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NDM-4 Metallo-β-Lactamase with Increased Carbapenemase Activity from *Escherichia coli*

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A clinical *Escherichia coli* isolate resistant to all β-lactams, including carbapenems, expressed a novel metallo-β-lactamase (MBL), NDM-4, differing from NDM-1 by a single amino acid substitution (Met154Leu). NDM-4 possessed increased hydrolytic activity toward carbapenems and several cephalosporins compared to that of NDM-1. This amino acid substitution was not located in the known active sites of NDM-1, indicating that remote amino acid substitutions might also play a role in the extended activity of this MBL.

Acquired metallo-β-lactamases (MBLs) are emerging resistance determinants in clinically relevant Gram-negative species (19). NDM-1 (New Delhi metallo-β-lactamase 1) has been recently identified, being first described from *Klebsiella pneumoniae* and *Escherichia coli* isolated in Sweden in 2008 from an Indian patient (23). NDM-1, as is the case for any MBL, confers a broad-spectrum β-lactam resistance, hydrolyzing penicillins, cephalosporins, and carbapenems but sparing monobactams (23). The rapid and large dissemination of NDM-1-producing Gram-negative species has been emphasized in many reports that have been published in the last 2 years (13, 16, 20). In *Enterobacteriaceae*, the $bla_{\text{NDM-1}}$ gene has been shown to be carried by different plasmid types (IncA/C, IncF, IncL/M, or untypeable) (13, 16, 17). Most $bla_{\text{NDM-1}}$-encoding plasmids coharbored multiple and variable resistance determinants, including those for β-lactams, quinolones, aminoglycosides, rifampin, chloramphenicol, and macrolides (13, 16, 17). The $bla_{\text{NDM-1}}$ gene has been widely identified in *Enterobacteriaceae* but also in *Acinetobacter baumannii* from Germany, India, the United Kingdom, and China (4, 5, 10, 11). In addition, NDM-2-producing *A. baumannii* isolates have been reported from Egypt and Israel (7, 10). NDM-2 differs from NDM-1 by a single amino acid substitution (Pro28Ala) located in the leader peptide of the enzyme that does not modify its hydrolytic properties compared to those of NDM-1 (7, 21). We report here the identification of a novel NDM variant that possesses extended hydrolytic properties.

E. coli I5 was recovered from a urinary culture of a patient hospitalized in India in January 2010. Susceptibility testing was performed by disk diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France) as previously described (6a). Results were interpreted according to the CLSI guidelines (6a). The MICs were determined by Etest (AB bioMérieux, Solna, Sweden) on Mueller-Hinton agar plates at 37°C. *E. coli* I5 was resistant to all β-lactams, including imipenem, meropenem, and ertapenem (Table 1). This isolate was additionally resistant to all tested aminoglycosides and fluoroquinolones. Production of MBL was assessed using Etest MBL (AB bioMérieux, Solna Sweden), which gave a positive result. Whole-cell DNA of *E. coli* isolate I5 was extracted using a QiaAmp minikit according to manufacturer recommendations (Qiagen, Courtaboeuf, France), and DNA was used as a template for the detection of different β-lactamases and 16S rRNA methylase genes using specific primers (1, 17). PCR amplification followed by sequencing identified the $bla_{\text{CTX-M-15}}$ gene together with the $bla_{\text{CMY-6}}$ gene. In addition, it revealed a novel bla_{NDM} type that was designated the $bla_{\text{NDM-4}}$ gene (http://www.lahey.org/Studies/). β-Lactamase NDM-4 differed by a single amino acid substitution (Met154Leu) from NDM-1 and by two substitutions (Ala28Pro and Met154Leu) from NDM-2. In addition, *E. coli* I5 harbored the *armA* gene, encoding a 16S rRNA methylase conferring high-level resistance to all aminoglycosides. Phylogenetic analysis using a multiplex PCR method as described previously (6) showed that isolate I5 belonged to phylogroup D, which includes extraintestinal isolates. Multilocus sequence typing analysis performed as described by Wirth et al. (22) showed that isolate I5 belonged to the ST648 sequence type. This is the first *E. coli* ST648 isolate found to be producing an NDM-1 enzyme, though an NDM-5-producing ST648 isolate was very recently identified (8).

In order to evaluate and compare the spectrum of hydrolysis of NDM-4 to that of NDM-1, cloning of the $bla_{\text{NDM-4}}$ and $bla_{\text{NDM-1}}$ genes was performed using a ZeroBlunt TOPO PCR cloning kit (Invitrogen, Cergy-Pontoise, France) followed by expression in the same *E. coli* TOP10 background (18). Selection was based on plates containing 100 μg of ticarcillin per ml and 30 μg of kanamycin per ml. The PCR amplicon encompassing the entire sequence of the bla_{NDM} genes used for cloning was obtained with the forward primer pre-NDM-for (5′-CACCTCATGTTTGAATTCGCC-3′) and reverse primer pre-NDM-rev (5′-CTCTGTCACATCGAAATCGC-3′). It gave rise to recombinant strains *E. coli* TOP10(pNDM-1) and *E. coli* TOP10(pNDM-4), expressing NDM-1 and NDM-4, respectively. Expression of the $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-4}}$ genes in *E. coli* TOP10 conferred resistance or reduced susceptibility to all β-lactams except aztreonam (Table 1). However, the MICs of imipenem and ertapenem were higher for *E. coli* expressing NDM-4 than that expressing NDM-1, suggesting that

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TABLE 1 MICs of β-lactams for *E. coli* clinical isolate and transformants[a]

β-Lactam(s)	MIC of indicated *E. coli* isolate				
	I5 (NDM-4)	TOP10 (NDM-4)	TOP10 (pNDM-4)	TOP10 (pNDM-1)	TOP10
Ticarcillin	>256	>256	>256	>256	4
Ticarcillin + CLA	>256	>256	>256	>256	4
Piperacillin	>256	>256	>256	>256	1
Piperacillin + TZB	>256	>256	>256	>256	1
Cefuroxime	>256	>256	>256	>256	2
Ceftazidime	>256	256	>256	>256	0.06
Cefotaxime	>256	256	>256	>256	0.12
Cefepime	>256	16	>256	>256	0.06
Cefoxitin	>512	>256	256	256	4
Aztreonam	>256	0.25	0.06	0.06	0.12
Imipenem	8	8	16	8	0.06
Meropenem	16	4	8	8	0.01
Ertapenem	>32	8	16	8	0.01
Doripenem	4	4	8	8	0.01

[a] Shown are MICs of β-lactams for the *E. coli* I5 clinical isolate, the transformant *E. coli* TOP10 harboring the natural plasmid from *E. coli* I5 and expressing NDM-4, and *E. coli* TOP10 strains harboring the recombinant plasmid pNDM-4 or pNDM-1 expressing β-lactamase NDM-4 or NDM-1, respectively.

the Leu154 residue was involved in the higher carbapenemase activity.

In order to determine whether NDM-4 might possess specific catalytic properties, a kinetic study was conducted. *E. coli* TOP10(pNDM-4) produced a β-lactamase with a theoretical pI value of 5.8. NDM-4 was purified to near homogeneity (>90% as estimated by SDS-PAGE analysis) from *E. coli* TOP10 pTOPO-NDM-4 crude extract by using a two-step chromatography process (anion exchange at pH 5.0 followed by anion exchange at pH 6.8 using Q-Sepharose columns) (18). The purification factor was estimated to be 40-fold.

β-Lactamase NDM-4 hydrolyzed all tested β-lactams except aztreonam, as was the case for other MBLs. Kinetic data showed that NDM-4 hydrolyzed imipenem at a higher level than did NDM-1 (Table 2). Similarly, the catalytic activity of NDM-4 was slightly higher than that of NDM-1 for meropenem (Table 2). Higher catalytic efficiencies were also observed for cefalotin, ceftazidime, and cefotaxime for NDM-4, whereas cefepime was less hydrolyzed (Table 2). The k_{cat} values were higher for NDM-4 than for NDM-1 for cefalotin and cefotaxime. In addition, NDM-4 showed a higher affinity for ceftazidime than that of NDM-1, with K_m values of 72 and 181 μM for NDM-4 and NDM-1, respectively.

Plasmid DNA of *E. coli* I5 was extracted by using the Kieser method (12). Plasmid DNA was analyzed by agarose gel electrophoresis, as described previously (14). Two plasmids were identified, being of ca. 120 kb and ca. 7 kb in size, respectively. Direct transfer of the β-lactam resistance markers into *E. coli* J53 was attempted by liquid mating-out assays at 37°C as described previously (2). Selection was performed using agar plates supplemented with cefoxitin (10 μg/ml) and azide (100 μg/ml). These conjugation experiments failed. However, electrotransformation experiments gave *E. coli* transformants harboring a single 120-kb plasmid that carried the bla_{NDM-4} gene. In addition to resistance to β-lactams, this plasmid conferred resistance to all aminoglycosides. A PCR-based replicon typing method (3) showed that this bla_{NDM-4}-positive plasmid belonged to the IncF incompatibility group. Genetic structures surrounding the bla_{NDM-4} gene performed by PCR mapping as described previously (17) identified a remnant of insertion sequence IS*Aba125* upstream of the bla_{NDM-4} gene. A bleomycin resistance gene, the ble_{MBL} gene, was

TABLE 2 Kinetic parameters of NDM-4 and NDM-1 enzymes[a]

β-Lactam	NDM-4			NDM-1[b]			k_{cat}/K_m ($\mu M^{-1} s^{-1}$) ratio for NDM-4/NDM-1
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	
Ampicillin	ND	ND	ND	22	15	0.66	—
Amoxicillin	3,400	1,007	0.3	NA	NA	NA	—
Cefoxitin	NH	NH	NH	49	1	0.02	—
Cephalotin	46	24	0.5	10	4	0.40	1.3
Cefotaxime	18	22	1.2	10	6	0.60	2.1
Ceftazidime	72	4	0.06	181	5	0.03	2
Cefepime	169	7	0.04	77	13	0.20	0.25
Aztreonam	NH	NH	NH	NH	NH	NH	—
Imipenem	86	40	0.46	94	20	0.20	2.20
Meropenem	95	30	0.31	49	12	0.25	1.25
Ertapenem	74	26	0.35	NA	NA	NA	—

[a] CLA, clavulanic acid; TZB, tazobactam; ND, not determined; NH, no hydrolysis detected with 200 μM substrate and 1 mg of purified enzyme; NA, not available; —, not possible to evaluate. Data are mean results of three independent experiments; standard deviations were within 15% of the means.
[b] From reference 22.

identified downstream of the bla_{NDM-4} gene. The same genetic environment has been observed for most of the analyzed NDM-1-positive enterobacterial isolates (17).

Conclusion. This study identified a novel NDM-type β-lactamase, NDM-4, possessing a high ability to hydrolyze carbapenems and several bulky cephalosporins. The Met154Leu substitution was responsible for this high carbapenemase activity. This amino acid substitution was not located in what has been identified as the active site of NDM-1 or the amino acid residue that binds to the zinc ions (21, 24). This is the second example of identification of amino acid substitution as a source of extended catalytic activity in an MBL structure. Actually, amino acid substitutions located outside the active site of VIM-type enzymes (VIM-11, VIM-19) have been shown to be able to modulate their hydrolytic activity (15, 18).

Since this work was in progress, NDM-5, another NDM variant which contains the same Met154Leu substitution in addition to a Val88Leu substitution, was reported (8). MICs of carbapenems for an NDM-5-producing isolate were reported to be higher than those for an NDM-1 producer, but no biochemical analysis of NDM-5 is available (8). Our work underlines the idea that NDM variants may possess an increased activity toward β-lactams and in particular toward carbapenems. The selection of these variants may have resulted from carbapenem-based therapy, taking into account the possibility that many NDM-1-producing isolates may exhibit only decreased susceptibility to carbapenems (16). Identification of NDM variants may signal an ongoing and rapid evolution of the NDM genes resulting from their large spread, at least in the Indian subcontinent.

Nucleotide sequence accession number. The sequence reported here has been deposited in GenBank with accession no. JQ348841.

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