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First Description of an *Escherichia coli* Strain Producing NDM-1 Carbapenemase in Spain▽

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A carbapenem-resistant *Escherichia coli* strain (DVR22) was recovered from a stool specimen from a patient with traveler's diarrhea who had traveled to India. Molecular screening led to the first identification of NDM-1 in Spain. The bla_{NDM-1} gene was located in a conjugative plasmid of ca. 300 kb that also contained the $bla_{CTX-M-15}$, bla_{TEM-1}, Δbla_{DHA-1}, and *armA* genes. In addition, bla_{NDM-1} was preceded by an IS*Aba125* insertion element only found in *Acinetobacter* spp.

The emergence of carbapenem resistance among *Enterobacteriaceae* is a major cause of concern since carbapenems currently represent the treatment of choice for severe infections caused by multidrug-resistant strains producing extended-spectrum β-lactamases (ESBLs) (8).

In addition to commonly known carbapenem-hydrolyzing enzymes in *Enterobacteriaceae* (IMP, VIM, KPC, and OXA-48), a novel class B metallo-β-lactamase (NDM-1) has recently been described. This enzyme, first identified in *Klebsiella pneumoniae* and *Escherichia coli* clinical isolates recovered in Sweden from a traveler returning from India, confers resistance to all β-lactams except aztreonam (22). Since then, several reports have identified bla_{NDM} genes worldwide that have typically been associated with multidrug-resistant strains (1, 5, 12, 16–18, 23).

A 40-year-old Spanish Caucasian male reported intermittent abdominal discomfort, fever, and bloody diarrhea about 5 days before returning from India. He visited a local dispensary in India where treatment with ofloxacin and ornidazole tablets (twice a day) was prescribed for 5 days. The patient reported to the Hospital Clinic of Barcelona 1 day after his return, still complaining of bloody diarrhea, although with fewer unformed stools. He was afebrile, without any sign of dehydration, and the rest of the physical examination was normal. The diarrhea resolved spontaneously over the next 9 days.

A carbapenem-resistant *E. coli* (DVR22) strain was recovered from the stool samples of the patient, and after isolation and identification, antimicrobial susceptibility profiling analysis performed with both BD Phoenix (Becton Dickinson, Franklin Lakes, NJ) and Etest strips (AB bioMérieux, Solna, Sweden) indicated that strain DVR22 was resistant to all the antibiotics tested except tigecycline (MIC of 0.75 μg/ml), fosfomycin (MIC of 32 μg/ml), and colistin (MIC of 0.5 μg/ml) (Table 1), presenting MICs of 8 μg/ml and 16 μg/ml for imipenem and meropenem, respectively, 24 μg/ml for ertapenem, and 6 μg/ml for doripenem (CLSI breakpoints from broth microdilution tests were used to classify the MICs obtained by Etest [7]). Screening for carbapenemase/MBL production yielded positive results when using either the cloverleaf test (modified Hodge test) or imipenem-EDTA Etest strips. PCR screening for β-lactamase genes followed by DNA sequencing using specific primers (NDM-1 F, 5′-CCAATATTATGCACC CGGTCG-3′, and NDM-1 R 5′-ATGCGGGCCGTATGAGT GATTG-3′) (2, 14, 21) identified the presence of bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}, and a partial sequence of the bla_{DHA-1} gene. In addition, screening for aminoglycoside resistance genes (21) identified the *armA* gene, encoding a 16S rRNA methylase conferring resistance to aminoglycosides.

In order to study the transferability of the resistance phenotype, a biparental mating between DVR22 and the *E. coli* strain J53AziR was conducted and transconjugants were selected on LB agar plates containing 1 μg/ml imipenem and 100 μg/ml sodium azide (Sigma Chemical Co., St. Louis, MO). PCR and susceptibility profiling showed that all transconjugants had become resistant to all the β-lactams and aminoglycosides tested (Table 1) and had also acquired the bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}, Δbla_{DHA-1}, and *armA* genes. Plasmid analysis by S1 nuclease–pulsed-field gel electrophoresis (PFGE) (20) was then performed on both the DVR22 strain and selected transconjugants, revealing the presence of a single plasmid of ca. 300 kb. Digoxigenin-labeled probes for the bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}, bla_{DHA-1}, and *armA* genes were hybridized against blotted nylon membranes from the S1-PFGE gels. All probes matched the band corresponding to the 300-kb plasmid. Altogether, these results indicate that all β-lactamases plus the *armA* gene are located in a single conjugative plasmid. Replicon typing classified this plasmid within the incompatibility group IncHI1 (3).

Previous reports have described the concurrence of bla_{NDM-1} together with additional ESBLs, mainly CTX-M-15 (1, 16–19), but this is the first time that they seem to be located on the same plasmid. Then again, this is also the first time that a bla_{NDM-1} gene has been found on such a large plasmid (NDM

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TABLE 1. *In vitro* susceptibilities of *E. coli* DVR22 and *E. coli* transconjugant expressing NDM-1 carbapenemase

Antibiotic(s)	MIC (μg/ml) in:		
	E. coli DVR22	*E. coli* J53 DVR22T	*E. coli* J53
Amoxicillin	>256	>256	4
Amoxicillin + clavulanate[a]	32	32	4
Piperacillin + tazobactam[b]	>256	>256	1
Cefoxitin	>256	>256	2
Cefotaxime	>256	>256	0.094
Ceftazidime	>256	>256	0.125
Cefepime	256	>256	0.25
Imipenem	8	16	0.19
Meropenem	16	12	0.023
Doripenem	6	4	0.032
Ertapenem	24	18	0.008
Aztreonam	>256	>256	64
Gentamicin	>8	>8	<1
Amikacin	>32	>32	<4
Tobramycin	>8	>8	<2
Ciprofloxacin	>32	0.008	0.008

[a] Clavulanate was used at a fixed concentration of 2 μg/ml.
[b] Tazobactam was used at a fixed concentration of 4 μg/ml.

enzyme genes are typically found in plasmids ranging from 50 to 200 kb) (1, 5, 16–18) and could reflect the formation of a cointegrate from two or more smaller plasmids carrying individual β-lactamase genes, such as $bla_{CTX\text{-}M\text{-}15}$ or $bla_{NDM\text{-}1}$. On the other hand, the *armA* gene appears to be commonly linked to $bla_{NDM\text{-}1}$ genes (1, 10, 13, 16, 19).

Multilocus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli) and PCR-based phylogroup analysis (6) revealed that *E. coli* DVR22 belonged to sequence type 156 (ST156) and phylogroup B1, respectively, differing from previously described NDM-carrying *E. coli* sequence types (16, 19, 22). PCR analysis to detect the presence of heat-stable (ST) and heat-labile (LT) toxins, verotoxins (VT), and enteroaggregative *E. coli* virulence factors were all negative.

In order to characterize the genetic environment of the $bla_{NDM\text{-}1}$ gene, outward NDM primers (NDMinv-R2, 5′-GGTCGCCAGTTTCCATTTGC-3′, and NDMinv-F2, 5′-TGCCGACACTGAGCACTAC-3′) were used to perform an inverse PCR over genomic DNA from strain DVR22 partially digested with Sau3AI and ligated with T4 DNA ligase (New England

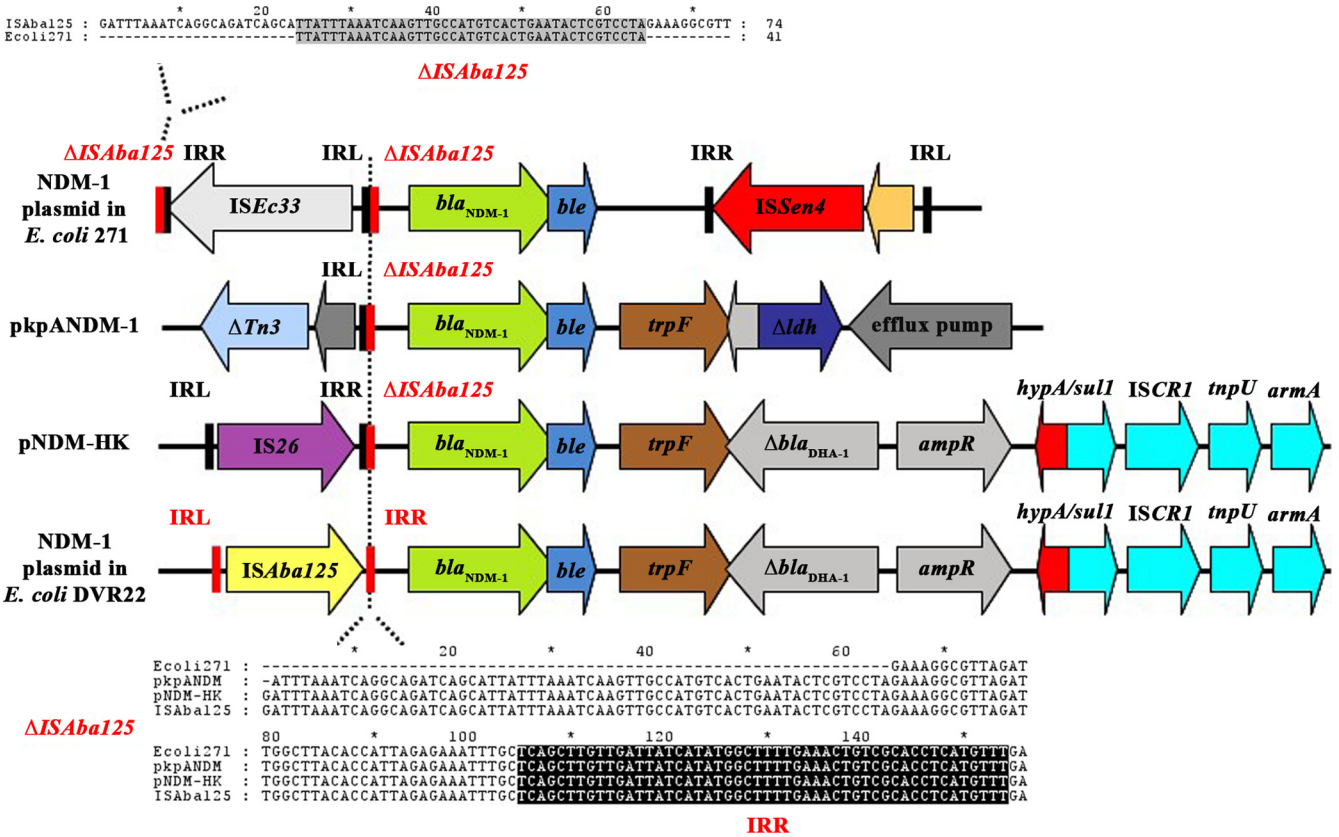

FIG. 1. Schematic drawing showing the genetic elements surrounding the $bla_{NDM\text{-}1}$ genes in *E. coli* 271, pkpANDM-1, pNDM-HK, and *E. coli* DVR22. Adapted from reference 10. The lengths of the arrows are proportional to the lengths of the genes or open reading frames (ORFs) except for the region spanning *hypA* to *armA* in both pNDM-HK and DVR22, which has been compressed to fit in the figure. The partial downstream region of IS*Aba125* containing the right inverted repeat (highlighted in black) found in all four sequences is shown in the lower alignment. The upper alignment shows the 42 missing base pairs from the downstream region of IS*Aba125* that are located upstream from the IS*Ec33* insertion element in the sequence from *E. coli* 271. Δ, truncated gene; *ampR*, LysR family $bla_{DHA\text{-}1}$ regulator; *armA*, 16S rRNA methylase gene; $bla_{DHA\text{-}1}$, class C β-lactamase gene; $bla_{NDM\text{-}1}$, New Delhi metallo-β-lactamase gene; *ble*, bleomycin resistance gene; *hypA*, putative hydrogenase nickel-incorporating gene; IRL, inverted repeat left; IRR, inverted repeat right; IS, insertion sequence; *ldh*, lactate dehydrogenase gene; *sul1*, sulfonamide resistance gene; Tn, transposon; *trpF*, phosphoribosylanthranilate isomerase gene. The GenBank accession numbers of the sequences are as follows: plasmid encoding NDM-1 in *E. coli* 271 (HQ162469), pkpANDM-1 (FN396877), pNDM-HK (HQ451074), and plasmid encoding NDM-1 in *E. coli* DVR22 (JF922606.1).

BioLabs, Ipswich, MA). Sequencing of the PCR products revealed that the region flanking the 3′ end of bla_{NDM-1} was very similar to that described for plasmid pNDM-HK, with a downstream *trpF* gene, encoding the *N*-(5′-phosphoribosyl)anthranilate isomerase, followed by a truncated bla_{DHA-1} gene (10). The published sequence of pNDM-HK was then used as a template to design specific primers to further span the downstream region. The PCR amplicons obtained with these primers always concurred with the expected size from the pNDM-HK sequence and allowed the bla_{NDM-1} gene to link up to the *armA* gene (Fig. 1). Sequencing of the bla_{NDM-1} upstream region identified the conserved putative promoter sequence described by Poirel et al. (16), as well as the presence of an IS*Aba125* insertion sequence (http://www-is.biotoul.fr). Although the presence of insertion sequences upstream from the bla_{NDM-1} gene has already been reported in *Enterobacteriaceae* (10, 16, 22), DVR22 is unique in the sense that IS*Aba125* (IS*30* family) has only been described in *Acinetobacter* spp. (9, 11, 13, 15, 24) and has not been found to be linked to bla_{NDM-1} before, suggesting horizontal transfer between *Acinetobacter* and *Enterobacteriaceae.* To further support this hypothesis, sequence comparison of the bla_{NDM-1} upstream sequences from *E. coli* strain 271 (16) and plasmids pNDM-HK and pKpANDM-1 (10, 22) identified a fragment of variable length containing the right-end repeat from IS*Aba125* in between the bla_{NDM-1} gene and the corresponding IS element as a remnant of IS*Aba125* insertion in all of these sequences. Moreover, the insertion sequence element IS*Ec33* from *E. coli* strain 271 is bracketed by the sequence upstream from the IS*Aba125* right end (Fig. 1). Reports describing NDM enzymes in *Acinetobacter* have already been published, but there is no information available regarding their genetic surroundings (5, 12, 13). In view of these results and also taking into account current investigations by our group concerning the identification of a chromosomally encoded IS*Aba125*-bla_{NDM-2} in *A. baumannii* (Paula Espinal, personal communication), it may be speculated that *Acinetobacter* is the source of the NDM enzymes found in *Enterobacteriaceae* that were originally spread by IS*Aba125*-mediated mobilization.

It is also worth mentioning that after a 4-month period, another stool specimen from the same patient was screened for bla_{NDM-1} carriage, but neither the DVR22 strain nor additional carbapenem-resistant strains could be isolated. These findings suggest that while carriage of multiple resistance determinants in a single plasmid might be beneficial under certain circumstances, in the absence of selective pressure, the burden associated with the replication and expression of extrachromosomal DNA involves a fitness cost that is not affordable, leading to the eradication of NDM-carrying bacteria (4). However, the simple clearance of DVR22 unrelated to its plasmid burden cannot be excluded.

This study reports the first identification of an *E. coli* strain producing NDM-1 in Spain and highlights the tremendous plasticity and disseminating potential of the bla_{NDM-1} gene.

Nucleotide sequence accession number. The sequence spanning from the IS*Aba125* to the *ampR* gene from the *E. coli* strain DVR22 has been submitted to GenBank and assigned sequence accession number JF922606.1.

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