Molecular characterization of *bla*$_{NDM-5}$ carried in an IncFII plasmid in *Escherichia coli* from a non-traveller patient in Spain

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Running title: NDM-5-producing *E. coli* in Spain

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Keywords: Antibiotic resistance, metallo-β-lactamase, *E. coli,* plasmid replicon typing

ABSTRACT

A carbapenem-resistant *Escherichia coli* (ST448) was recovered from a urine culture of a female patient with no recent travelling record. PCR screening identified the presence of $bla_{\text{NDM-5}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CMY-42}}$ and *rmtB*. $bla_{\text{NDM-5}}$ was carried in a conjugative IncFII-type plasmid (90 Kb) together with $bla_{\text{TEM-1}}$ and *rmtB*. The genetic surrounding of $bla_{\text{NDM-5}}$ showed structural similarities to those of pMC-NDM and pGUE-NDM, identified in Poland and France in *E. coli* of African and Indian origin, respectively.

Carbapenemase-producing *Enterobacteriaceae* (CPE) constitute a public health problem regarding both hospital and community-acquired infections (1). Despite increasing reports of CPE in Europe and, predominantly in southern European countries, the prevalence of CPE in Spain still remains relatively low although is steadily increasing, with only a few outbreaks or sporadic isolates harboring VIM, KPC, OXA-48 or NDM-1 being reported (2). After the identification of NDM-1 in a Swedish traveller returning from India (3), NDM enzymes have gathered special attention due to their rapid global spread and frequent association with additional resistance genes (4). There are currently twelve NDM variants (http://www.lahey.org) differing by one or two amino acid substitutions that may display slightly different hydrolytic capabilities (4).

The aim of this study was to characterize an NDM-5-producing *E. coli* recovered in Spain from a non-traveller patient.

A 78-years-old Spanish Caucasian female with a history of acute pyelonephritis treated with ceftriaxone (1g/24h) was positive for a carbapenem-resistant *E. coli* strain (HC105) in urine 15 days after the end of treatment. The patient was asymptomatic at this stage. Susceptibility testing using Etest strips (AB-bioMérieux, Solna, Sweden) and interpreted according to EUCAST guidelines (version 4.0, 2014, http://www.eucast.org) indicated that HC105 was resistant to all the antibiotics tested except tigecycline, fosfomycin and colistin (MIC of 0.38 μg/ml, 0.5 μg/ml and 0.125 μg/ml, respectively) with ertapenem, imipenem and meropenem MICs > 32 μg/ml (Table 1). The patient was successfully treated with fosfomycin (2g/8h) and no additional CPE were recovered. Screening of HC105 for carbapenemase/metallo-β-lactamase (MBL)-production yielded positive results using either the modified Hodge test or imipenem-EDTA Etest strips, suggesting carriage of an MBL. PCR and sequence analysis for carbapenemase, ESBL,

and plasmid-mediated AmpC cephalosporinase-encoding genes (5, 6) identified the presence of bla_{NDM-5}, bla_{TEM-1}, bla_{OXA-1} and bla_{CMY-42}. Screening for 16S rRNA-methylase genes (7) also identified the *rmtB* gene conferring high-level resistance to all aminoglycosides, in agreement with the susceptibility profile (Table 1). Multilocus sequence typing (http://mlst.ucc.ie/mlst/dbs/Ecoli) and PCR-based phylogroup analysis (8) assigned *E. coli* HC105 to sequence type 448 (ST448) and phylogroup B1, respectively.

To study the transferability of the resistance phenotype, a biparental mating between HC105 and *E. coli* J53AziR was conducted and transconjugants were selected on LB agar plates containing 1 μg/ml meropenem and 100 μg/ml sodium azide. All transconjugants acquired resistance to all aminoglycosides and β-lactams tested but aztreonam (Table 1), concomitant with the acquisition of bla_{NDM-5}, bla_{TEM-1} and *rmtB* but not of bla_{OXA-1}, and bla_{CMY-42}. S1 nuclease–pulsed-field gel electrophoresis (PFGE) (6) revealed two plasmids of circa 50 and 90kb in HC105 but only a single plasmid of 90kb in the transconjugant strains. Plasmid replicon typing (9, 10) showed that HC105 contained plasmid-types belonging to IncFII and I1 incompatibility groups while transconjugant strains had only acquired the IncFII-type. Digoxigenin-labeled probes against bla_{NDM-5}, bla_{TEM-1} and *rmtB* hybridized against blotted nylon membranes from the S1-PFGE gels matched with the band corresponding to the 90kb plasmid, demonstrating co-carriage of all three resistance mechanisms in a single conjugative IncFII plasmid of circa 90kb, designated as pHC105-NDM.

Inverse PCR over genomic DNA from HC105 (6) and DNA sequencing showed the presence of a remnant IS*Aba125* sequence immediately upstream from bla_{NDM-5} as well as the *ble*, *trpF* and *tat* genes downstream from bla_{NDM-5} (Fig.1), altogether comprising a genetic arrangement highly conserved among NDM-producing isolates

82 from different Gram-negative species (11). Subsequent PCR mapping of the bla_{NDM-5}
83 genetic surrounding showed that this region was bracketed by two IS*26* insertion
84 sequences constituting a putative composite transposon also containing an IS*CR1*
85 element and a class I integron whose *int1* gene was truncated by the downstream IS*26*
86 copy. In addition, bla_{TEM-1} and *rmtB* were located adjacent to the upstream IS*26* copy
87 (Fig.1).

88 The genetic surrounding of the bla_{NDM-5} gene in pHC105-NDM is almost
89 identical to that of the bla_{NDM-1} gene present in pMC-NDM (12), which contains an
90 additional IS*26* truncating IS*CR1*, and is very similar to that of bla_{NDM-1} in pGUE-NDM
91 (13), except for the upstream bla_{TEM-1} and *rmtB* genes that are missing in pGUE-NDM.
92 Both pMC-NDM and pGUE-NDM are IncFII plasmids of approximately 90kb that were
93 identified in Poland and France from *E. coli* isolates of African and Indian origin and
94 belonging to ST complexes ST23 and ST131, respectively.

95 NDM enzymes encoded in IncFII plasmids, with a narrow host range, are being
96 increasingly reported (12-17), an observation that deserves further attention since IncFII
97 plasmid scaffolds have been linked to the worldwide spread of CTX-M-15 (18). Of
98 note, the genetic array bla_{TEM-1}-*rmtB*-IS*26* upstream from bla_{NDM} in both pHC105-NDM
99 and pMC-NDM has typically been associated with the Tn*3* transposon in IncFII
100 plasmids as well (19-22), and it is not unlikely that linkage of this array with the NDM
101 module originated from an IS*26*-mediated recombination event, a common mechanism
102 in the mobilization and genetic rearrangement of NDM-related structures (13, 14).

103 In this study we report the molecular characterization of an *E. coli* (ST448)
104 clinical isolate carrying a NDM-5 metallo-β-lactamase in a ~90kb IncFII plasmid,
105 representing the fourth NDM-producing isolate described in Spain. Previous isolates
106 carried the NDM-1 variant and were associated with recent travels to India (6, 23, 24),

while HC105 represents the first NDM-5 reported in Spain and it was recovered from a non-traveller whose close relatives reported no recent travelling history either, thus suggesting that it was community-acquired and autochthonous. This is also the first ST448 *E. coli* strain harboring an NDM enzyme. Interestingly, $bla_{\text{NDM-5}}$ was harbored by IncFII-type plasmids in isolates from India and the UK also bearing $bla_{\text{TEM-1}}$ and *rmtB* genes, suggesting that $bla_{\text{NDM-5}}$ genes might share a common genetic platform (25, 26).

The nosocomial spread of NDM-producing isolates has only been sporadically reported and was likely related to particular clonal lineages (17, 27-29). Multiple reports, however, have identified community-acquired NDM-producing isolates, suggesting the existence of a hidden reservoir and transmission among colonized carriers (30). The present study highlights that, while NDM enzymes are still rarely reported in the clinical setting in Spain, attention should be paid to the prevalence of NDM enzymes within the community to monitor future trends and prevent their further spread into epidemic clonal lineages.

The sequence of $bla_{\text{NDM-5}}$ and its genetic environment has been deposited at the NCBI GenBank under accession number KM598665.

This study was supported by grant 2014-SGR0653 from the Departament de Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya, by the Ministerio de Economía y Competitividad, Instituto de Salud Carlos III, co-financed by European Regional Development Fund (ERDF) "A Way to Achieve Europe," the Spanish Network for Research in Infectious Diseases (REIPI RD12/0015) and by funding from the European Community (MagicBullet contract HEALTH-F3-2011-278232).

We would like to thank Dr. Alessandra Carattoli for kindly providing the control plasmids for replicon typing of incompatibility groups.

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FIGURE LEGENDS AND TABLES

Fig. 1. Schematic drawing showing the genetic elements surrounding the bla_{NDM} genes in pHC105-NDM (KM598665), pMC-NDM (HG003695) and pGUE-NDM (JQ364967). The lengths of the arrows are not proportional to the lengths of the genes or open reading frames (ORFs). Red arrow indicates the remnant IS*Aba125* fragment providing the -35 promoter region for transcription of bla_{NDM}. Abbreviations and symbols are: bla_{TEM}, TEM β-lactamase; *rmtB*, 16S rRNA methylase gene; IS, insertion sequence; bla_{NDM}, New Delhi metallo-β-lactamase gene; *ble*, bleomycin resistance gene; *trpF*, phosphoribosylanthranilate isomerase gene; *tat*, twin-arginine translocation pathway signal protein gene; *sul1*, sulfonamide resistance gene; *qac*, quaternary ammonium compounds resistance gene; *aadA2*, aminoglycoside adenyltransferase gene; *orf1*, open reading frame; *dhfrA12*, dihydrofolate reductase gene; *int1*, integrase gene; *tnpM*, transposition modulator gene; *aacC2,* aminoglycoside acetyltransferase gene; IRL, inverted repeat left; IRR, inverted repeat right; Tn, transposon; Δ, truncated gene.

262 **Table 1.** *In vitro* susceptibilities of *E. coli* HC105 and *E. coli* J53AziR HC105
263 transconjugant expressing NDM-5 carbapenemase.

Antimicrobial Agents	MIC (μg/ml) in:		
	***E. coli* HC105**	***E. coli* J53 AziR HC105T**	***E. coli* J53AziR**
Cefoxitin	> 256	> 256	2
Cefotaxime	> 256	> 256	0.094
Ceftazidime	> 256	> 256	0.25
Cefepime	> 256	128	0.25
Imipenem	> 32	> 32	0.19
Meropenem	> 32	> 32	0.023
Ertapenem	> 32	> 32	0.008
Aztreonam	64	0.19	0.047
Gentamicin	> 256	> 256	0.19
Amikacin	> 256	> 256	0.75
Tobramycin	> 256	> 256	0.19
Tigecycline	0.38	0.38	0.38
Colistin	0.125	0.125	0.125
Fosfomycin	0.5	0.5	0.5
Levofloxacin	>32	0.047	0.047
Ciprofloxacin	> 32	0.032	0.008

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