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Novel IncFII plasmid harbouring bla_{NDM-4} in a carbapenem-resistant *Escherichia coli* of pig origin, Italy

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Objectives: To the best of our knowledge, we describe the first evidence in Europe of an MDR, bla_{NDM-4}-positive *Escherichia coli* isolated from a food-producing animal, harboured by a novel IncFII plasmid of which we report the complete sequence.

Methods: One bla_{NDM-4}-positive *E. coli* isolated in 2019 from the caecal contents of a fattening pig in Italy was in-depth characterized by combined bioinformatic analysis of Oxford Nanopore long reads and Illumina short reads, for *in silico* typing, determination of the bla_{NDM-4} genetic context and full reconstruction of the bla_{NDM-4}–carrying plasmid.

Results: The isolate belonged to ST641 and to the genoserotype O108:H23 and tested positive for different virulence genes and plasmid replicons. The MDR phenotype of resistance to all β-lactams, carbapenems, sulfamethoxazole and trimethoprim was mediated by bla_{TEM-1B}, bla_{NDM-4}, *sul1/sul3* and *dfrA12*, respectively. The bla_{NDM-4} gene was harboured by a novel 53 043 bp IncFII plasmid (pMOL412_FII) composed of four main genetic regions, including an MDR region (MRR-NDM-4) of 16 kb carrying bla_{NDM-4} and several antimicrobial resistance genes located in a class 1 integron. pMOL412_FII was closely related to another ~90.3 kb plasmid (pM109_FII) harbouring bla_{NDM-4} in an *E. coli* isolated from a human patient in Myanmar.

Conclusions: To the best of our knowledge, we have identified for the first time in Europe an NDM-producing Enterobacterales in livestock and resolved the complete sequence of the novel pMOL412_FII plasmid harbouring bla_{NDM-4} in an MRR. A global One Health approach, comparing genomic data from different sources and geographical areas, may help to trace back and control possible plasmid-borne carbapenemase gene transmission between animals and humans and along the food chain at international level.

Introduction

New Delhi MBL 4 (NDM-4), which differs from NDM-1 by a single amino acid substitution (Met154Leu), was demonstrated to increase carbapenemase activity.[1] At present, only sporadic cases of human infections associated with NDM-4-producing *Escherichia coli* have been described.[1,2] Additionally, reports on the occurrence of all carbapenemases in bacteria from food-producing animals and food of animal origin in Europe are very scarce.[3,4]

In this study, to the best of our knowledge, we report the first evidence in Europe of an NDM-4-producing *E. coli* isolated from a farmed animal (a fattening pig), harboured by a novel plasmid of 53 043 bp, of which we report the complete sequence by using a combined approach of Illumina–Oxford Nanopore sequencing and bioinformatics analysis.

Materials and methods

In 2019, one carbapenem-resistant *E. coli* was isolated by the National Reference Laboratory for Antimicrobial Resistance, Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Rome, Italy. It was detected from the caecal contents of a fattening pig sampled at slaughter, in the framework of EU-harmonized antimicrobial resistance (AMR) monitoring activities (according to Decision 2013/652/EU, https://eur-lex.europa.eu/eli/dec_impl/2013/652/oj). The slaughtered pig belonged to a holding located in Northwest Italy.

For the isolation, we used the 'specific isolation of carbapenemase-producing *E. coli*' method according to the protocol of the European Union Reference Laboratory for Antimicrobial Resistance (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/530_esbl-ampc-cpeprotocol-version-caecal-v7-09-12-19.pdf). One presumptive carbapenemase-producing isolate was then subjected to species identification by means of routine biochemical assays.

Antimicrobial susceptibility was assessed via MIC determination using the broth microdilution method and consensus 96-well microtitre plates (TREK Diagnostic Systems, Westlake, OH, USA). Antimicrobials tested, dilution ranges and interpretation of MIC values were in accordance with Decision 2013/652/EU. Based on the phenotype of carbapenem resistance, the isolate was screened by a consensus PCR for various carbapenemase genes.[5] Subsequently, it was in-depth characterized by WGS and bioinformatics analysis.

WGS was first performed using an Illumina platform (MiSeq). DNA extraction, library preparation, trimming and *de novo* assembly of raw reads were performed as reported by Alba *et al.*[6] The assembly obtained was annotated using the RAST Server.[7] Additionally, a manual curation for the obtained annotation was performed, especially for the ISs by using the ISfinder database.[8] Molecular characterization was performed as reported by Alba *et al.*,[6] using different CGE tools to assign STs, for the genetic basis of AMR and for the detection of plasmid replicons. SerotypeFinder 2.0,[9] VirulenceFinder 2.0[10] and VF database (http://www.mgc.ac.cn/VFs/) were also used for *in silico* serotyping and virulence gene detection. The sequence obtained was compared with those available in public repositories (GenBank). Conjugation and transformation were not attempted; however, in order to close any remaining gaps and precisely identify and locate all bla_{NDM-4}-harbouring plasmid regions (including the transfer region), the isolate was also sequenced using the nanopore-based MinION device (Oxford Nanopore Technologies) with the rapid barcoding kit (SQK-RBK004). A hybrid (Illumina–Oxford Nanopore) assembly was performed using the Unicycler pipeline.[11]

Results

The *E. coli* isolate shows MDR, carbapenem resistance and wide β-lactam resistance with ampicillin, cefotaxime, ceftazidime, meropenem, imipenem and ertapenem MIC values of >64, >64, >128, >16, 8 and >2 mg/L, respectively, and tested positive by PCR for the presence of the bla_{NDM} gene family. Details on the results of genetic characterization by WGS and the AMR phenotype are reported in Table S1 (available as Supplementary data at *JAC* Online). The AMR phenotype was confirmed by the genotype, the isolate being concomitantly resistant to all β-lactams, carbapenems, sulfamethoxazole and trimethoprim, mediated by bla_{TEM-1B}, bla_{NDM-4}, *sul1/sul3* and *dfrA12* genes, respectively (Table S1). No ESBL/AmpC genes were detected. The isolate tested positive for different plasmid replicons, with IncFII and IncR types showing 100% coverage and identity (Table S1). Thanks to the hybrid (Illumina–Oxford Nanopore) assembly approach, bla_{NDM-4} was demonstrated to be located on a novel IncFII plasmid, named pMOL412_FII, with a size of 53 043 bp. Annotation of the plasmid sequence identified four main genetic regions. The plasmid backbone (69.8% of the total plasmid size) included a replication region containing the *repA* gene encoding a replication initiation protein of the IncFII family, a stability region and the IncF transfer region (Figure 1a and b and Table S1). Results of BLAST analysis (considering 90% as the coverage threshold) revealed that the pMOL412_FII backbone sequence matched (identity >99.8%) with six complete IncFII backbone sequences previously detected in

E. coli (Table S1). The variable region was an MDR region (MRR) of 16 kb harbouring bla_{NDM-4} (named MRR-NDM-4) and bracketed by two copies of IS*26* and transposons (Figure 1a and b and Table S1).

Besides bla_{NDM-4}, the bla_{NDM-4}-containing region also contained several other AMR determinants (Figure 1b), including *dfrA12*, *sul1*, *qacEΔ1* (quaternary ammonium compound resistance gene) and *aadA2* (streptomycin resistance gene), which were located on a gene cassette array inserted into a class 1 integron (Figure 1b and Table S1). The complete sequence of the resolved plasmid was submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number ERZ1392780. Comparison with public databases revealed that the pMOL412_FII plasmid was most closely related (57% coverage and 99.82% identity) to an ~90.3 kb plasmid (pM109_FII) harbouring bla_{NDM-4} of a carbapenem-resistant *E. coli* that was previously isolated from a human patient in Myanmar (Figure 2).[12] Additionally, MRR-NDM-4 was identical to the bla_{NDM-4} region (12388:22899) of the above mentioned carbapenem-resistant *E. coli* (accession number: AP018139).[12]

Discussion

To the best of our knowledge, this represents the first description of an NDM-producing *E. coli* and Enterobacterales in a food-producing animal in Europe. Indeed, in primary production, bla_{NDM-4} has so far only been detected in two *E. coli* isolated from a diseased chicken and from a broiler faeces sample in China.[2,13]

In this study, we have also identified and resolved the complete sequence of a novel IncFII plasmid (pMOL412_FII) of 53 043 bp harbouring the carbapenemase gene bla_{NDM-4}. Overall, different combinations of plasmid types and bla_{NDM} gene variants have been previously described,[2,12] with bla_{NDM-4} mainly associated in human patients with IncFIA or IncFII, but also with IncX3 and IncK plasmid types.[1,2,14] Conversely, in livestock, bla_{NDM-4} had only been detected in IncHI2 plasmids, co-existing with the *mcr-1* gene in *E. coli* isolated from poultry samples in China.[2,13] Notably, the complete sequences of plasmids carrying such carbapenemases are usually not available for comparison. Indeed, the most closely related to pMOL412_FII was pM109_FII from a carbapenem-resistant *E. coli* of human origin in Myanmar.[12] Interestingly, both pMOL412_FII and pM109_FII harboured an identical MRR with the bla_{NDM-4} gene bracketed by two copies of IS*26*, potentially able to mobilize this gene and possibly capable of involving different plasmids as vehicles of dissemination.[12]

In Italy, Coppo *et al.*[15] described, by means of classical typing methods, a variable region of a class 1 integron containing bla_{NDM-4}, similar to our MRR-NDM-4. In this previous study, the bla_{NDM-4} region was located on an IncF plasmid of an ST405 *E. coli* identified in a human patient, earlier hospitalized in India. However, in that study the plasmid was not resolved and the complete sequence was not available for comparison.

Importantly, our results proved how the combined bioinformatics analysis of Oxford Nanopore long reads with Illumina short reads has been decisive to precisely locate a class 1 integron containing bla_{NDM-4} in an MRR (MRR-NDM-4) and to resolve the IncFII plasmid (pMOL412_FII).

Carbapenem-resistant Enterobacteriaceae have been globally isolated from the environment, livestock, pets, wildlife and food,[16]
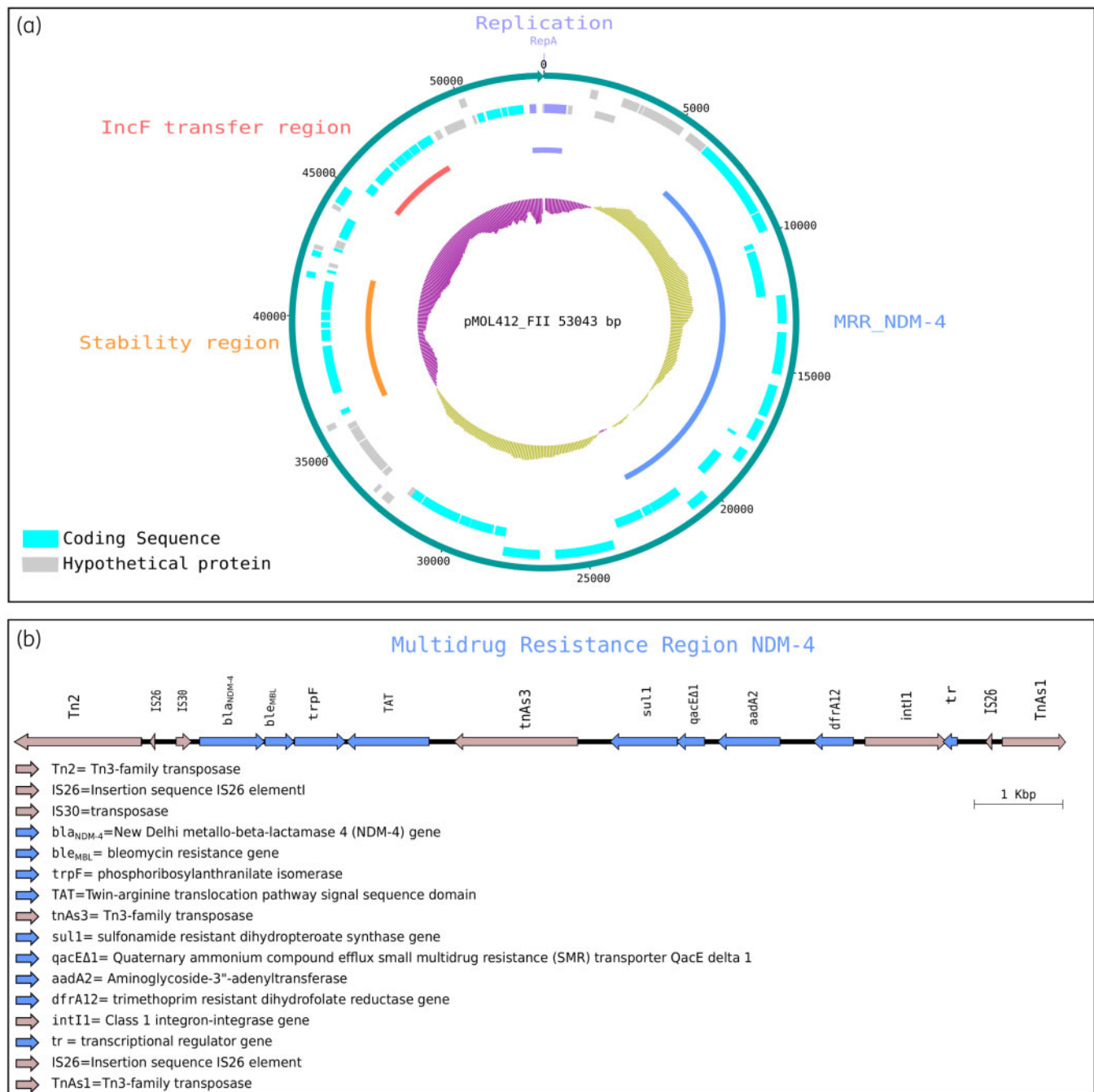
Figure 1. (a) Circular representation (ring diagram) of plasmid pMOL412_FII. Circles indicate, from the innermost to the outermost: plot of the G + C content, specific plasmid regions having important functions, plus and minus strands (third and fourth circles, respectively) with all the annotated regions located on the plasmid and the fifth circle represents the scale. (b) Linear representation of MRR-NDM-4. All the genetic elements of MRR-NDM-4 are represented proportionally with their length and orientation. The full names of the genetic elements are listed below the diagram. Coding sequences and resistance genes are indicated by blue arrows, while IS elements are indicated by pink arrows. This figure appears in colour in the on-line version of *JAC* and in black and white in the print version of *JAC*.

with an increased prevalence of NDM-producing *E. coli* reported from food in China.[17]

The *E. coli* isolate described herein belonged to ST641, an ST not previously associated with NDM-mediated carbapenem resistance. Indeed, this ST had been already detected in animal samples, including pig samples.[18,19]

In Europe, according to the EU-harmonized AMR monitoring programme, the prevalence of carbapenemase-producing *E. coli*
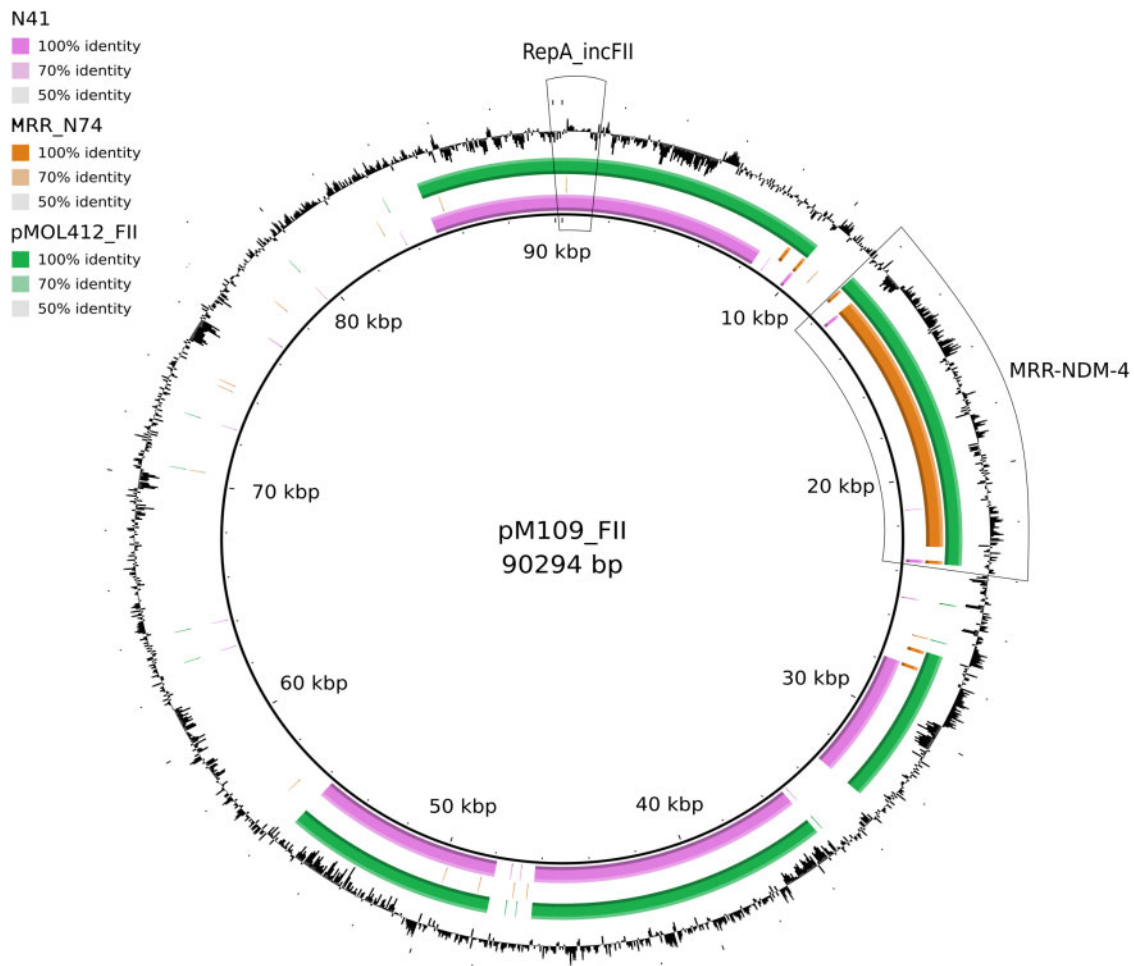
Figure 2. Comparative analysis of closely related plasmids pMOL412_FII and pM109_FII harbouring bla_{NDM-4}. From the outside inwards, the outer circle shows a plot of the G + C content, the second circle shows the homologous regions of pMOL412_FII and pM109_FII, the third and fourth circles show the two contigs (MRR_N74 and N41) obtained with Illumina short-read assembly that partially reconstruct the pMOL412_FII sequence and the fifth circle shows the size (kb) of the resolved pM109_FII. Two framed areas indicate the replication region (RepA_incFII) and the MRR (MRR-NDM-4). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and *Salmonella* spp. among livestock in Europe has so far remained very low (<1%), with few bla_{OXA-48} and bla_{VIM-1}-positive isolates so far detected.[18,19] It is noteworthy that the NDM-4-positive isolate was detected from the pig caecal sample only by the isolation method specific for carbapenemase-producing *E. coli*. Conversely, it was not detected by the ESBL/AmpC/ carbapenemase-producing *E. coli* method, which uses MacConkey agar supplemented with 1 mg/L cefotaxime as the selective agar, on which it may have been overgrown by other ESBL/AmpC-producing *E. coli*. Interestingly, by using this latter method we only cultured an ESBL-producing *E. coli* (CTX-M-32 type) from the same sample (data not shown).

In conclusion, these findings confirm the importance of continuous and specific monitoring of carbapenem-producing Enterobacterales in food-producing animals and along the food chain in Europe, even though carbapenems have never been licensed for veterinary use and are not used in animal farming. It is well known that the use of third- and fourth-generation cephalosporins in animals can select for most carbapenemases (except

perhaps the OXA-type carbapenemases). Even the extensive and continuous oral usage of aminopenicillins may be of selective advantage, since carbapenemases are very versatile enzymes and also inactivate penicillins, cephalosporins and monobactams, beside extended-spectrum cephalosporins.[20]

Although, in our case, possible transmission pathways among farms, between animals and in-contact humans are yet to be investigated, a human source for the introduction of the NDM-4 gene, its plasmid or even the bla_{NDM-4}-positive ST641 *E. coli* isolate within the farm, e.g. by in-contact personnel, cannot be ruled out. In this regard, further investigations at farm level (including personnel), will be useful to assess possible carbapenem-resistant Enterobacteriaceae (and bla_{NDM-4}) transmission routes.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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