNDM-JN01, and the plasmid harboring bla_{NDM-1} from strain JN247 was named pNDM-JN02.

Plasmid sequencing revealed that pNDM-JN01 (harboring bla_{NDM-14}) is 41,084 bp long with a GC content of 38%. Plasmid pNDM-JN02 (harboring bla_{NDM-1}) was identical to pNDM-JN01, with the exception of a single nucleotide change in the *ndm* gene. A BLAST search showed that pNDM-JN01/pNDM-JN02 was similar to previously published plasmid pNDM-BJ01 (15). Sequence comparison revealed that a 6,187-bp region containing the genes *groS*, *groEL*, and *insE* and insertion sequence IS*Aba125* was absent from pNDM-JN01 and pNDM-JN02. We also found the same deletion in the bla_{NDM-1} downstream region in pNDM-44551 (GenBank accession no. KF208467.1) from *A. pittii* 445511 with a partial sequence in the NCBI database (Fig. 2). It was probable that pNDM-JN01, harboring the novel bla_{NDM-14} gene, evolved from pNDM-BJ01.

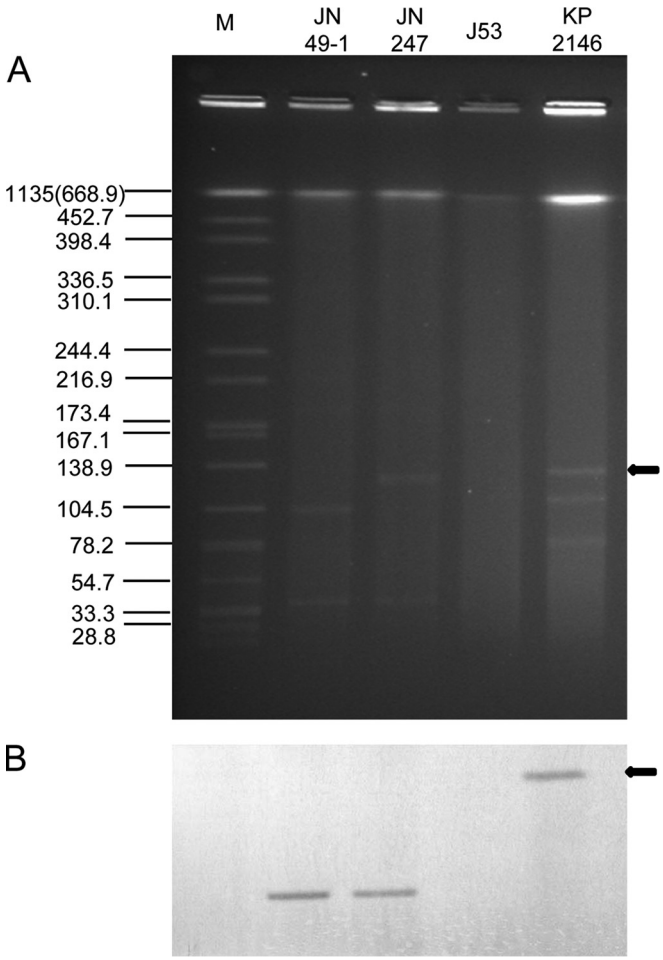

FIG 1 Identification of bla_{NDM}-positive plasmids. (A) S1 nuclease plasmid pulsed-field gel electrophoresis profiles. (B) Southern blot hybridization for bla_{NDM}. The arrows on the right indicate the positive control, *K. pneumoniae* ATCC BAA-2146 carrying an NDM-1-positive plasmid with a size of 140,825 bp. Lane M, reference standard strain H9812 restricted with XbaI. The molecular sizes on the left are in kilobases.

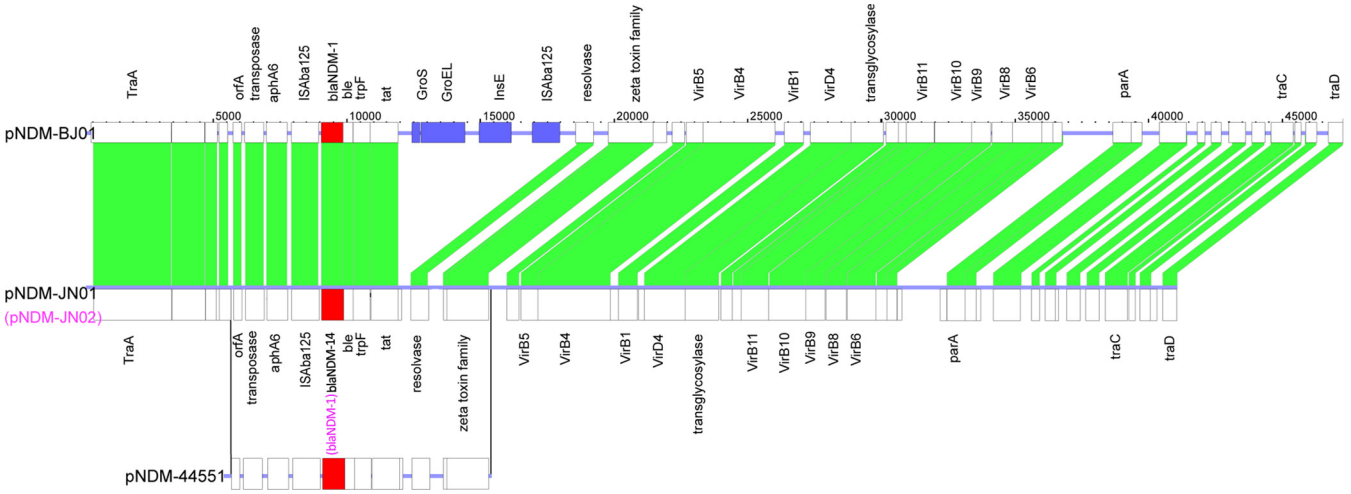

FIG 2 Sequence comparison of bla_{NDM}-harboring plasmids pNDM-BJ01, pNDM-JN01/pNDM-JN02, and pNDM-44551 (only partial sequences of pNDM-44551 were accessible in the NCBI database). Boxes indicate ORFs identified by sequence analysis, and all regions are drawn to scale. Similar structures and high sequence homology are indicated by white boxes. Blue boxes indicate ORFs found only in pNDM-BJ01. Solid lines represent highly homologous sequences. The bla_{NDM} genes are represented by red boxes.

Twelve NDM variants have been reported in several different countries (see www.lahey.org/studies). Upon sequence analysis of all of the variants, we determined that bla_{NDM-14} had a close relationship with bla_{NDM-8}, as NDM-14 had only one amino acid difference (Asp130Gly) and NDM-8 had two differences (Asp130Gly and Met154Lys) from NDM-1.

It is interesting that while *E. coli* DH5α transformants without their native promoter (pHSG398-NDM-1 and pHSG398-NDM-14) exhibited resistance to ampicillin, ceftazidime, and cefotaxime but showed susceptibility to meropenem and imipenem, other transformants (pHSG398-NP-NDM-1 and pHSG398-NP-NDM-14) exhibited resistance to all β-lactams, including meropenem and imipenem, only when expressed under the control of their native promoters (Table 1). This is consistent with previous reports (10, 16, 17).

The most intriguing finding from our observations was that *E. coli* DH5α carrying pHSG398-NP-NDM-14 conferred meropenem and imipenem resistance higher than that of *E. coli* DH5α carrying pHSG398-NP-NDM-1 (Table 1). It was concluded that the differences in carbapenem MICs were caused by mutations outside the promoter region. As was the case for other MBLs, NDM-14 hydrolyzed all of the β-lactams tested except aztreonam (Table 2). Kinetic data showed that NDM-14 had an affinity for imipenem, meropenem, and ampicillin higher than that of NDM-1, with K_m values reduced by 18 μM for imipenem, 16 μM for meropenem, and 74 μM for ampicillin, whereas slightly lower affinities of NDM-14 than of NDM-1 for cefotaxime, ceftazidime, and cefuroxime were observed. In addition, NDM-14 had an affinity for penicillin G significantly lower than that of NDM-1, with K_m values of 186 and 58 μM for NDM-14 and NDM-1, respectively. Small differences (1- to 2-fold) between the k_{cat}/K_m values of NDM-1 and NDM-14 were observed (Table 2) (16, 18).

The amino acid substitution at position 130 (Asp130Gly) appears to confer higher carbapenemase activity, even though it is located outside the active center. This substitution is similar to those in NDM-7 (Asp130Asn and Met154Lys) and NDM-8 (Asp130Gly and Met154Lys) (10, 18). It remains unclear which sites play an important role in enzymatic activity. The crystal structure of NDM-1 shows that the active site of NDM-1 is decided at the bottom of a shallow groove enclosed by two important loops, L3 and L10 (19, 20). However, residue 130 is not located in these loops. We suggest that the residue may have an indirect

TABLE 2 Kinetic parameters of NDM-14 and NDM-1 enzymes[a]

β-Lactam	NDM-14			NDM-1		
	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1}s^{-1}$) ratio	K_m (μM)[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($\mu M^{-1}s^{-1}$) ratio
Ampicillin	80 ± 14	102 ± 7	1.28	154 ± 10	182 ± 5	1.18
Penicillin G	186 ± 9	230 ± 10	1.24	58 ± 5	142 ± 8	2.45
Cefotaxime	46 ± 5	63 ± 4	1.37	26 ± 4	49 ± 3	1.88
Ceftazidime	72 ± 4	16 ± 1	0.22	51 ± 3	22 ± 1	0.43
Cefuroxime	44 ± 2	15 ± 1	0.34	31 ± 1	20 ± 1	0.65
Aztreonam	NH[c]	NH	NH	NH	NH	NH
Imipenem	90 ± 4	42 ± 2	0.47	108 ± 5	58 ± 3	0.54
Meropenem	53 ± 2	60 ± 3	1.13	69 ± 7	72 ± 9	1.04

[a] The proteins were initially modified by adding 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 of up to 1 mM and enzyme concentrations of up to 700 nM.

effect on the formation of the active site. It has previously been proven that NDM-7 has increased carbapenemase activity (10). Thus, these data suggest that amino acid substitutions at position 130 contribute to the carbapenemase activity of NDM proteins.

In summary, we identified a novel NDM variant in *A. lwoffii*, NDM-14, possessing increased carbapenemase activity. In addition, another *A. lwoffii* isolate carrying bla_{NDM-1} was confirmed. Two bla_{NDM}-positive plasmids, which were extracted from clinical isolates JN49-1 and JN247, harbored nearly identical sequences (one nucleotide difference between the bla_{NDM-1} genes). Taken together, these data suggest that the emergence of *Acinetobacter* spp. with similar NDM-positive plasmids promotes dissemination of the bla_{NDM} gene, resulting in antibiotic resistance.

Nucleotide sequence accession numbers. Plasmid pNDM-JN01 and pNDM-JN02 sequences have been deposited in the GenBank database under accession numbers KM210086 and KM210088, respectively.

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We have no conflicts of interest to declare.

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