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1 **The coexistence of two *bla*<sub>NDM-5</sub> genes on an IncF plasmid**  
2 **as revealed by nanopore sequencing**

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10  
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12  
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14  
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18

19 **Abstract**

20 In a carbapenem-resistant *Escherichia coli* clinical isolate of sequence type 167, two  
21 copies of *bla*<sub>NDM-5</sub> were found on a 144,225-bp IncF self-transmissible plasmid of the  
22 F36:A4:B- type. Both *bla*<sub>NDM-5</sub> genes were located in 11,065-bp regions flanked by  
23 two copies of IS26. The two regions were identical in sequence but were present at  
24 different locations on the plasmid, suggesting a duplication of the same region. This  
25 study highlights the complex genetic contexts of *bla*<sub>NDM-5</sub>.

26 New Delhi metallo- $\beta$ -lactamase (NDM) is a type of carbapenem-hydrolysing enzymes  
27 (carbapenemases) with the ability to hydrolyze all  $\beta$ -lactams except monobactams (1),  
28 representing a serious challenge for treatment of bacterial infections, infection control  
29 and public health. ~~Up to now~~To date, there are 21 variants of NDM, ~~among which~~with  
30 NDM-5 ~~is~~ one of the most common variants encountered in the Enterobacteriaceae  
31 (2-5). The NDM-5-encoding gene, *bla*<sub>NDM-5</sub>, usually exists in a single copy on  
32 plasmids. However, we have found the peculiar presence of two copies of *bla*<sub>NDM-5</sub> on  
33 a single plasmid within an *Escherichia coli* clinical isolate, which is reported here.

34  
35 *E. coli* strain SCEC020007 was recovered from urine of a female outpatient with  
36 urinary tract infection in October 2016 in China. The strain was resistant to amikacin  
37 (MIC, >512  $\mu$ g/ml), ceftazidime (>512  $\mu$ g/ml), ceftazidime-avibactam (>512/4  $\mu$ g/ml),  
38 ciprofloxacin (256  $\mu$ g/ml), imipenem (64  $\mu$ g/ml), meropenem (256  $\mu$ g/ml),  
39 piperacillin-tazobactam (>512/4  $\mu$ g/ml) and trimethoprim-sulfamethoxazole  
40 (128/2,432  $\mu$ g/ml), but was susceptible to aztreonam (8  $\mu$ g/ml), colistin (2  $\mu$ g/ml) and  
41 tigecycline (0.25  $\mu$ g/ml) as determined using the broth dilution method of the Clinical  
42 Laboratory Standards Institute (6). As there are no breakpoints of colistin and  
43 tigecycline from CLSI, those defined by EUCAST (<http://www.eucast.org/>) were  
44 applied.

45  
46 A draft genome sequence of the strain was generated on the Illumina HiSeq X10  
47 platform, which generated 5,557,833 clean reads and 1.67 Gb clean bases. A total of  
48 113 contigs (102 >1,000 bp; *N*50 126,680 bp) with a 50.76% GC content were *de*  
49 *novo* assembled using SPAdes (7). Strain SCEC020007 belonged to phylogenetic  
50 group A as determined using PCR as described previously (8) and sequence type

51 167 (ST167) as determined using the genomic sequence to query the *E. coli*  
52 multi-locus sequence typing database  
53 (<http://enterobase.warwick.ac.uk/species/index/ecoli>). Antimicrobial resistance genes  
54 were identified from genome sequences using the ABRicate program  
55 (<https://github.com/tseemann/abricate>) to query the ResFinder database  
56 (<http://genomicepidemiology.org/>). Strain SCEC020007 had 9 antimicrobial  
57 resistance genes mediating resistance to aminoglycosides (*aadA2*, *aadA5*, *rmtB*),  
58  $\beta$ -lactams (*bla*<sub>NDM-5</sub> and *bla*<sub>TEM-1</sub>), tetracycline (*tet(A)*), sulphonamides (*sul1*) and  
59 trimethoprim (*dfrA12* and *dfrA17*). Plasmid replicon types within strain SCEC020007  
60 were determined using ~~by~~ the PlasmidFinder tool at <http://genomicepidemiology.org/>.  
61 Surprisingly, strain SCEC020007 had an IncFIA, an IncFII and an IncB/O/K/Z replicon  
62 but no IncX3, which is the common replicon type of plasmids associated with *bla*<sub>NDM-5</sub>.  
63  
64 To untangle the genetic context of *bla*<sub>NDM-5</sub>, strain SCEC020007 was subjected to  
65 sequencing using the long-read ~~real-time~~ MinION Sequencer (Nanopore, Oxford, UK).  
66 ~~The A~~ *de novo* hybrid assembly of both short Illumina reads and long MinION reads  
67 was ~~performed-constructed~~ using Unicycler v0.4.3 (9) under conservative mode for  
68 ~~an~~ increased accuracy. ~~The C~~ complete circular contigs generated were then  
69 corrected using Plion v1.22 (10) with Illumina reads for several rounds until no  
70 change was detected. The hybrid assembly of Illumina and MinION reads revealed  
71 that strain SCEC020007 had a 4.8-Mb circular chromosome, a 144,225-bp plasmid  
72 containing ~~an~~ IncFIA and ~~a~~ FII replicon~~s~~ (designated pNDM5\_020007) and an  
73 84,952-bp plasmid with an IncB/O/K/Z replicon (designated pBOKZ\_020007).  
74 Surprisingly, there were two copies of *bla*<sub>NDM-5</sub> in strain SCEC020007, both of which  
75 were present on pNDM5\_020007. Both *bla*<sub>NDM-5</sub> genes were located in 11,065-bp

76 regions flanked by two copies of IS26 and the two regions were identical in sequence  
77 but were present at different locations on pNDM5\_020007 (Figure 1), suggesting that  
78 the 11,065-bp region is duplicated. The presence of the two *bla*<sub>NDM-5</sub> genes and their  
79 locations on pNDM5\_020007 were confirmed by PCR. The 11,065-bp region  
80 contained a complex class 1 integron with a *dfrA17-aadA5* cassette array and *ISCR1*  
81 (insertion sequence common region 1), which is truncated by IS26 at its 5' conserved  
82 segment, a 69-bp remnant of *ctuA1* (encoding an ion tolerant protein), *dsbC*  
83 (encoding an oxidoreductase), *trpF* (encoding a phosphoribosylanthranilate  
84 isomerase), *ble* (mediating bleomycin resistance), *bla*<sub>NDM-5</sub>, a truncated *ISAbA125*  
85 and a truncated *ISEcp1/ISEc9* element (Figure 1). The co-existence of two *bla*<sub>NDM-5</sub>  
86 genes has not been reported before but the co-existence of two *bla*<sub>NDM-1</sub> genes has  
87 been described previously (11, 12). Two tandem copies of *bla*<sub>NDM-1</sub> genes have been  
88 found in the chromosome of an ST167 *E. coli* in China (11) and a *Pseudomonas*  
89 *aeruginosa* strain in Serbia (12). In both cases, the tandem copies of *bla*<sub>NDM-1</sub> are  
90 associated with *ISCR1* but not IS26. It is known that *ISCR1* uses the rolling circle  
91 mechanism for transposition and may generate tandem duplication of its mobilized  
92 sequence via homologous recombination (13). However, the duplication of the  
93 11,065-bp region carrying *bla*<sub>NDM-5</sub> on pNDM5\_020007 is not tandem, suggesting that  
94 the duplication might not result from the action of *ISCR1* but could be mediated by  
95 IS26. The exact mechanism for the duplication of such a large region warrants further  
96 studies.

97  
98 Assembly based on Illumina reads alone generated only a single contig containing  
99 *bla*<sub>NDM-5</sub> and was unable to reveal that there were actually ~~were~~ two identical copies of  
100 the same contig. This imposes difficulties for completing the *bla*<sub>NDM-5</sub>-carrying plasmid

101 | sequence by convention<sup>al</sup> methods including PCR and Sanger sequencing to close  
102 | gaps between contigs. By contrast, MinION sequencing ~~is~~was able to resolve the  
103 | copy numbers of genes and contigs and their exact position on the plasmid relative to  
104 | each other.

105

106 | Plasmid multi-locus sequence typing (pMLST) was performed using the pMLST tool  
107 | (<https://cge.cbs.dtu.dk/services/pMLST/>). pNDM5\_020007 belongs to the F36:A4:B-  
108 | type. pNDM5\_020007 ~~was~~has closest similarity (97% coverage and 99% identity) to  
109 | a 149.5-kb unnamed plasmid (GenBank accession no. CP023871) from *E. coli* strain  
110 | FDAARGOS\_434, which was recovered from a human rectal swab in British  
111 | Columbia, Canada, in 2014. This unnamed plasmid also carries *bla*<sub>NDM-5</sub> (a single  
112 | copy) and belongs to the F36:A4:B- type. Backbones of pNDM5\_020007 and the  
113 | unnamed plasmid of strain FDAARGOS\_434 are almost identical, suggesting that  
114 | they might have originated from a common plasmid. Conjugation experiments were  
115 | carried out in broth and on filters with the azide-resistant *E. coli* strain J53 as the  
116 | recipient. pNDM5\_020007 was able to be transferred by conjugation, suggesting that  
117 | it is self-transmissible.

118

119 | In conclusion, we identified the presence of two *bla*<sub>NDM-5</sub> genes on an F36:A4:B-  
120 | self-transmissible plasmid. The co-existence of two *bla*<sub>NDM-5</sub> genes was due to the  
121 | duplication of an IS26-bracketed region containing *ISCR1*.

122

123 | **Nucleotide sequence accession numbers.** The complete sequence of  
124 | pBOKZ\_020007, pNDM5\_020007 and the chromosome of strain SCEC020007 has

125 been deposited into GenBank under the accession no. CP025625, CP025626 and  
126 CP025627, respectively.

127

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133

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177

178 **Figure legend**

179 **Figure 1.** pNDM5\_020007 and the genetic context of *bla*<sub>NDM-5</sub>. The two  
180 11,065-bp *bla*<sub>NDM-5</sub>-containing regions bracketed by IS26 are indicated by  
181 orange circles in the map of pNDM5\_020007 and are shown in detail at the  
182 bottom. Δ represents truncated genes or mobile genetic elements.