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Outbreak Caused by NDM-1- and RmtB-Producing *Escherichia coli* in Bulgaria

Laurent Poirel,[a,b] Encho Savov,[c] Arzu Nazli,[a] Angelina Trifonova,[c] Iva Todorova,[c] Ivanka Gergova,[c] Patrice Nordmann[a,b]

Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland[a]; INSERM U914, South-Paris Medical School, K.-Bicêtre, France[b]; Laboratory of Microbiology, Military Medical Academy, Sofia, Bulgaria[c]

Twelve consecutive carbapenem-resistant *Escherichia coli* isolates were recovered from patients (infection or colonization) hospitalized between March and September 2012 in different units at a hospital in Bulgaria. They all produced the carbapenemase NDM-1 and the extended-spectrum-β-lactamase CTX-M-15, together with the 16S rRNA methylase RmtB, conferring high-level resistance to all aminoglycosides. All those isolates were clonally related and belonged to the same sequence type, ST101. In addition to being the first to identify NDM-producing isolates in Bulgaria, this is the very first study reporting an outbreak of NDM-1-producing *E. coli* in the world.

Worldwide occurrence of carbapenemase producers among *Enterobacteriaceae* is now well recognized (1, 2). In Europe, there are distinct epidemiological situations corresponding mainly to the diffusion of OXA-48 producers in France, Belgium, The Netherlands, and Turkey (3, 4) and KPC-producing isolates in Italy and Greece (5), while some countries show more diverse distributions, such as in the United Kingdom, where KPC, OXA-48, VIM, and NDM-1 producers are being found (6). The recent emergence of NDM-1 producers (mostly *Klebsiella pneumoniae* isolates) is often related to imported cases, with a link to the Indian subcontinent (7). In addition, there are sporadic reports of NDM-1 producers (*Enterobacteriaceae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) originating from countries of the Middle East or the Balkan region, including Croatia, Kosovo, and Serbia (7, 8). Very little information is available regarding the diffusion of carbapenemase producers in Eastern Europe.

Therefore, our study aimed to characterize the mechanisms responsible for carbapenem resistance by investigating a collection of enterobacterial isolates recovered in Bulgaria. From March to September 2012, 12 *Escherichia coli* isolates being resistant to carbapenems were recovered at the Military Medical Academy hospital of Sofia, Bulgaria. Susceptibility testing was performed by disk diffusion on solid agar plates following the CLSI recommendations (9). Those isolates had been recovered from urine ($n = 4$), blood ($n = 1$), respiratory specimens ($n = 2$), wound ($n = 1$), catheter ($n = 1$), abdominal exudate ($n = 1$), and rectal swabs ($n = 2$). The MICs of carbapenems were determined by Etest (AB bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates at 37°C, and results of susceptibility testing were interpreted according to the CLSI guidelines (10). The MICs of imipenem, meropenem, and ertapenem were 32, >32, and >32 μg/ml for all the *E. coli* isolates, respectively. They were resistant to all β-lactams, including broad-spectrum cephalosporins (MICs of ceftazidime and cefotaxime being >32 μg/ml) (Table 1). In addition, they were resistant to all tested aminoglycosides (amikacin, gentamicin, netilmicin, kanamycin) and to fluoroquinolones and sulfonamides. Using the Etest, the MIC of rifampin was elevated (128 μg/ml), and that of colistin was found to be 0.5 μg/ml.

Carbapenemase detection was performed by using the Carba NP test (11), and positive results were obtained for all isolates. PCR assays were performed to identify the type of carbapenemase produced, with a series of primers designed for the detection of bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, and bla_{OXA-48} carbapenemase genes (12). They revealed that all isolates were positive for a bla_{NDM}-like gene, and sequencing always identified bla_{NDM-1}. Then, double-disk synergy testing was performed as described previously (10) and showed that all *E. coli* isolates produced an extended-spectrum β-lactamase (ESBL). PCR experiments performed as described previously (13) followed by sequencing identified the $bla_{CTX-M-15}$ gene in all isolates.

Considering the high-level resistance to all aminoglycosides observed for all *E. coli* isolates, a search of 16S rRNA methylase-encoding genes was performed by multiplex PCR as described previously (14). Results showed that all isolates were positive for the 16S rRNA methylase RmtB-encoding gene.

Genotyping was performed by pulsed-field gel electrophoresis as described previously (15) and showed indistinguishable patterns among all the *E. coli* isolates (data not shown). This suggested that the isolates had been recovered during an epidemic context. Then, multilocus sequence typing was performed as described (http://mlst.ucc.ie/mlst/dbs/Ecoli) and showed that this clone belonged to sequence type 101 (ST101).

Interestingly, all the patients infected or colonized with that clone had been hospitalized in different hospitalization units during a short period of time (Table 1). The index case was not identified, and the source of that strain in the hospital setting could not be determined. Of note, whereas there have been several reported outbreaks involving NDM-1-producing *K. pneumoniae* in different countries, such as Turkey (16, 17), Colombia (18), Canada (19), Kenya (20), Italy (21), and South Korea (22), reports of NDM-1-producing *E. coli* have been scattered (13, 23–26), including isolates recovered from companion animals in the United States (27). However, nosocomial transmissions of NDM-1-pro-

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Address correspondence to Patrice Nordmann, patrice.nordmann@unifr.ch.

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TABLE 1 Features of the NDM-1- and RmtC-producing *E. coli* isolates and patient characteristics[a]

Patient/strain no.	Date of isolation (mo/day/yr)	Site of isolation	Status of the patient	Gender	Hospitalization unit	Underlying disease	Treatment	Outcome
1	02/27/2012	Urine	Infected	F	ICU-1	Pneumonia	Ampicillin-sulbactam	Died
2	02/27/2012	Urine	Infected	M	ICU-1	Pneumonia	Aztreonam	Died
3	03/05/2012	Body fluid	Colonized	F	ICU-1	Unknown	None	Discharged
4	03/10/2012	ETA	Colonized	F	ICU-1	Unknown	None	Discharged
5	03/14/2012	ETA	Infected	F	ICU-1	Pancreatitis/septic shock	Cefepime and amikacin	Died
6	03/19/2012	Body fluid	Colonized	M	ICU-1	Unknown	None	Discharged
7	03/19/2012	Rectal swab	Colonized	M	Hepatology	Unknown	None	Discharged
8	03/19/2012	Urine	Colonized	M	ICU-1	Unknown	None	Discharged
9	03/23/2012	Stool	Infected	M	Hepatology	Rectal fistula	Tigecycline	Died
10	05/23/2012	Blood	Infected	M	Gastroenterology	Postcholecystectomy sepsis	Meropenem	Discharged
11	08/08/2012	Wound	Colonized	M	Vascular surgery	Unknown	None	Discharged
12	08/09/2012	Urine	Colonized	M	ICU-2	Unknown	None	Discharged

[a] ETA; endotracheal aspirate; ICU; intensive care unit; M, male; F, female.

ducing *E. coli* are extremely rare, with one recent report from South Korea (28). Of note, that latter study also showed that an ST101 *E. coli* clone was involved, even though the authors highlighted the poor standards of hygiene observed in the corresponding hospital (28). As known for ESBL producers, outbreaks due to multidrug-resistant *Enterobacteriaceae* are related mainly to the diffusion of *Klebsiella pneumoniae*. It remains to determine whether NDM-1-producing *E. coli* could eventually be widespread in the community in the same geographical area, constituting a significant reservoir which has been shown to often lead to recurrent importations in the hospital settings (29).

Of note, we characterized here another enterobacterial strain coharboring $bla_{\text{NDM-1}}$ together with a 16S rRNA methylase-encoding gene, such association being extremely frequent, regardless of the type of methylase gene (13, 30). However, this specific association between the $bla_{\text{NDM-1}}$ and *rmtB* genes has been very rarely reported, and mainly in *E. coli*, with a single isolate from Australia (25) and a single isolate from Belgium (Pakistan origin) (31).

In order to evaluate whether the cooccurrence of the $bla_{\text{NDM-1}}$ and the *rmtC* genes could be related to the spread of a specific plasmid, mating-out assays were performed using all those isolates as donors and with *E. coli* J53 as the recipient, as described previously (25). Interestingly, *E. coli* transconjugants coproducing NDM-1 and RmtC were obtained for all isolates, and a single plasmid of ca. 150 kb was identified in all transconjugants. That plasmid additionally conferred elevated MICs of rifampin to all transconjugants (64 μg/ml). However, it did not harbor the $bla_{\text{CTX-M-15}}$ gene, in accordance with the susceptibility to aztreonam (a substrate spared by NDM-1) (32) observed for the *E. coli* transconjugants. Attempts to type this plasmid by using the PCR-based replicon typing (33) remained unsuccessful. PCR mapping was performed to identify the genetic sequences surrounding the $bla_{\text{NDM-1}}$ gene in all positive isolates. The same structure was found, with the $bla_{\text{NDM-1}}$ gene preceded by a truncated version of IS*Aba125*, followed by the ble_{MBL} gene encoding resistance to bleomycin (34). Similar structures have been identified previously in different species from different countries (7).

Our study further highlights the spread of the $bla_{\text{NDM-1}}$ gene in Balkan countries and also further highlights the accumulation of resistance traits that may be found among most NDM-1 producers. This *E. coli*-related nosocomial outbreak is intriguing since *E. coli* is rarely found as a source of nosocomial infections. Further investigations will be performed in order to evaluate whether this ST101 *E. coli* clone might possess some specific features enhancing its spread or persistence, knowing that ST101 NDM-1-producing *E. coli* strains have been formerly reported in South Korea (28), Canada (35), England (36), Pakistan (36), and India (37).

In addition, further investigations are now required to better evaluate to what extent the $bla_{\text{NDM-1}}$ gene might be disseminated in Bulgaria. Interestingly, while this work was in progress, the first studies documenting the occurrence of carbapenemase-producing *Enterobacteriaceae* in that country have been published, including VIM-1-producing *K. pneumoniae* and *Proteus mirabilis* (38) and KPC-2-producing *K. pneumoniae* (39), thus suggesting a heterogeneous distribution of those emerging resistance traits.

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