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Occurrence and characterisation of *Escherichia coli* Sequence Type 410 co-harbouring $bla_{\text{NDM-5}}$, $bla_{\text{CMY-42}}$ and $bla_{\text{TEM-190}}$ in a dog from the United Kingdom

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Running title: NDM-5 producing *Escherichia coli* ST410 in a UK dog

SYNOPSIS

Background/ Objectives

Carbapenemase-producing Enterobacteriaceae (CPE) are a public health threat, and have been found in humans, animals and the environment. Carbapenems are not authorised for use in EU or UK companion animals, and the prevalence of carbapenem-resistant Gram-negative bacilli (CRGNB) in this population is unknown.

Methods

We investigated CRGNB isolated from animal specimens received by one diagnostic laboratory from 34 UK veterinary practices (Sept 2015-Dec 2016). Any Gram-negative isolates from clinical specimens showing reduced susceptibility to fluoroquinolones and/or aminoglycosides and/or cephalosporins were investigated phenotypically and genotypically for carbapenemases. A complete genome assembly (Illumina/Nanopore) was generated for the single isolate identified to investigate the genetic context for carbapenem resistance.

Results

One ST410 *Escherichia coli* isolate [(CARB35); 1/191, 0.5%)], cultured from a wound in a springer spaniel, harboured a known carbapenem-resistance gene ($bla_{\text{NDM-5}}$). The gene was located in the chromosome on an integrated 100kb IncF plasmid, also harbouring other drug resistance genes (*mrx*, *sul1*, *ant1*, *dfrA*). The isolate also contained $bla_{\text{CMY-42}}$ and $bla_{\text{TEM-190}}$ on two separate plasmids (IncI1 and IncFII, respectively), which showed homology with other publicly available plasmid sequences from Italy and Myanmar.

Conclusions

Even though the use of carbapenems in companion animals is restricted, the concurrent presence of $bla_{\text{CMY-42}}$ and other antimicrobial resistance genes could lead to co-selection of

carbapenemase genes in this population. Further studies investigating the selection and flow of plasmids carrying important resistance genes amongst humans and companion animals are needed.

Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) are a serious public health problem due to limited therapeutic options for CPE-associated infections.[1] Increased carbapenem use, especially for treating infections caused by ESBL-producers, is a significant driver of CPE emergence in human medicine.[2] In contrast, carbapenems are not authorised for veterinary use[3,4] except for the management of MDR Gram-negative infections under the prescribing cascade (https://www.gov.uk/guidance/the-cascade-prescribing-unauthorised-medicines#special-considerations-for-the-responsible-use-of-antibiotics-under-the-cascade).

Furthermore, there is limited carbapenem resistance testing and no UK national surveillance of CPE prevalence in companion animals. Consequently, occurrence of carbapenemase-producing bacteria in animals may remain undetected. Here, we report surveillance data from a UK Veterinary Diagnostics Laboratory which introduced carbapenem-resistance screening for Gram-negative bacteria. We also describe the molecular characterisation of a NDM-5 producing E. coli isolates isolated from a wound in a dog.

Materials and methods

Bacterial isolates cultured from clinical specimens submitted September 2015-December 2016 to one UK diagnostic laboratory were included in this study. Clinical specimens were

received from 34 veterinary practices across England (n=29), Wales (n=4) and Ireland (n=1) and included swabs, urine, tissues, sterile fluids, bronchoalveolar lavages, faecal samples (cats, dogs) and bovine milk samples. To increase detection of all carbapenemase-producing isolates (including OXA-48 producers), any Gram-negative bacteria with reduced susceptibility to fluoroquinolones, aminoglycosides and/or cephalosporins cultured were tested using chromID Carba SMART agar bi-plates (bioMerieux, Basingstoke, UK).

Each half of the bi-plates was inoculated with 10µl of fresh, pure culture (0.5 MacFarland suspension), and incubated aerobically (37±2°C, 22-24 hours). *Klebsiella pneumoniae* NCTC13368 (SHV-18 [ESBL]) was used as a negative control and *K. pneumoniae* NCTC13438 (KPC-3), NCTC13440 (VIM-1) and NCTC13442 (OXA-48) as positive controls. Isolates exhibiting characteristic growth were identified using either API (20E/20NE, bioMérieux UK Ltd) or MALDI-TOF (Laboklin, Germany).

Susceptibility testing for the carbapenem-resistant isolate identified in this study was performed by broth microdilution (TREK Diagnostic System, West Sussex, UK), interpreted according to the European Committee on Antimicrobial Susceptibility testing guidelines (version v7.0, at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf).

Bacterial DNA was extracted by heat lysis and centrifugation, and used to screen for: carbapenemases (*bla*$_{NDM}$, *bla*$_{OXA-48-like}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{KPC}$); ESBLs (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ *bla*$_{GES}$, *bla*$_{PER}$, *bla*$_{VEB}$); plasmid-mediated pAmpC-group genes,[5, 6] and colistin

resistance (*bla*$_{MCR-1}$, *bla*$_{MCR-2}$; https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/278_mcr-multiplex-pcr-protocol-v2-oct16.pdf) as previously described.

Whole genome sequencing and analysis

The carbapenem-resistant *E. coli* isolate was re-cultured from stock; DNA was extracted using the Qiagen Genomic-tip 100/G kit (Qiagen, Hilden, Germany). Aliquots of the same DNA extract were sequenced on both the Illumina HiSeq 4000 and Oxford Nanopore Technologies' MinION (library preparation kit: SQK-LSK208, flowcell: FLO-MIN106 R9.4, as in Phan HTT *et al*.[7] Sequence data have been deposited in the NCBI (BioProject: PRJNA473397).

A hybrid, complete genome assembly was constructed from the two sequencing datasets using Unicycler (v4.1; parameters: --no_correct --min_component_size 500 --min_dead_end_size 500 --verbosity 1 --mode bold). The Unicycler assembly was also compared with a hybridSPAdes (v.3.6; default parameters, "careful" option) assembly to verify the genome structure using a different assembly method.

In silico MLST typing was performed using BLASTn against the PubMLST allele databases (available at https://pubmlst.org/general.shtml). Plasmid typing, insertion sequence typing and resistance gene characterisation were carried out using PlasmidFinder, the ISFinder database and an in-house script (ResistType), as previously described.[7] The chromosomally integrated plasmid sequence and multi-drug resistance region harbouring *bla*$_{NDM-5}$ were compared with publicly available plasmid sequences in GenBank using BLASTn, with

default settings. Data visualisations were created using the GenomeDiagram module in Biopython.

Results

One hundred and ninety-one Gram-negative isolates from dogs (n=158), cats (n=27), cattle (n=4), a rabbit and a guinea pig, were sub-cultured onto chromID Carba SMART bi-plates; of these, 28 isolates generated moderate-heavy growth, where *Acinetobacter* spp. (n=4) and *Pseudomonas* spp. (n=23) grew on one or both halves, whilst *Escherichia coli* (1/191) grew on the CARB side only.

No bla_{ESBL}/pAmpC or carbapenem-resistance genes were identified in cultured *Pseudomonas* spp. and *Acinetobacter* spp. which were most likely selected due to their intrinsic resistance (decreased permeability and/or expression of efflux pumps) to the agents included in the CARBA-SMART plates. However, the *E. coli* isolate (isolate CARB35) harbored bla_{NDM} (confirmed on sequencing to be $bla_{\text{NDM-5}}$), bla_{CMY} and bla_{TEM}. The NDM-producing *E. coli* was cultured (pure growth) from a foreleg wound on the 5th digit of a 7-year-old English springer spaniel. The dog had a history of foot lacerations, dog bite wounds and urinary tract infections, for which he received multiple courses of amoxicillin/clavulanate, cefovecin, doxycycline and enrofloxacin in the preceding six years.

The NDM-producing *E. coli* was resistant to ampicillin, cefoxitin, aztreonam, cefazolin, cefepime, cefpodoxime (all >16µg/mL), amoxicillin/clavulanate (>32 µg/mL), piperacillin/tazobactam, ticarcillin/clavulanate (>64 µg/mL), meropenem, imipenem

(4μg/mL), ciprofloxacin(2μg/mL), levofloxacin (>8 μg/mL), and trimethoprim/sulfamethoxazole (>4 μg/mL). The isolate remained susceptible to gentamicin, amikacin (≤1 and ≤4 μg/mL, respectively), tigecycline (≤0.25 μg/mL), and colistin/polymyxin B (≤0.25 μg/mL).

The two assemblers agreed on a complete assembly for the NDM-5-producing *E. coli* isolate which included a chromosome (~4.9Mb; ST410) and five plasmids (~3kb, 4kb, 59kb [IncI1], 89kb [IncFII], 90kb [IncY). $bla_{\text{NDM-5}}$ was chromosomally integrated into the *E. coli* genome, flanked by multiple IS elements, in an 18.6kb configuration harbouring other drug resistance genes (*mrx*, *sul1*, *ant1*, *dfrA*) (Fig.1). A similar flanking sequence for $bla_{\text{NDM-5}}$ has been observed in only a handful of publicly available but largely unpublished sequences, including submissions from the US, China and Germany (Fig.S1). This multi-drug resistance region was nested within a 100kb region encoding plasmid-associated genes, including IncFII, IncFIA and IncFIB replicons, and flanked by two IS*150* insertion sequences in the same orientation, most consistent with it representing an integrated plasmid (Fig. 1). This 100kb region had 99% sequence identity over 86% of its length to the reference plasmid sequences CP024860.1 (172kb; submitted Nov-2017 by NIH, USA; isolation source unknown) and KP789020.1 (*E. coli* WCHEC13-8 plasmid pCTXM15 harbouring $bla_{\text{NDM-1}}$ and $bla_{\text{CMY-42}}$ from Chengdu, China; 56kb; submitted Nov-2015; human clinical isolate).

The IncI1 plasmid (59kb), carrying $bla_{\text{CMY-42}}$, had a 99% sequence match to *E.coli* plasmid tig00001287_pilon (GenBank accession: CP021882.1, 68kb);the IncFII plasmid (89kb), carrying $bla_{\text{TEM-190}}$, shared 99% sequence identity over 93% of the sequence query with two *E.coli* plasmids (GenBank accessions: KY463220.1, AP018147.1).

Genes/gene mutations encoding resistance to spectinomycin/streptomycin (*aadA2* - chromosome, positions: 194104-194895), trimethoprim/trimethoprim-sulfamethoxazole (*dfrA12* - chromosome, positions: 193199-193696; *sul1* - chromosome, positions: 195400-196239; *folP* - chromosome, positions: 680079-680927), macrolides-lincosamide-streptogramin (*mphA* - choromosome, positions: 202749-203654 and plasmid pCARB35-2, positions: 83836-84741), nitrofurantoin (in *nfsA*: chromosome, positions: 3510946-3511599) and fluoroquinolones (*gyrA* - chromosome, positions: 1676727-1679354; *parC* - chromosome, positions: 841664-843922; *parE* - chromosome, positions: 831111-833003), and *fimE* (chromosome, positions: 4197047-4197643) and *usp* (chromosome, positions: 2077529-2077957, 2620969-2621403, and 3475153-3475581) virulence factors, were also identified.

Discussion

ESBL/AmpC-producing Enterobacteriaceae have emerged in food-producing and companion animals over the past two decades.[8] Although still rare, carbapenemase-producers, mainly NDM-1 *Acinetobacter* spp., VIM-1 *E. coli* and *Salmonella* spp., have been reported worldwide in livestock.[9] However, there are very few reports of CPE in companion animals. NDM-1-producing *E. coli* was first described in dogs and cats from the US in 2013, only four years after the first description of NDM-1-producing bacteria in humans.[10] OXA-48-producing *E. coli* and *Klebsiella* spp. were first reported in dogs from Germany and subsequently also in clinical canine isolates in the US, including pandemic strains such as *E. coli* ST648.[11, 12] However, a low prevalence (0.6%, n=160) of CPE, consisting of a single VIM-1-producing *K. pneumoniae* isolate, was found amongst companion animals from

Spain.[13] More recently, IMP-4-producing *Salmonella* Typhimurium was isolated from a cat with persistent haemorrhagic diarrhoea in Australia[14] and OXA-23-producing *A. baumannii* associated with urinary tract infection was detected in a cat from Portugal.[15]

NDM-5 differs from NDM-1 by two amino acid substitutions and has been described in Enterobacteriaceae from both humans and livestock, mainly in Asian countries, including Myanmar.[16] NDM-5-producing *E. coli* was also recently reported in clinical isolates from Italian patients, one of which had a history of travel to Thailand,[17] and also in Spain in a patient who had not travelled abroad.[18] In companion animals, NDM-5-producing *E. coli* ST1284 was isolated form a rectal swab in a dog from Algeria; molecular characterisation suggested that $bla_{\text{NDM-5}}$ was likely to be chromosomally located.[19]

The NDM-5-producing *E. coli* isolate in our study was ST410, and harboured $bla_{\text{NDM-5}}$ on a plasmid integrated into the chromosome. ST410 is an emerging clone with worldwide distribution, associated with MDR human infections, including bloodstream infections and demonstrating potential for nosocomial spread.[20] $bla_{\text{TEM-190}}$ was present on an IncFII plasmid highly similar to IncFII $bla_{\text{NDM-5}}$ plasmids found in human clinical isolates from Italy (ST405) and Myanmar (ST410).[16, 18] Our isolate also harboured $bla_{\text{CMY-42}}$ located on an IncI1 plasmid, similarly present in the Italian NDM-5 producing *E. coli* isolates,[17] suggesting a shared plasmid population amongst which $bla_{\text{NDM-5}}$, $bla_{\text{TEM-190}}$ and $bla_{\text{CMY-42}}$ are circulating.

Although $bla_{\text{NDM-1}}$ is common amongst human carbapenem-resistant isolates in the UK, $bla_{\text{NDM-5}}$ has been reported on only a small number of occasions: once, in 2011, also on an IncFII plasmid in a ST648 *E. coli* recovered from a patient recently hospitalised in India,[21]

and in 2014, in four ST410 isolates, for which there are limited additional metadata, and in which the genetic location of *bla*$_{NDM-5}$ could not be determined.[20] Given the similarity of its genetic background with previously described human isolates, the low prevalence of CPE in animals, and the increasing evidence of environmental contamination with CPE by human hospital effluents,[8] it is possible that the NDM-5-*E. coli* isolated in this study might be of human origin. Our study was limited with respect to the sampling frame and lack of available epidemiological data, but suggests that further detailed studies on the selection and flow of important resistance genes, including carbapenemases, amongst humans and animals are needed.

Although the use of carbapenems in companion animals is uncommon, the concurrent presence of *bla*$_{CMY-42}$ and *bla*$_{TEM-190}$ on common plasmids could lead to rapid co-selection of *bla*$_{NDM}$ in this population. In addition, the detection of a carbapenemase producing *E. coli* ST410 recently described as a high-risk MDR clone with increased potential for inter-species transmission,[22] in companion animals is concerning. Hence, improved antimicrobial stewardship as well as introducing routine detection of carbapenem-resistance in animal isolates is warranted to reduce the risk of zoonotic transmission and will contribute to concerted "One Health" efforts in containing the spread of resistance to last resort antimicrobials.

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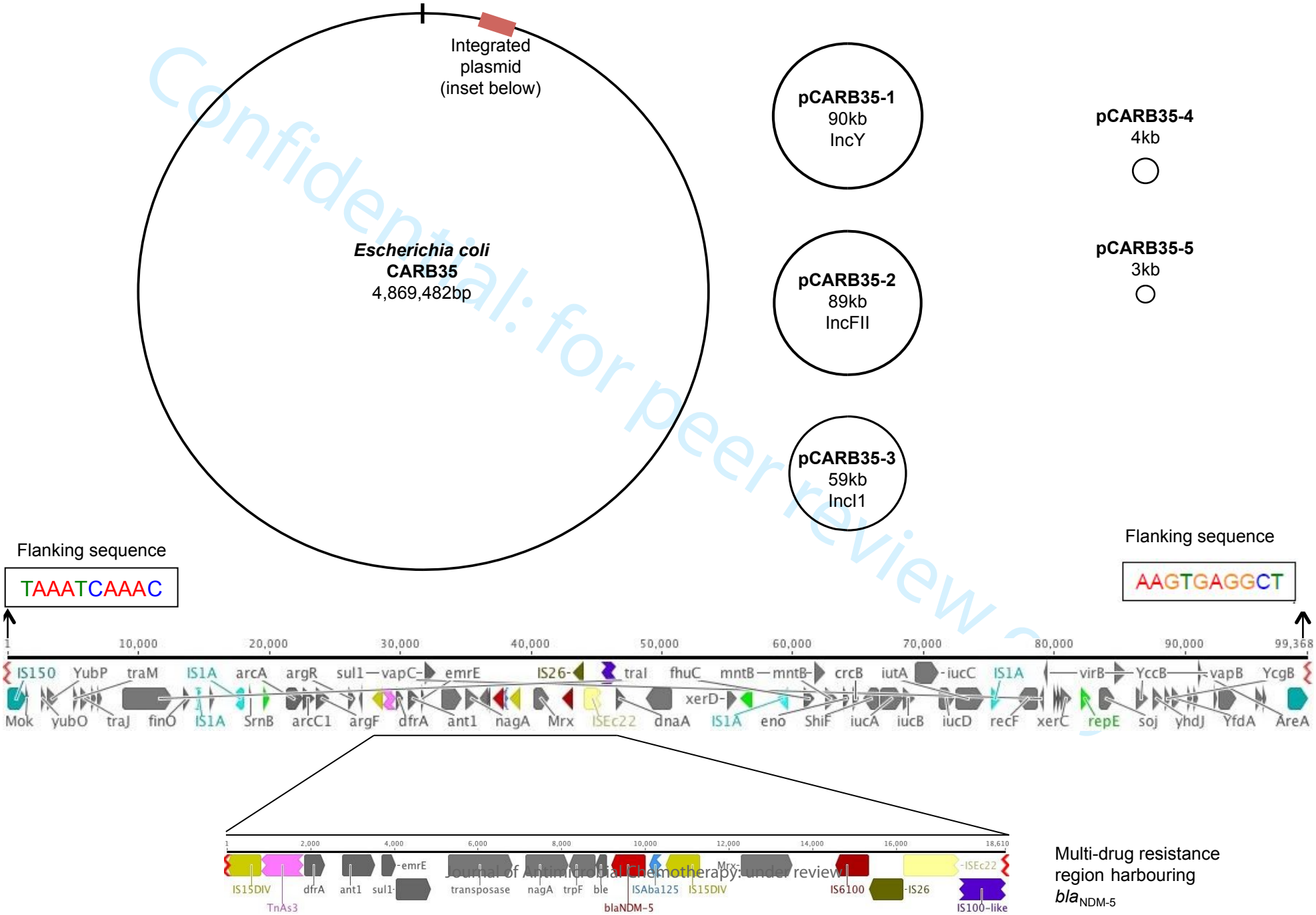
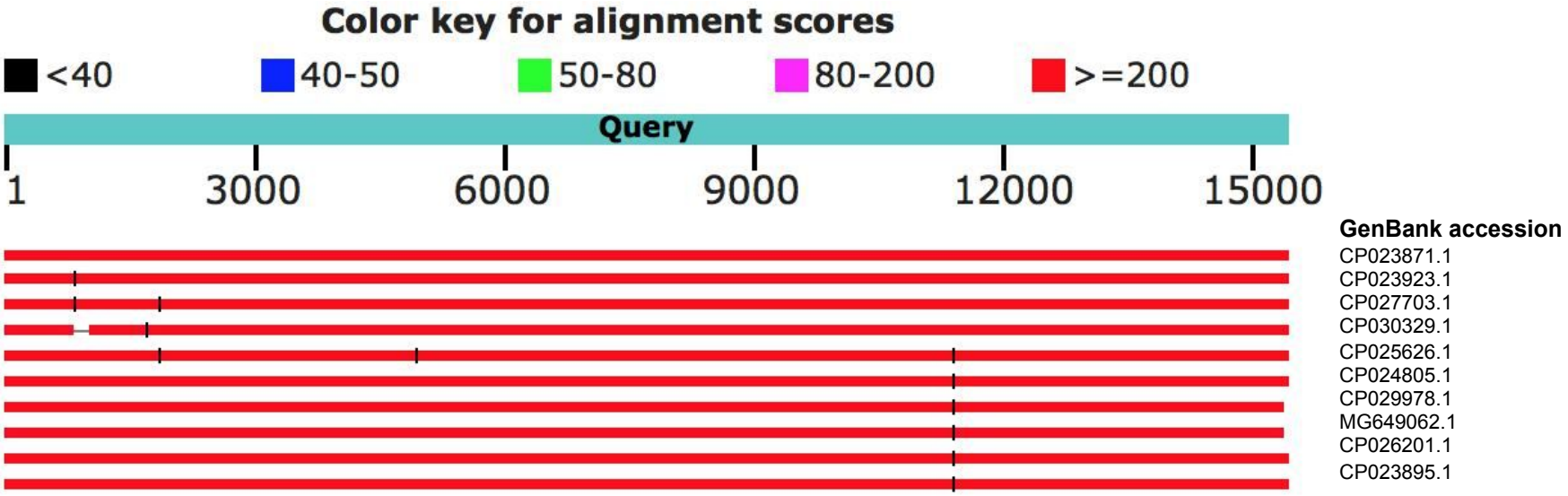
Fig 1: Genomic structure of *Escherichia coli* CARB35, with the bla_{NDM-5}-containing plasmid-like structure integrated into the chromosomal sequence. Insets show the structures flanking the bla_{NDM-5} gene in greater detail, highlighting the presence of multiple other co-localised drug resistance genes and insertion sequences (This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*)



Multi-drug resistance region harbouring *bla*$_{NDM-5}$

Fig S1. Blastn alignments to multi-drug-resistance region of pCARB35-1 (last accessed: 10/Aug/2018) (last accessed: 10/Aug/2018)

Fig S1. Blastn alignments to multi-drug-resistance region of pCARB35-1 (last accessed: 10/Aug/2018) (This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*)



GenBank accession	Strain	Chromosome/Plasmid	Species	Country	Source
CP023871.1	FDAARGOS_434	Plasmid	*E. coli*		Unknown (unpublished)
CP023923.1	FDAARGOS_440	Plasmid	*K. pneumoniae*		Unknown (unpublished)
CP027703.1	675SK2	Plasmid	*E. coli*	Switzerland	Wastewater
CP030329.1	AR_452	Plasmid	*E. coli*		Unknown (unpublished)
CP025626.1	SCEC020007	Plasmid	*E. coli*		Unknown (unpublished)
CP024805.1	AMA1167	Plasmid	*E. coli*		Unknown (unpublished)
CP029978.1	51008369SK1	Plasmid	*E. coli*		Unknown (unpublished)
MG649062.1	Ec001	Plasmid	*E. coli*	Italy	Urine, human PMID: 29501819
CP026201.1	ECONIH6	Plasmid	*E. coli*	USA	Unknown (unpublished)
CP023895.1	FDAARGOS_433	Plasmid	*E. coli*		Unknown (unpublished)