The First NDM Metallo-β-Lactamase-Producing *Enterobacteriaceae* Isolate in Poland: Evolution of IncFII-Type Plasmids Carrying the *bla*$_{NDM-1}$ Gene

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Poland's first *Enterobacteriaceae* isolate producing the New Delhi metallo-β-lactamase (NDM) was identified in August 2011. *Escherichia coli* sequence type ST410 NDM-1 was cultured from a critically ill patient who had been transferred directly from the Congo. The *bla*$_{NDM-1}$ gene was carried by conjugative IncFII-type plasmid pMC-NDM (87,619 bp), which showed structural similarity to plasmid pGUE-NDM, which was identified earlier in France in an *E. coli* ST131 isolate of Indian origin.

Multidrug-resistant, carbapenemase-producing members of the family *Enterobacteriaceae* (CPE) are a public health problem of the highest global concern today [1, 2]. They express diverse carbapenem-hydrolyzing enzymes, including metallo-β-lactamases (MBLs) [1, 2]. Of a few MBL types observed in CPE so far, the NDM (New Delhi MBL) enzymes were described first in 2009 [3], and this commenced an avalanche of reports on their spread [4]. NDM-producing organisms are endemic on the Indian subcontinent and likely also in some Middle Eastern and Balkan countries, from where numerous transfers have been recorded worldwide [4, 5]. The *bla*$_{NDM}$ genes have a chimeric structure [6] and are linked with the bleomycin resistance gene *ble*$_{MBL}$ [7]. The *bla*$_{NDM}$-*ble*$_{MBL}$ operon (plus some other genes) was found in transposon Tn*125* formed by two IS*Aba125* insertion elements and was probably preassembled in an *Acinetobacter* before transmission to *Enterobacteriaceae* [8]. The presence of Tn*125*-like elements in diverse plasmids and a variety of Gram-negative species and clones [9–19] indicates that they play a key role in the spread of *bla*$_{NDM}$ genes. However, in contrast to *Acinetobacter baumannii*, in *Enterobacteriaceae*, the Tn*125* structure has usually been altered into a variety of truncated variants [1, 4]. The aim of this study was to analyze the first NDM case identified in Poland, which was probably linked to travel to equatorial Africa.

A 53-year-old male Polish citizen traveled to the Congo in 2011. He was admitted to an intensive care unit (ICU) because of sudden cardiac arrest on 24 July. After 24 h, the critically ill patient was transferred to Poland by air and directly admitted to the ICU of a Warsaw hospital (26 July). A week later, a sample of his urine yielded *Escherichia coli* isolate 5428/11, which was identified by Vitek 2 (bioMérieux, Marcy l'Etoile, France) as resistant to carbapenems. The MBL-positive phenotype of the isolate was determined by the EDTA double-disk test [20]. MICs of antimicrobials were evaluated with Etest (bioMérieux) and interpreted according to EUCAST (http://www.eucast.org/). *E. coli* 5428/11 was susceptible only to tigecycline and colistin of all of the drugs tested (Table 1). The patient was treated with colistin for 12 days and isolated for his entire stay. However, despite these efforts, he died of multiorgan failure on 20 August without infection symptoms. There was no transmission of the MBL-producing *E. coli* strain to other ICU patients.

PCR screening of *E. coli* 5428/11 for *bla*$_{VIM}$-, *bla*$_{IMP}$-, and *bla*$_{NDM}$-like MBL genes was positive with *bla*$_{NDM}$-specific primers [9], and sequencing of the amplicon revealed the presence of NDM-1 [3]. Isoelectric focusing [21] showed that the isolate also produced β-lactamases with pIs of 8.9, 7.4, and 5.4 that were identified by PCR and sequencing [22] as CTX-M-15, OXA-1, and TEM-1, respectively (PCR screening for *bla*$_{CMY-2}$- and *bla*$_{OXA-48}$-like genes was negative). The presence of multiple β-lactamases in NDM producers, including those found in *E. coli* 5428/11, is the characteristic they have in common [3, 12, 23–25].

E. coli 5428/11 was subjected to multilocus sequence typing [26]; the database available at http://mlst.ucc.ie/mlst/dbs/Ecoli was used to assign its sequence type (ST). The isolate belonged to ST410, clonal complex ST23, and by PCR assay [27] it was classified in *E. coli* phylogroup A. ST410 isolates have been recovered worldwide from human, meat, and animal samples with various resistance traits, such as extended-spectrum β-lactamases (ESBLs) of the CTX-M type [28–32]. ST410 acquired the KPC-2 carbapenemase in Greece [33], and it has also been identified with NDM-1, as reported in Norway (patient from India) [34], the United Kingdom [9], Switzerland (patient from Serbia), France, and the United States [12].

E. coli 5428/11 (rifampin MIC, 12 μg/ml) was mated with *E. coli* A15 Rif^r [21] by using 100 μg/ml rifampin and 0.5 μg/ml imipenem as selective agents. Transconjugants were obtained with high efficiency ($\sim 2 \times 10^{-3}$ per donor cell). The PCR-based replicon typing (PBRT) of plasmids [35] showed replicons FIA, FIB, and FII in the clinical isolate and only FII in the transconjugant. Plasmid profiling was carried out by pulsed-field gel electrophoresis (PFGE) of total DNA cut with nuclease S1 (TaKaRa, Otsu, Japan) [36]. Following PFGE, the DNA was hybridized separately with seven probes by using the ECL Random-Prime Labeling and Detection system (Amersham Pharmacia Biotech, Little

TABLE 1 Susceptibilities of the *E. coli* 5428/11 isolate producing NDM-1, CTX-M-15, TEM-1, and OXA-1 and its *E. coli* A15 Rif[r] transconjugant producing NDM-1 and TEM-1

Isolate	MIC (µg/ml)[a]																
	AMX	AMC	PIP	TZP	CAZ	CTX	FEP	ATM	ERT	IPM	MEM	AMK	GEN	CIP	TET	TGC	CST
E. coli 5428/11	>256	>256	>256	>256	>256	>256	32	32	>32[b]	>32[b]	>32[b]	>256	>256	>32	>256	0.19	0.38
R[+] [5428/11][c]	>256	>256	>256	24	>256	32	6	0.064	2	2	4	>256	>256	0.016	2	0.19	0.38
E. coli A15 Rif[r]	4	4	1.5	0.5	0.125	0.032	≤0.016	0.047	0.008	0.19	0.032	1.5	0.5	0.032	2	0.25	0.38

[a] Abbreviations: AMK, amikacin; AMC; amoxicillin-clavulanate; AMX, amoxicillin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; ERT, ertapenem; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TET, tetracycline; TGC, tigecycline; TZP, piperacillin-tazobactam.
[b] With carbapenems, multiple scattered colonies were observed inside the growth inhibition zones around Etest strips.
[c] R[+] [5428/11], transconjugant of *E. coli* 5428/11 obtained by using *E. coli* A15 Rif[r] as the recipient.

Chalfont, United Kingdom). The probes were the FIA, FIB, and FII PBRT amplicons, and PCR products specific for all of the β-lactamase genes were detected. Two plasmids were found in *E. coli* 5428/11, one of ~90 kb with replicon FII and one of ~110 kb with replicons FIA and FIB; only the first one was present in the transconjugant. Replicon sequence typing (37) showed that the ~90-kb plasmid was of the F2:A⁻:B⁻ type, whereas the ~110 kb molecule was of the F⁻:A1:B1 type. Of the β-lactamase genes, *bla*~NDM-1~ hybridized only with the F2:A⁻:B⁻ plasmid, whereas *bla*~TEM-1~ was located on both. The *bla*~CTX-M-15~ and *bla*~OXA-1~ genes did not hybridize with any of the plasmids but only with the *E. coli* 5428/11's highest-molecular-weight DNA band. The following I-Ceu I analysis (38) demonstrated cohybridization of the *bla*~CTX-M-15~ and rRNA gene probes with an ~800-kb DNA fragment, indicating the chromosomal location of *bla*~CTX-M-15~ (results not shown). This has been another report of *bla*~NDM-1~ on an IncFII-type plasmid, following those from India, France (isolate of Indian origin), Switzerland (Serbian origin) and Turkey (12, 39). One of these plasmids, pGUE-NDM from an *E. coli* ST131 strain imported to France from India (40), has been well characterized, including its entire sequence (15, 41).

The ~90-kb F2:A⁻:B⁻ plasmid, named pMC-NDM, was pu-

rified from the transconjugant with the Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) and sequenced with the 454 GS Junior instrument (Roche Diagnostics, Indianapolis, IN) by Genomed (Warsaw, Poland). Sequence reads were assembled into 11 contigs by the CLC Genomics Workbench software (version 4.7.2; CLC Bio, Aarhus, Denmark). The contigs were closed by Sanger sequencing of PCR products. The sequence was analyzed with the DNA Lasergene (DNASTAR, Madison, WI), BLAST (http://blast .ncbi.nlm.nih.gov/Blast.cgi), and RAST (42) tools.

pMC-NDM is a molecule of 87,619 bp with a GC content of 51%. It is similar to 87,022-bp pGUE-NDM (GenBank accession no. JQ364967) (15), with the high-homology part covering almost the entire backbone and a relatively large fragment of multidrug resistance regions in both plasmids (Fig. 1). This part comprises 87.0 and 90.6% of the pMC-NDM and pGUE-NDM structures, respectively. The IncFII replicon region from the *repA2* gene to *oriR* is the same in both plasmids. However, 21 single nucleotide polymorphisms (SNPs) were found within an ~500-bp-long fragment downstream of *oriR*, including *repA4* (43), and in fact, pMC-NDM is more similar in this fragment to some other IncFII-type plasmids than to pGUE-NDM (e.g., Rsc13; GenBank accession no. J01783) (44). The conjugative transfer loci *tra-trb* differ only
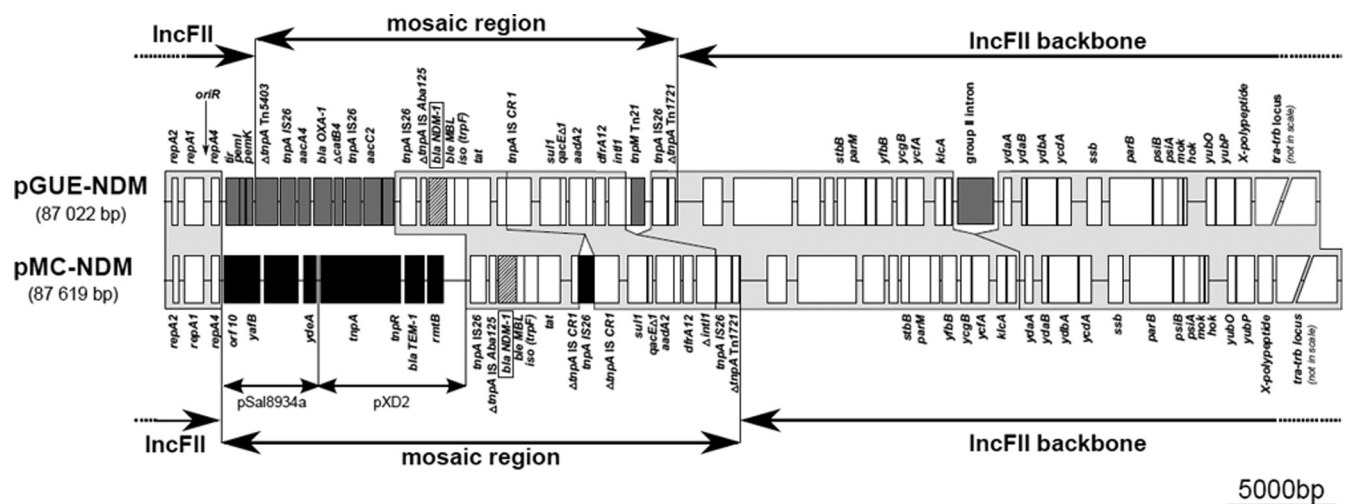

FIG 1 Comparison of IncFII-type plasmids pMC-NDM and pGUE-NDM carrying *bla*~NDM-1~ genes. Boxes indicate ORFs identified by sequence analysis. All regions are drawn to scale, except for the *tra-trb* locus. Gene designations refer to ORFs with putative functions that were assigned on the basis of homology searches performed by Bonnin et al. (15) or in this study. Parts of both plasmids showing similar structures and high sequence homology are indicated by frames shaded light gray. Dark gray and black boxes indicate ORFs found only in pGUE-NDM and pMC-NDM, respectively. The *bla*~NDM-1~ genes are represented by hatched boxes. The IncFII backbone, mosaic regions, and the pMC-NDM fragments that are highly homologous to plasmids pSal8934a and pXD2 are indicated by horizontal arrows.

by two SNPs and the number of CAACAGCCG tandem repeats in the *traD* gene (11 and 10 repeats in pMC-NDM and pGUE-NDM, respectively). Of the two fragments associated with stable inheritance in pGUE-NDM, the region located downstream of *repA4* and carrying the toxin-antitoxin type II system *pemI/K* is missing from pMC-NDM (see below). However, the extensive backbone fragment upstream of *tra-trb* with the toxin-antitoxin type I system *hok/mok* and the *parB* and *parM* partition genes is present in both plasmids. The differences between pMC-NDM and pGUE-NDM in this region included 27 point changes and different numbers of TACTGC repeats (six and seven repeats, respectively) and a group II intron inserted into pGUE-NDM but not into pMC-NDM.

pMC-NDM and pGUE-NDM share a unique arrangement of mobile elements that in both plasmids constitutes a large part of their mosaic regions. It is located directly upstream of the common stable inheritance region and contains remnants of three transposons, namely, Tn*1721*-, Tn*21*-, and Tn*125*-like elements, and two IS*26* copies located upstream of ΔTn*125* and ΔTn*1721*, respectively. Major differences between pMC-NDM and pGUE-NDM concern the Tn*21*-like element, containing a class 1 integron with the *dfrA12-orfF-aadA2* cassette array and the IS*CR1* element (45) behind the 3′-CS segment. In pMC-NDM, this element is truncated further by an ~1.1-kb deletion covering *tnpM* and a fragment of the integrase gene *intI1*. pMC-NDM also has one more IS*26* element inserted within IS*CR1* by transposition, as indicated by the presence of 8-bp direct repeats at its boundaries. In addition, the ΔTn*125*-adjacent IS*26* element has two SNPs when the two plasmids are compared. The remnant of Tn*125* (8) containing the bla_{NDM-1}-ble_{MBL} operon is the same in pMC-NDM and pGUE-NDM.

The remaining parts of the mosaic regions in pMC-NDM and pGUE-NDM are different. Instead of an ~9-kb fragment with the genes *aacA4*, bla_{OXA-1}, Δ*catB4*, and *aacC2* in pGUE-NDM, pMC-NDM carries an ~11.4-kb-long segment consisting of two parts. The first of these (~6.9 kb) is located upstream of IS*26*-ΔTn*125* and encompasses the Tn*3* transposon with bla_{TEM-1} and the 16S rRNA methylase gene *rmtB*, conferring resistance to aminoglycosides (46). The same arrangement of Tn*3* and *rmtB* was found in some other plasmids, including IncFII-type plasmid pXD2 (GenBank accession no. JN315966) (47). The second part (~4.5 kb) is located between Tn*3* and the replicon region and is almost identical (one SNP) to a fragment of IncI-type plasmids pSal8934a (GenBank accession no. JF274993) and Plm (GenBank accession no. JQ901381). This part contains several open reading frames (ORFs), including the gene *yafB*, which codes for a putative conjugation repressor domain, similar to FinO in IncFII plasmids (48), as has been annotated for numerous IncI-like plasmids, e.g., pSal8934a and ColIb-P9 (GenBank accession no. AB021078). A sequence of ~280 bp upstream of *yafB* (and *orf10*) in pSal8934a is highly homologous to the region between the *oriR* and *pemI/K* genes in IncFII plasmids covering a fragment of *repA4*. Therefore, it might have been a site of recombination between IncFII- and IncI-type molecules during pMC-NDM evolution, resulting in the acquisition of the IncI-backbone fragment and deletion of the *pemI/K* toxin-antitoxin genes. Similar events between IncFII- and IncI-like plasmids have been observed before (49, 50).

A comparison of pMC-NDM and pGUE-NDM revealed differences but also clear similarities between the two IncFII-like plasmids with bla_{NDM-1}, carried by unrelated *E. coli* isolates of

different geographic and epidemiological origins. The key common element is essentially the same composition of the truncated Tn*1721*-, Tn*21*-, and Tn*125*-like transposons and their identical location with respect to the backbone. The described architecture of this region must have resulted from a series of DNA rearrangements that are rather unlikely to have occurred independently several times. Therefore, pMC-NDM and pGUE-NDM may have an ancestor in common and, with time, may have differentiated from each other by multiple events.

This work documents the first NDM-positive CPE in Poland, which was possibly imported from Africa, as suggested by the clinical data. Earlier studies showed the presence of NDM CPE in Cameroon (51), Kenya (24), Morocco (25, 52), and South Africa (53), while North Africa has also been described as a region where *A. baumannii* with NDMs has spread (54, 55). Comparative analysis of the pMC-NDM and pGUE-NDM plasmids indicates the probable evolution of NDM-1 genetic determinants proceeding on a large geographic scale.

Nucleotide sequence accession number. The complete sequence of plasmid pMC-NDM has been submitted to the EMBL database and assigned accession number HG003695.

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