

RESEARCH ARTICLE

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# Identification of a New Delhi metallo- $\beta$ -lactamase-4 (NDM-4)-producing *Escherichia coli* in Italy

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## Abstract

**Background:** During June-July 2012, six imipenem-resistant *Escherichia coli* isolates were isolated from two patients hospitalized in a ward of one large tertiary-care hospital in Genoa, Italy. Genetic features associated with *bla*<sub>NDM-4</sub> gene were investigated.

**Results:** The isolates exhibited the same PFGE profile and a multidrug-resistant (MDR) phenotype to aminoglycosides, fluoroquinolones, and  $\beta$ -lactams. The strains produced the NDM-4 carbapenemase and the *bla*<sub>NDM-4</sub> gene was part of the variable region of a class 1 integron. MLST analysis revealed that all isolates belonged to sequence type 405 (ST405).

**Conclusions:** This is the first report on the emergence of an MDR strain of *E. coli* producing the NDM-4 MBL in Italy.

**Keywords:** Carbapenemases, *E. coli*, ST405, Class 1 integron

## Background

The emerging New Delhi metallo- $\beta$ -lactamase (NDM), an acquired class B carbapenemase that was first detected in *Klebsiella pneumoniae* isolate from a Swedish patient of Indian origin has become a major public health concern worldwide [1].

Two cases of the new variant, NDM-4, have been recently described in isolated recovered from patients previously hospitalized in India and Cameroon [2,3]. In Italy, a few cases of NDM-1 producing *E. coli* and *K. pneumoniae* have been reported during 2009 and 2011 [4,5].

In this study we described six NDM-4-producing *E. coli* isolates obtained from two patients admitted to an Italian hospital. We also present data on the localization and the genetic environment of the *bla*<sub>NDM-4</sub> gene.

## Methods

### Bacterial strains

Six *E. coli* isolated from urine samples of two inpatients at the San Martino-IST University Hospital (Genoa, Italy) were studied. Isolates were taken as part of standard patient

care and informed consent for the use of clinical data has been obtained by both patients.

### Strain identification, antibiotic susceptibility testing and phenotypic screening for MBL production

Routine identification and antibiotic susceptibility testing were carried out using the Vitek-2 automated system (BioMérieux, Marcy-L'etoile, France). *In vitro* activity of carbapenems, aztreonam, fosfomycin and nitrofurantoin was further determined by the broth microdilution method and interpreted according to the of European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Version 4.0, 2014) [6]. To detect metallo- $\beta$ -lactamase (MBL) production, a synergy test using imipenem and EDTA discs was used [7].

### Pulsed-field gel electrophoresis (PFGE)

Genomic DNA was prepared, digested with *Xba*I (New England Biolabs Inc., MA, USA) and subjected to PFGE with the CHEF DRII device (Bio-rad, Milan, Italy), as described previously [8]. Fingerprinting pattern was interpreted according to the method of Tenover *et al.* [9].

### Multilocus sequence typing (MLST)

MLST was carried out using protocols and conditions described on the *E. coli* MLST website (<http://mlst>.

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warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi.html). Sequence types were assigned using the website interface.

### Molecular analysis techniques

Polymerase chain reaction (PCR) amplification of the *bla*<sub>NDM</sub> gene and direct sequencing of the PCR products was performed as previously described [10]. Screening for resistance genes was carried out using primers and conditions previously described [11-13]. Phylogenetic analysis using multiplex PCR method as described previously [14] was used. PCR experiments were performed to identify the upstream- and downstream-located regions of the *bla*<sub>NDM-4</sub> gene [15]. Mapping of the variable region of class 1 integron was performed by PCR as described previously [16]. The genetic environment of *bla*<sub>NDM-4</sub> was studied by PCR mapping and sequencing as described previously [13].

### Conjugation assay and plasmid study

Plasmid transfer was attempted by conjugation, using *E. coli* J53 as the recipient, as described previously [17]. Plasmid DNA, isolated from *E. coli*, was obtained by the alkaline lysis method and was used as a template in PCR analysis with primers that are specific for *bla*<sub>NDM</sub> and *bla*<sub>CTX-M</sub> [18]. To rule out chromosomal DNA contamination the template was used to amplify an internal fragment of the house-keeping *recA* gene. A PCR-based replicon typing method was used to identify the incompatibility group [19].

## Results

### Bacteria and patients

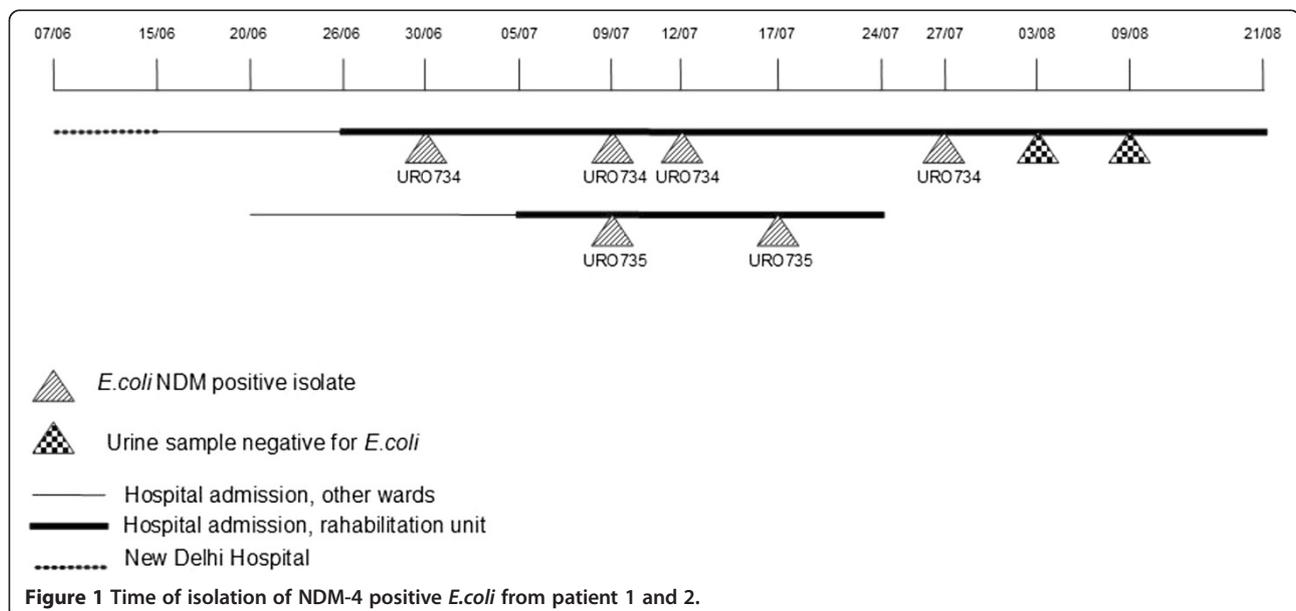
The first NDM-4-positive *E. coli* isolate (URO734, index strain) was detected from the urine of a 61-year-old male inpatient (patient 1) of the rehabilitation unit of the San

Martino-IST Hospital on 30 June 2012 (Figure 1). At the beginning of June, the patient was hospitalized for 7 days, in a hospital in New Delhi, India, with a history of right middle cerebral artery ischemic stroke and left-sided hemiparesis. On 15 June 2012 the patient was admitted to San Martino-IST stroke center and on 26 June he was transferred in the rehabilitation unit for 57 days. Subsequent urine samples, collected during the hospitalization period (9 July, 12 July, 27 July), continued to yield NDM-4-positive *E. coli* showing the same MDR phenotype as URO734 until 27 July. The patient was empirically treated with colistin. Subsequent urine samples (03 August, 09 August) were negative for *E. coli*.

A second case of urinary tract infection sustained by NDM-4-positive *E. coli* was detected in July 2012 in another inpatient (patient 2), a 79-year-old man, with a history of hip replacement, who was admitted to the same rehabilitation unit during a period overlapping the admittance of the index case. The first isolate from patient 2 (isolate URO735) was contemporary with the second isolate from patient 1. Subsequent urine sample, collected during the admission period (17 July), continued to yield NDM-4-positive *E. coli*, showing the same MDR phenotype as URO734. Initially, the patient was empirically treated with piperimic acid and then, after antimicrobial susceptibility results were available, with nitrofurantoin. The clinical condition of the patient improved and the patient was discharged, without further positive urine culture. No history of travel in India or other NDM endemic areas was reported for this patient.

### Antimicrobial susceptibility

The NDM-4-positive *E. coli* isolates exhibited a MDR phenotype to aminoglycosides, fluoroquinolones, and all



**Table 1 Minimum Inhibitory Concentrations of selected antimicrobials agents against NDM-4-producing *E.coli* isolates**

Isolates	Patients	Sample collection date	Sample	MIC (µg/ml)													
				AK	AMC	CAZ	LEV	CO	IPM	MER	GM	TYG	SXT	CTX	NIT	FOS	AZT
URO734	1	30 Jun	Urine	>64	>32	>64	>8	<0,5	16	>128	>16	<0,5	>320	>64	64	<2	>64
URO734	1	09 Jul	Urine	>64	>32	>64	>8	<0,5	16	>128	>16	<0,5	>320	>64	64	<2	>64
URO734	1	12 Jul	Urine	>64	>32	>64	>8	<0,5	16	>128	>16	<0,5	>320	>64	64	<2	>64
URO734	1	27 Jul	Urine	>64	>32	>64	>8	<0,5	16	>128	>16	<0,5	>320	>64	64	<2	>64
URO735	2	09 Jul	Urine	>64	>32	>64	>8	<0,5	16	>128	>16	<0,5	>320	>64	64	<2	>64
URO735	2	17 Jul	Urine	>64	>32	>64	>8	<0,5	16	>128	>16	<0,5	>320	>64	64	<2	>64

AK: amikacin; AMC: amoxicillin/clavulanic acid; CAZ: ceftazidime; LEV: levofloxacin; CO: colistin; IPM: imipenem; MER: meropenem; GM: gentamicin; TYG: tigecycline; SXT: thrimethoprim/sulfamethoxazole; CTX: cefotaxime; NIT: nitrofurantoin; FOS: fosfomycin, AZT: aztreonam.

β-lactams tested. The strains were susceptible to colistin, nitrofurantoin, fosfomycin and tigecycline (Table 1). All NDM-4-positive isolates produced metallo-β-lactamase (MBL) activity by the imipenem-EDTA double-disk synergy test.

#### Phylogenetic group and PFGE

*E. coli* can be classified as phylogroup A, B1, B2 or D according to the phylogenetic relationship of the sequences. Phylogenetic analysis showed that isolates belonged to the phylogenetic group D, which includes extra-intestinal isolate. All isolates exhibited the same PFGE macrorestriction profile (Figure 2).

#### MLST

All the NDM4-positive isolates were designated to a certain MLST sequence type by the combination of the seven allelic housekeeping genes. MLST analysis revealed that all isolates belonged to sequence type 405 (ST405).

#### Genetic context of *bla*<sub>NDM4</sub>

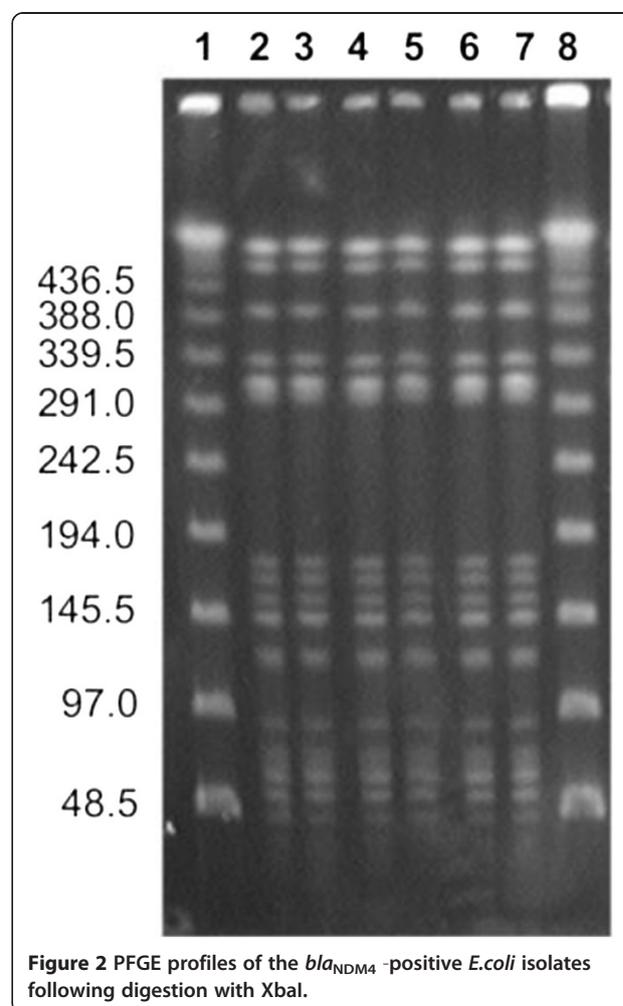
In the index isolate, PCR and sequencing analysis detected the presence of *bla*<sub>NDM-4</sub> and of the following acquired resistance genes: *bla*<sub>TEM-1</sub>, *bla*<sub>CTX-M-15</sub>, *dfrA12*, *aac* (3)-II, *aadA2*. No other carbapenemase genes (OXA-48 or VIM types) were identified in these isolate. The resistance determinants *dfrA12* and *aadA2* were carried on gene cassette inserted into a class 1 integron (Figure 3), resulting in a cassette array identical to that previously described in *E.coli* GUE-NDM isolate from India (accession number JQ364967).

Genetic structures surrounding the *bla*<sub>NDM-4</sub> gene performed by PCR identified immediately upstream of the gene the *ISAbal25* insertion sequence and downstream of the gene was identified the *ble*<sub>MBL</sub> gene encoding the resistance to bleomycin (Figure 3).

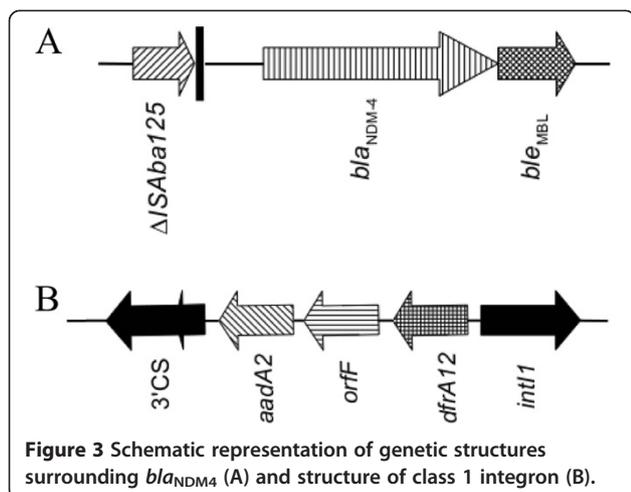
#### Plasmid features

The *bla*<sub>NDM</sub> gene could not be transferred by conjugation to *E.coli* J53 recipient. All strains carried a large plasmid

(>23 Kb) and when the plasmid band was extracted from the gel and used as templates for the amplification of the *bla*<sub>NDM</sub> and *bla*<sub>CTX-M</sub> genes, the specific products were detected, suggesting that both resistance determinants resided in this plasmid. The PCR-based replicon typing method showed that *bla*<sub>NDM-4</sub> -positive plasmid belonged to the IncF incompatibility group.



**Figure 2** PFGE profiles of the *bla*<sub>NDM4</sub> -positive *E.coli* isolates following digestion with XbaI.



## Discussion

In this communication, we described the first isolation of NDM-4 producing *E. coli* in Italy, represented by *E. coli* of sequence type 405(ST405). *E. coli* ST405 belonging to phylogenetic group D is increasingly reported as multi-drug resistant strains causing extra-intestinal infections [20] and is a well-known pandemic clonal lineage implicated as vehicles driving the international spread of *bla*<sub>CTX-M</sub> [21].

NDM is not associated with certain clones, plasmids or transposons [13], our *bla*<sub>NDM4</sub>-positive plasmid belonged to the IncF incompatibility group which is known to be a major vehicle for dissemination of the *bla*<sub>CTX-M-15</sub> gene [22].

By analyzing genetic structures surrounding the *bla*<sub>NDM4</sub> gene, we identified insertion sequence ISAbal25 upstream and the bleomycin resistance gene downstream of the *bla*<sub>NDM4</sub> gene, this genetic environment of our NDM isolates was the same observed for most NDM-1 positive enterobacterial isolates [13].

In both patients, after treatment with *in vitro* active antimicrobial agents (colistin and nitrofurantoin), clinical improvement was observed and in subsequent urine samples of patient 1 *E. coli* NDM-4 was no longer isolated. Patient 2 was discharged without further microbiological investigation.

Patient 1 was previously hospitalized in India, a geographical region with high prevalence of NDM-producing isolates. This is the first example of importation of an Indian NDM-4-producing isolate in Italy following a hospital transfer, confirming the recent observations suggesting that the Indian subcontinent may represent an important reservoir of NDM producers. Because patient 2 had not a history of travel to NDM endemic areas and PFGE profile of the strains was identical, it is plausible that a spread of NDM-4-producing *E. coli* from patient 1 to patient 2 occurred.

According to the hospital microbiology laboratory records, no further isolation of NDM-4-positive bacteria was reported to date in our hospital. To our knowledge, we report here the first NDM-4 producing *E. coli* detected in Italy and the fourth worldwide [2,3,23]. NDM-4 producing *E. coli* strains have been previously described in patients from India, Cameroon and Denmark. In this last case, the Danish patient was previously hospitalized in Vietnam. In three cases (Cameroon, Denmark and Italy), isolates belonged to the ST405 sequence type. This finding is alarming because, ST405, has been previously identified as a successful international sequence type and it could favor the spread of NDM producers.

## Conclusions

This is the first report on the emergence of an MDR strain of *E. coli* producing the NDM-4 MBL in Italy as the result of importation of an Indian NDM-4-producing isolate following a hospital transfer. The isolate belonged to a well-known international sequence type (ST405) able to spread and cause outbreak.

Our data confirms the need for a systematic screening to rapidly detect NDM-producing strains especially among patients previously hospitalized in the endemic geographic areas to avoid dissemination of carbapenemase-producing Enterobacteriaceae.

## Abbreviations

NDM: New Dehli Metallo-beta-Lactamase; MBL: Metallo-beta-Lactamase; PFGE: Pulse-Field Gel Electrophoresis; PCR: Polymerase Chain Reaction; MDR: Multidrug resistant; EUCAST: European Committee on Antimicrobial Susceptibility Testing; MLST: Multi Locus Sequence Typing; MIC: Minimum Inhibitory Concentration.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

EC carry out the experiments AM carried out microbiological diagnostic analysis, designed the study and wrote the manuscript; FV, VDB and MC produced clinical and infectious diseases data and revised the manuscript, GO implemented microbiological procedures to detect carbapenemase producing strains and monitored their emergence during the study period. CV critically revised the manuscript. All authors read and approved the final version for publication.

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