Molecular Epidemiology of NDM-5-producing *Escherichia coli* high-risk clones identified in two Italian Hospitals in 2017-2019

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Running head: Genomics of the NDM-5 *E.coli* ST617 clone

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Abstract

Between November 2018 and October 2019, carbapenem-resistant *Enterobacterales* carrying New Delhi Metallo-β-lactamase (NDM) caused one of the largest and persistent outbreaks occurred in Italy and intensified surveillance measures have been taken in all Italian hospitals.

In this study we analyzed NDM-5- producing *Escherichia coli* identified in two hospitals of the Lazio region in Italy.

Epidemiological and microbiological data demonstrated that in 2018-2019 the NDM-5-producing high-risk *E. coli* ST167 clone circulated in patients from both hospitals. In 2019, another NDM-5-producing *E. coli* clone identified by MLST as ST617 was introduced in one of the two hospitals and caused an outbreak.

This study describes an application of genomics as a useful method to discern endemic and outbreak clones when applied to strains of the same species (*E. coli*) with the same resistance determinant (NDM-5) and the relevance of screening patients admitted in critical units for carbapenemase producers to prevent outbreaks.

INTRODUCTION

Until November 2018, carbapenem-resistant *Enterobacterales* carrying New Delhi Metallo-β-lactamase (NDM) have rarely been reported in Italy [1]. However, between November 2018 and October 2019, one of the largest and persistent outbreaks caused by NDM-producing *Enterobacterales* occurred in Tuscany, Italy with a total of 1,645 cases. NDM-1-positive strains isolated from patients involved in the outbreak were at 90.9% *Klebsiella pneumoniae* and 4.2% *Escherichia coli* [2]. To counteract the expansion of the outbreak in other Italian regions, intensified surveillance measures have been taken in all the Italian hospitals. Rapid identification of colonized patients and screening for carbapenemase producers have been routinely performed by rectal swabs on patients admitted in critical units. In a previous study we reported about the emergence of ST167 NDM-5-producers colonizing or infecting patients recovered in different wards of Policlinico Umberto I (PUI) of Rome, Italy. Two pairs of strains (91, 92 and 100, 311, respectively) belonged to two different variants of ST167: one variant was characterized by the K48 capsular synthesis cluster of *Klebsiella pneumoniae* and the Integrative Conjugative Elements (ICEs), and one variant was negative to both genetic determinants [6].

In this study we report of a retrospective analysis performed on *E. coli* NDM-producers collected in 2017-2019 in two hospitals of Lazio Region, the PUI and the Santa Maria Goretti Hospital of Latina (SMG). During the study period, a total of 19 patients were infected or colonized by *E. coli* producing NDM. Epidemiological and microbiological data were collected in both hospitals. Genomic approach was used to characterize the NDM-5 producing *E. coli* collection.

METHODS

Epidemiological data

The isolation of the strains took place between November 2017 and December 2019 at the Microbiology Unit of PUI and at the Microbiology Laboratory of SMG. Data of patients were extracted from the electronic medical records. The study was presented to the ethical board of PUI

63 according to the Italian government regulation. Based on this examination, the study received
64 written consent and was approved by the local Ethics Committee (approval no. 449/19).

65 **Bacterial isolation**

66 Bacteria investigated were isolated from samples processed during the routine analysis, specifically
67 rectal swabs, urine, and blood cultures. Rectal swabs were directly plated on Brilliance™ CRE
68 medium plates (Oxoid LTD). Blood culture bottles were incubated in the automatic
69 VirtuoBacT/ALERT system (BioMérieux, Inc. Marcy l'Etoile, France). Positive blood cultures and
70 urine samples were plated on BD Columbia Agar with 5% Sheep Blood, MacConkey agar (Becton
71 Dickinson, Heidelberg, Germany). Isolated colonies were identified by the MALDI-TOF MS
72 system (Bruker Daltonik GmbH, Bremen, Germany).

73 **Susceptibility testing and Bacterial typing**

74 Antimicrobial susceptibility was tested by Vitek2 system (BioMérieux). The minimum inhibitory
75 concentration (MIC) values of antibiotics were assessed following breakpoint tables for
76 interpretation of MICs and zone diameters Version 9.0, valid from 2019-01-02
77 (http://www.eucast.org/clinical_breakpoints). *E. coli* strains isolated from rectal swabs showing
78 growth on Brilliance™ CRE medium plates (Oxoid LTD) and showing meropenem MIC≥ 4 mg/L
79 (from intermediate to resistant phenotype, according to EUCAST
80 http://www.eucast.org/clinical_breakpoints/) and all *E. coli* from positive blood cultures were tested
81 using the real-time PCR assay Xpert® Carba-R kit for GeneXpert® System (Cepheid) to evaluate
82 the presence of the bla_{VIM}, bla_{IMP-1}, bla_{KPC}, bla_{OXA-48} and bla_{NDM} carbapenemase genes.

83 **Whole-genome sequencing**

84 Genomic DNA was purified from bacterial strains using the Macherey Nagel DNA extraction kit
85 (Düren, Germany), paired-end libraries were generated using the Nextera XT DNA sample
86 preparation kit (Illumina Inc, San Diego, CA, USA) and sequenced using the Illumina MiSeq
87 instrument with 2x300PE protocol (Illumina Inc). *De novo* assembly of Illumina reads was

88 performed using the SPADES 3.8 software through the ARIES public Galaxy server
89 (https://w3.iss.it/site/aries/). Antimicrobial resistance and replicon genes were detected using the
90 ResFinder [3] and PlasmidFinder [4] online tools, respectively (https://cge.cbs.dtu.dk/services/).
91 Insertion sequences were identified by ISFinder (https://isfinder.biotoul.fr)[5]. Multilocus sequence
92 typing (MLST) was performed at the Enterobase (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli)
93 website. For the assembly of the IncF-NDM-LT-1 and IncI-Latina1 plasmids, order and orientation
94 of contigs with overlapping paired-ends was initially performed following the assembly of reference
95 plasmids: p91_NDM-5 (MN007141), p91_CMY-42 (MN007140) identified in *E. coli* ST167 in
96 patient P4 from Rome [6]. Complete plasmid sequences were confirmed by PCR-based gap closure
97 method and Sanger sequencing of the amplicons. Plasmid sequences were annotated at the RAST
98 server (http://rast.nmpdr.org/). Plasmid allele numbers and sequence types were assigned by
99 plasmid Multilocus Sequence typing (pMLST; https://cge.cbs.dtu.dk/services/).

100 **Bacterial typing**

101 All NDM-producing strains isolated at the PUI and SMG hospitals that were not subjected to WGS
102 were typed designing a new PCR test based on the capsular *wzi* type 53 gene identified by genomics
103 as a marker specific for tracing the ST617 outbreak clone (Suppl. Table 1). All strains were also
104 tested for the presence of the bla_{CMY} gene (Suppl. Table 1). Amplicons obtained with this PCR were
105 verified by Sanger sequencing. MLST was also used to confirm sequence types and plasmid
106 identity among the isolates was checked by PCR-based tests performed with primers listed in
107 supplementary table 1. All strains were analyzed by the PBRT-KIT 2.0 (Diatheva srl, Cartoceto,
108 IT).

109 **Phylogenesis and capsular cluster analysis**

110 Phylogenetic analysis of ST167 and ST617 genomic sequences were performed by building a
111 maximum likelihood (ML) tree on a SNP analysis performed by the kSNP version 3.0 software at
112 the ARIES public Galaxy server (https://w3.iss.it/site/aries/). For comparison, 50 complete
113 reference genomes of ST167 and 71 ST617 were identified and downloaded from Enterobase

114 (https://enterobase.warwick.ac.uk/) and included in the comparison. The phylogenetic tree was
115 visualized using the Fig Tree program version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).
116 One-hundred-thirty-five capsular synthesis cluster DNA sequences were downloaded from
117 supplementary material in Brisse *et al*., 2013 [7] and used by BLASTN against the LT-1 ST617
118 genome. The *wzi* K53, K48, K41and bla_{NDM}-gene probes were used by BLASTN against a
119 collection of 287 ST617 genomes downloaded from the Enterobase database. A parsimony
120 phylogenetic tree was built with the kSNP version 3.0 software at the ARIES public Galaxy server
121 (https://w3.iss.it/site/aries/) and the presence of the K- and NDM-genetic determinants was
122 highlighted in the branches of the phylogenetic tree.
123 **Accession numbers**
124 IncF-NDM-LT-1 and IncI-CMY-LT-1 plasmid sequences have been deposited in GenBank,
125 accession numbers: MW048884, MW048885, respectively. Genome and plasmid sequences of *E.*
126 *coli* ST617 LT-1 have been released in BioProject PRJNA663762; BioSample SAMN16178545.
127
128 **RESULTS**
129 **Epidemiological analysis of patients positive for NDM-producing *E. coli***
130 The first patient (P1) colonized by an NDM-positive *E. coli* strain was identified in Rome on 24
131 November 2017 at the PUI (Table 1, Figure 1). There were no further NDM-positive cases until
132 October 2018. Six cases were identified in 2018, 1 at the SMG (P2) and 5 at the PUI (P3-P7).
133 Among these patients, three (P4, P5, P6) were hospitalized at the same ward of the hospital
134 (Internal Medicine, IM) in the first 2 weeks of October, whereas P3, which harbored the strain in
135 the urine, was hospitalized in a different ward (Nephrology) also on September-October 2018.
136 There were not identifiable common links with the three previous cases. Interestingly, P7 was a
137 young healthy female who developed diarrhea and intestinal symptoms after returning from India
138 and found colonized on October 2018 by NDM-producer *E. coli*. In the same period (Sept-Oct
139 2018) a patient hospitalized at the Hematology ward of SMG (P2) had a bloodstream infection

140 sustained by an NDM-producing *E. coli*. Two further sporadic cases of NDM-positive *E. coli*
141 strains were identified at PUI on February-March 2019 period (P8-P9). In detail, P8 was
142 hospitalized at Geriatrics ward and during hospitalization developed a urinary tract infection
143 whereas P9 was found to be colonized after ICU admission. Of note, both subjects came from other
144 hospitals in Rome.

145 In October 2019, an outbreak of NDM-producing *E. coli* occurred at the SMG hospital. Ten patients
146 were involved (P10-P19). All strains were isolated from stools and did not give infections,
147 including patient P19 that had a sepsis and meningitis sustained by *Klebsiella pneumoniae*. The first
148 patient (P10) was a foreigner driver who was hospitalized in sub-ICU and then transferred to the
149 surgery ward. Rectal swab screening for carbapenem-producing *Enterobacterales* was performed
150 on this patient at the admission to the sub-ICU but the results of cultivation, revealing colonization
151 by NDM-positive *E. coli*, arrived some days after the transfer to the surgery ward. A woman (P12)
152 was transferred from the geriatric ward to the surgery ward on the same day of patient P10 and
153 resulted colonized by NDM *E. coli*. Active surveillance was activated in surgery and geriatric wards
154 discovering 8 further cases. Three wards experienced intra-ward transmission: surgery, urology, and
155 the sub-intensive unit. Measures were taken to control the outbreak with implementation of the
156 MDR bundle of the hospital that included active surveillance, identification and isolation of cases
157 and contacts, hand hygiene assessment and supportive courses. No further NDM-positive cases
158 were detected after 6 November 2019.

159 **Molecular epidemiology of NDM-5-producing *Escherichia coli***

160 All NDM-positive *E. coli* strains isolated from patients hospitalized in the two hospitals since
161 October 2018 were positives for the *bla*$_{\text{NDM-5}}$ gene. This information suggested that an inter-hospital
162 circulation of NDM-5-producing *E. coli* occurred in the two hospitals.

163 Whole genome sequences (WGSs) were obtained for strain LT-1 from patient P18 hospitalized in
164 Sub-ICU at SMG during the outbreak, strain 301, isolated from patient P8 at PUI and from the
165 historical strain 112 isolated in 2017 at PUI.
166 *In silico* MLST assigned strain LT-1 to Sequence Type 617 and strain 301 to ST167. Strain 122
167 isolated in November 2017 was assigned to ST205 and produced NDM-7. WGSs were compared
168 with previously described genomes of strains 100, 91, 92 and 311 isolated at PUI [6].
169 A total of 50 ST167 and 71 ST617 genomes available from the EnteroBase database were
170 downloaded and compared with WGSs obtained in this study, generating a SNP-based ML-
171 phylogenetic tree. ST167 and ST617 clustered on two different branches with multiple clades (Fig.
172 2A). ST167 WGSs differed from ST617 WGSs for >29000 total SNPs.

173 **Specific virulence markers identified in NDM-5-positive *E. coli* ST617**

174 In the LT-1 ST617 outbreak strain, the capsular synthesis cluster, showing 98.8% nucleotide
175 identity, 99% coverage with the K53 capsule of *Klebsiella quasipneumoniae KL130* (CP029597)
176 was identified. *In silico* analysis performed on 287 ST617 genomes downloaded from the
177 Enterobase database demonstrated that the K53 capsular cluster was present only in 6 genomes
178 within this *E. coli* sequence type (Figure 2B). Among genomes available in GenBank, very few
179 matches were obtained by BLASTN using the K53 capsular cluster DNA sequence from strain LT-
180 1 as the query sequence. These were *Klebsiella quasipneumoniae* KL130 (CP029597), *E. coli*
181 ST744 (CP016182) and *Klebsiella variicola* (CP017289) genomes, respectively [8].
182 The K53 cluster sequence was therefore evaluated as molecular marker for a rapid PCR-based
183 screening of NDM-5-producing *E. coli* strains of our collection that were not subjected to WGS.
184 A specific PCR was devised for detection of the *wzi* K53 gene (primers described in Supplementary
185 table 1) and used on ST167 and ST617 listed in table 1.
186 The *wzi* K53 marker was identified in all ST617 strain from the SMG outbreak and in any ST167
187 strain (Table 1).

188 **Common features shared by *Escherichia coli* ST617 and ST167 high-risk clones**

189 Both ST167 and ST617 genomes carried a highly related Integrative Conjugative Element
190 integrated in the asparagine t-DNA (data not shown). This ICE is constituted by a Type IV secretion
191 system (T4SS), associated with the cluster encoding the yersiniabactin (Ybt) virulence trait,
192 encoding for an efficient iron uptake system [9][10].
193 Complete plasmid sequences were obtained for both IncF-NDM-LT-1 (Acc. No. MW048884) and
194 IncI-CMY-LT plasmids (acc. No MW048885) from isolate P18. The IncF carried replicons FIA,
195 FII, FAB formula [F36:A4:B-], and ResFinder identified $bla_{\text{NDM-5}}$, *mph(A), tet(A), aadA2* and
196 *dfrA12,*and *sul1* resistance genes.The IncI-γ plasmid showed pMLST alleles [A5-R4-T15] and
197 carried the $bla_{\text{CMY-42}}$, resistance gene. ResFinder also identified *tet(B)* gene in the ST617 genome
198 probably located in the chromosome.
199 The $bla_{\text{NDM-5}}$ gene was found in a complex integron, bracketed by two IS*26*, containing an ISCR1
200 element and a class I integron with the *aadA2, dfrA12* resistance gene cassettes (Figure 3).

201 By BlastN, the best match with IncF-NDM-LT-1 (99,99% nucleotide identity and 79% coverage)
202 was observed with plasmid p91_NDM-5 (MN007141), identified in P4, P5 ST167 *E. coli* strains,
203 isolated at PUI on October 2018 (Figure 3) [6]. Both plasmids originated from plasmid pSJ_94
204 (CP011064) identified in a ST167 *E. coli* strain from pheasant in China[11]. The comparative
205 analysis between IncF plasmids showed that the scaffold was highly conserved, including the same
206 replicons, the iron/manganese ABC transporter-aerobactin system, and the lactose fermenting
207 operon (Figure 3). Both plasmids carried the *dfrA12-aadA2*-ISCR1-$bla_{\text{NDM-5}}$ complex integron, the
208 macrolide (*mphA-mphR* genes), but plasmid p91_NDM-5 also carried the $bla_{\text{TEM-1b}}$ and the 16S
209 RNA methylase *rmtB* genes that were not found in IncF-NDM-LT-1.

210 The IncI-CMY-LT carrying the $bla_{\text{CMY-42}}$ gene was identical to the same plasmid identified as
211 p91_CMY-42 (MN007140) and detected in P4 and P5 strains [6]. They were both characterized by
212 the loss of the *pilV* and *sogS* genes and by the same ardA-5, repI1-4, trbA-15 (A5-R4-T15) pMLST
213 alleles. All ST617 strains were positive for the $bla_{\text{CMY-42}}$ gene (Table 1).

214

Discussion

PUI is a 1500-bed hospital in Rome, and SMG is a 406-bed hospital in Latina. The two cities are 73 kms distant and no patient exchanges occurred between the two hospitals during the period of the study. The identification of twelve NDM-producing *E. coli* in 2019 in these two hospitals was suggestive of an inter-hospital outbreak. The ten patients involved in the SMG outbreak were from different wards of the hospital but epidemiologically linked by ward transfer or subjected to common invasive procedures. Following the directives of the Italian Ministry of Health enacted to counteract the NDM-outbreak occurred in Tuscany, molecular epidemiology of strains isolated in 2019 was performed. These strains were compared with 7 cryo-conserved strains from sporadic NDM-positive episodes occurred in the two hospitals in previous years, for a total of 19 NDM-positive *E. coli* from 19 infected or colonized patients hospitalized in the November 2017-November 2019 period at PUI and SMG hospitals of Lazio Region. Bacterial typing demonstrated that strains isolated from October 2018 to March 2019 from both hospitals were ST167 producing NDM-5. These were of two types, distinguished by the presence or absence of the K48 capsular cluster [6]. In October-November 2019, ST167 *E. coli* producing NDM-5 was suspected to sustain an outbreak occurred at SMG. Instead, all the NDM-5 positive *E. coli* of the outbreak clone were ST617.

ST167 is well known to be a high-risk clone associated with $bla_{\text{NDM-5}}$ carbapenemase type and was described globally, predominantly in China [12,13]. This clone has been detected also in non-human sources, animals, wastewater, rivers and wildlife [14, 15].

The co-occurrence of ST167 and ST617 *E. coli* lineages, both belonging to the ST10 Clonal Complex, was described responsible for a cluster of carbapenem-resistant infections in a Chinese hospital during a 7-month period from May to November 2014 [12]. These strains were phylogenetically distant but as observed in PUI and SMG, isolates shared identical or highly related NDM-5-plasmids and virulence determinants.

240 In this study, the two clones were successfully discerned by analyzing capsular genetic
241 determinants. Both strains imported capsular clusters from *K. pneumoniae*. As previously described,
242 one lineage of ST167 at PUI carried the *wzi* K48 capsular cluster [6], while ST617 causing the
243 outbreak at the SMG hospital was positive for the rarely identified *wzi* K53 capsular type, which
244 was used as a specific molecular marker for tracing the outbreak isolates.
245 Molecular and epidemiological evidences suggested that *E. coli* ST167 circulated in the two
246 hospitals for several years and in other Italian hospitals in 2018 but did not cause evident outbreaks
247 [16]. The admission of a foreign traveler introduced the ST617 strain at the SMG hospital and gave
248 the outbreak. ST167 and ST617 *E. coli* clones are good examples to sustain the necessity to perform
249 accurate screening of patients admitted in critical units to prevent outbreaks.

250 This study also highlights that the identification of the same bacterial species (*E. coli*) with the same
251 resistance determinant (NDM-5) in two hospitals may be confounding and recognized as an inter-
252 hospital outbreak. In fact, there are globally diffused major high-risk clones that share common
253 genetic markers that need deeper molecular tracing to be recognized and contained.

254

255 Conflict of interest statement

256 Authors have no conflict of interest to declare with respect to the content of this paper

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311

Legends to Figures

Fig. 1 Epidemiological curve of NDM-positive *Escherichia coli*

The graph reports the numbers of patients colonized or infected with NDM-5 *Escherichia coli* in two hospitals in Rome and Latina and the type of strains is indicated above the graph

Fig. 2. Panel A: Neighbor-Joining phylogenetic tree of ST617 and ST167 *E. coli* genomes. The NJ- tree was built on a SNP analysis performed on 50 ST617 and 70 ST167 *E. coli* genomes, determined in this study and downloaded from the EnteroBase database.

Panel B: Parsimony phylogenetic tree of ST617 genomes and presence of K53- and NDM-genes. The Parsimony- tree was built on a SNP analysis performed on 287 ST617 genomes, determined in this study and downloaded from the EnteroBase database. BLASTN analysis was performed at the Galaxy server (https://w3.iss.it/site/aries/) against K53-, K41- and K48-capsular synthesis clusters and bla_{NDM}-gene variants. Branches colored in orange represent NDM-positive ST617 genomes, respectively; branches marked by green squares represent the K-positive genomes. An asterisk represents the position of the K53-ST617 clone analyzed in this study.

Fig. 3. Major structural features of NDM-5 IncF plasmids

Predicted coding sequences are indicated by coloured arrows oriented as the direction of transcription of each respective gene: resistance genes, red; transposon-related genes and insertion sequences, yellow; replicons, orange; ABC and iron uptake clusters, brown, ADI and Lactose operons, pale green; transfer locus dark green; toxin-antitoxin genes, blue