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SHORT RESEARCH AND DISCUSSION ARTICLE

Genomic features of an extensively drug-resistant and NDM-1–positive *Klebsiella pneumoniae* ST340 isolated from river water

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Abstract

The environmental contamination plays a significant role in the emergence of antimicrobial resistance. In this study, we report a genomic analysis of an extensively drug-resistant and bla_{NDM-1}-producing *Klebsiella pneumoniae* (EW807) strain recovered from a surface water sample. Strain EW807 belonged to sequence type (ST) 340 and serotype O4:KL15, a high-risk clone of the clonal group 258. This strain carried a broad resistome, including bla_{NDM-1} and $bla_{CTX-M-15}$. The core genome multilocus sequence typing phylogenetic analysis revealed that the EW807 strain was most related to strains from Brazil and the USA. An IncX3 plasmid was identified harboring the bla_{NDM-1} gene, while an IncFIB(K) plasmid was detected carrying the $bla_{CTX-M-15}$ in addition to multidrug resistance and multimetal tolerance regions. IncX3 and IncFIB(K) plasmids shared high similarity with plasmids from a human in China and a dog in Brazil, respectively. The regions harboring the bla_{NDM-1} and $bla_{CTX-M-15}$ genes contained sequences from the Tn*3* family. These findings suggest that IncX3 plasmid could play a role in the spread of NDM-1 in a post-pandemic scenario. To the best of our knowledge, this is the first report of bla_{NDM-1}-producing *K. pneumoniae* ST340 O4:KL15 strain in the environment. Therefore, the presence of high-risk clones of *K. pneumoniae* carrying carbapenemases in the environment requires strict surveillance.

Keywords *Enterobacterales* · High-risk clone · Extended-spectrum β-lactamase · Metallo-β-lactamase · Plasmid · Environmental contamination

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* strains are listed as priority pathogens by the World Health Organization (WHO) and are among the major pathogens causing healthcare-associated infections worldwide. High-risk clones of *K. pneumoniae* possess various virulence and antimicrobial resistance determinants, leading to a limitation of therapeutic options and an increase in persistent infections (Campos et al. 2020; Antimicrobial Resistance Collaborators 2022). Furthermore, carbapenem-resistant strains typically harbor several other antimicrobial resistance genes (ARGs), spotlighting extended-spectrum β-lactamase (ESBL)-encoding genes, and may even be resistant to colistin, leading to serious public health implications. Worrying, plasmid-mediated carbapenemases threaten the effectiveness of carbapenems, which are first-line agents used to treat severe bacterial infections (Bonomo et al. 2018; Perez-Palacios et al. 2021).

New Delhi metallo-β-lactamase (NDM), encoded by the bla_{NDM} gene, is one of the most widespread metallo-β-lactamase (MBL). Currently, there are more than 50 bla_{NDM} variants with the bla_{NDM-1} gene being the most identified (Naas et al. 2017; Khan et al. 2017). This gene has been described on different plasmids and is most associated with intact or truncated IS*Aba125*, ble_{MBL}, and a set of other genes (Partridge and Iredell 2012; Kopotsa et al. 2019). In Brazil, clinical strains harboring the bla_{NDM} gene have been

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identified in different regions, but this gene is infrequent in environmental strains (Campana et al. 2017; Furlan et al. 2018; Corrêa et al. 2021; Camargo 2022). The transmission risk of carbapenem-resistant strains from surface water to humans can occur through several routes, including recreational activities, drinking water, and food chain. Indeed, the presence of these strains in aquatic ecosystems accelerates their transfer to different sources (Serwecińska 2020; Larsson and Flach 2022). Here, we report a genomic analysis of $bla_{\text{NDM-1}}$- and $bla_{\text{CTX-M-15}}$-positive *K. pneumoniae* ST340 from surface water.

Materials and methods

Between February and March 2020, a study of carbapenem-resistant *Enterobacterales* strains in aquatic ecosystems ($n = 54$) of São Paulo State, Brazil, was conducted. In this context, EW807 strain, the only MBL producer in this investigation, was recovered from a sampling site of Pardo River (20 27 01S 48 27 15W) in a rural area ~ 20 km away from Guaíra City (total area of 362,183 km^2 with 18,606 inhabitants), São Paulo State, using MacConkey agar (Oxoid, UK) plus 4 mg/L of meropenem. In addition to mucoviscous phenotype, the EW807 strain was molecularly identified as *K. pneumoniae* using species-specific (bla_{SHV}) and universal (16S rRNA) targets (Weisburg et al. 1991; Fonseca et al. 2017). MBL production was initially identified using the Carbapenemase Detection Kit (Cecon, Brazil). Then, the bla_{NDM} gene was molecularly confirmed by conventional PCR followed by Sanger sequencing (Peirano et al. 2011). Conjugation assay using *Escherichia* coli J53 resistant to sodium azide as a recipient strain was performed to determine transferability of the bla_{NDM} gene. Plasmid stability was evaluated (Furlan et al. 2023) and plasmid extraction was carried out using QIAGEN® Plasmid Midi Kit (QIAGEN, Germany).

The disk diffusion, broth microdilution, or E-test® methods were used to determine the antimicrobial susceptibility (CLSI 2020; Lima et al. 2022; EUCAST 2020), and the resistance pattern was evaluated as described by Magiorakos et al. (2012). Whole-genome sequencing was carried out using the Illumina MiSeq platform (Illumina Inc., USA) with 251 bp paired-end reads. The draft genome was de novo assembled using SPAdes v.3.15.2. (Bankevich et al. 2012). Resistome, plasmid replicons, and multilocus sequence typing were identified using ResFinder v.4.1, PlasmidFinder v.2.1, and MLST v.2.0, respectively, with default parameters, available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/).

Serotyping, virulome, and metal tolerance genes were determined by Kleborate (https://github.com/katholt/Kleborate) and BIGSdb-Pasteur (https://bigsdb.pasteur.fr/klebsiella/). A phylogenetic tree was constructed based on core genome multilocus sequence typing (cgMLST) scheme using strains available at Pathogenwatch platform (https://pathogen.watch/) and OneBR database (http://onehealthbr.com/) and visualized with iTOL v.6.6 (https://itol.embl.de/).

Nonsynonymous mutations in determinants of colistin resistance were identified in-house using *K. pneumoniae* MGH 78578 (GenBank accession number CP000647) as a reference. Plasmid contigs were predicted by mlplasmids (Arredondo-Alonso et al. 2018), and the scaffolds and gaps were refined and closed, respectively, with BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Geneious Prime® v.2022.2.2. The plasmids were visualized and compared using Easyfig (https://mjsull.github.io/Easyfig/) and BLAST Ring Image Generator (https://brig.sourceforge.net).

Results and discussion

Strain EW807 was recovered from a sampling site that presented good annual water quality index averages during 2016 and 2020 and had a trophic state index at the mesotrophic level (CETESB 2020). These data evidence that an unpolluted river harbored carbapenem-resistant genes and suggest that WHO critical priority pathogens are spreading silently throughout natural environments. This strain was resistant to critically important antimicrobials (e.g., polymyxins, carbapenems, extended-spectrum cephalosporins, fluoroquinolones, and aminoglycosides) but susceptible to aztreonam/avibactam and tigecycline, supporting its classification as extensively drug-resistant (XDR) (Table 1).

Molecular typing showed that EW806 strain belonged to ST340 O4:KL15, a high-risk clone of the clonal group (CG) 258 closely related to multidrug resistance and high pathogenicity. Phylogenetic relatedness among 204 genomes of globally reported ST340 O4:KL15 revealed that the EW807 strain was most related to strains from South America (Brazil) and North America (USA) (Fig. 1). This clone has been recovered mainly from humans and emerged especially in Asia and Europe. Besides, ST340 is mainly associated with ESBL- and carbapenemase-encoding genes, supporting the epidemiological success of XDR strains in human clinical settings. Virulome analysis showed gene-encoded aerobactin (*iutA*), enterobactin (*entB*), and type 3 fimbriae (*mrk*), which are pathogenicity factors usually identified in CG258 strains (Cerdeira et al. 2017; Zhao et al. 2021; Nakamura-Silva et al. 2022).

In addition to $bla_{\text{NDM-1}}$, the EW807 strain also carried acquired ARGs to β-lactams ($bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$), fluoroquinolones [*aac(6′)-Ib-cr*], tetracyclines (*tetA*), aminoglycosides [*aph(3″)-Ib*, *aac(3)-IIa*, *aph(3′)*-VIa, *aph(6)-Id*, *aadA1*, *aadA2*], folate pathway antagonists (*sul1*, *sul2*), phenicols (*catA1*, *catA3*), trimethoprim (*dfrA12*,

Table 1 Antimicrobial susceptibility of EW807 strain

Antimicrobial agent	Antimicrobial susceptibility (MIC mg/L)[1,2]
Colistin	R (8)
Amoxicillin/clavulanate	R (> 256)
Piperacillin/tazobactam	R
Cefazolin	R
Cefoxitin	R
Ceftazidime	R (> 256)
Ceftazidime/avibactam	R (> 256)
Ceftriaxone	R (> 256)
Ceftaroline	R
Cefepime	R (> 256)
Aztreonam	R (128)
Aztreonam/avibactam	S (≤ 1)
Imipenem	R (256)
Meropenem	R (128)
Nalidixic acid	R
Ciprofloxacin	R (> 64)
Gentamicin	R (128)
Amikacin	R
Tetracycline	R (256)
Minocycline	R
Sulfamethoxazole/trimethoprim	R
Chloramphenicol	R (> 256)
Nitrofurantoin	R
Tigecycline	S (1)

All results were interpreted according to CLSI (CLSI 2020), except for tigecycline that used EUCAST (EUCAST 2020) and aztreonam/avibactam that used the guidelines of Lima et al. (2022)

R resistant, *S* susceptible, *MIC* minimum inhibitory concentration

dfrA14), and macrolides [*mph(A)*]. Intrinsic ARGs, including $bla_{\text{SHV-11}}$, *oqxA*, *oqxB*, and *fosA*, were also detected. Known mutations that contribute to increased resistance for cephalosporins (OmpK36: Asn49Ser, Leu59Val, Gly189Thr, Phe198Tyr, Phe207Tyr, Ala217Ser, Thr222Leu, Asp223Gly, Glu232Arg, Asn304Glu), carbapenems (OmpK37: Ile70Met, Ile128Met, Asn230Gly), fluoroquinolones (GyrA: Ser83Ile; ParC: Ser80Ile), and colistin (PmrB: Arg256Gly) were identified. Besides, tolerance genes to copper (*pcoABCDRSE*), arsenic (*arsRDABC*), and silver (*silESRCBAP*) were also found.

Several plasmid replicon types, including IncFII(K1), IncFII(Yp), IncFIA(HI1), IncFIB(K), IncHI2(pST1), IncHI2A IncQ1, IncX3, Col440I, Col440II, ColpVC, and Col(pHAD28), were identified, and ten plasmids were visualized after plasmid extraction. In this context, two plasmids, pEW807-1 and pEW807-2, were identified bearing $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$, respectively, and were maintained for 30 days after daily serial passages in antimicrobial-free culture medium. The $bla_{\text{NDM-1}}$ gene was successfully transferred to the J53 strain. In addition to ARGs, these plasmids also harbored genes related to conjugal transfer, replication, stability, partition, maintenance, metal tolerance, and mobile genetic elements.

The pEW807-1 belonged to IncX3 and was 50,456 bp in length with 47.2% GC content. This plasmid was closely related (99% query coverage and 99.9% nucleotide identity) to pNDM-1-C37 plasmid from a strain of *Enterobacter hormaechei* subsp. *hoffmannii* recovered from a sputum sample in China. IncX3 plasmids are important hosts of carbapenem resistance and are closely related to the spread of bla_{NDM} at the human-animal-environmental interface. In South America, Brazil stands out regarding the presence of IncX3 plasmids associated with carbapenemases (Kopotsa et al. 2019; Guo et al. 2022). The region harboring the $bla_{\text{NDM-1}}$ gene contained a Tn*3*-family unit transposon, named Tn*3000*, with the core sequence composed by IS*3000*-ΔIS*Aba125*-$bla_{\text{NDM-1}}$-ble_{MBL}-*trpF-tat-cutA1-groES*-Δ*groEL-tnpA*, which is linked to the dissemination of $bla_{\text{NDM-1}}$ among *Enterobacteriaceae* from different countries, including Brazil (Fig. 2A) (Partridge and Iredell 2012; Campos et al. 2015).

The pEW807-2, a multidrug-resistant and multimetal-tolerant plasmid, co-harbored $bla_{\text{CTX-M-15}}$, *aadA2*, aac*(3)-IIa*, *sul1*, *dfrA12*, and *mph(A)*, as well as *sil*, *pco*, and *ars* genes. This plasmid belonged to IncFIB(K) and was 163,853 bp in length, containing 52.9% GC content. Comparative analysis showed that pEW807-2 was most similar (99% query coverage and 99.9% nucleotide identity) with pPVT01_P1 in the genome of *K. pneumoniae* ST11/CG258 isolated from an infected dog in Brazil (Fig. 2B) (Sellera et al. 2021). The IncF group is one of the most frequent plasmid incompatibility types and has been commonly reported harboring ESBL-encoding genes with $bla_{\text{CTX-M-15}}$/IncFIB(K) being recently identified in Brazilian birds (Kopotsa et al. 2019; Davies et al. 2022). Class 1 integron (In*27*) with the *dfrA12-orfF-aadA2* cassette array was found. The genetic context of $bla_{\text{CTX-M-15}}$ was ΔIS*26*-ΔTn*3*-ΔIS*26*-$bla_{\text{CTX-M-15}}$-*wbuC*-ΔTn*3*-ΔIS*26*, which shared high nucleotide identity (> 99.9%) with others available at GenBank®.

In general, ST340 O4:KL15 strains are associated with $bla_{\text{CTX-M}}$ and/or $bla_{\text{KPC-2}}$ genes, while $bla_{\text{NDM-1}}$-positive strains are unusual. Although there were reports of clinical ST340 strains harboring bla_{NDM} in Brazil (Aires et al. 2017; Boszczowski et al. 2019; Camargo 2022; Camargo et al. 2022), a complete genomic characterization was lacking. These findings support the expansion of WHO critical priority pathogen related to outbreaks, exhibiting resistance to carbapenems and colistin, and producing $bla_{\text{NDM-1}}$ and ESBL in Brazil (Monteiro et al. 2019; Rodrigues et al. 2021). Despite the high prevalence of $bla_{\text{CTX-M}}$ variants (94%) in all genomes analyzed, only 14% carried the $bla_{\text{NDM-1}}$ gene, being EW807 strain the only one from the environment

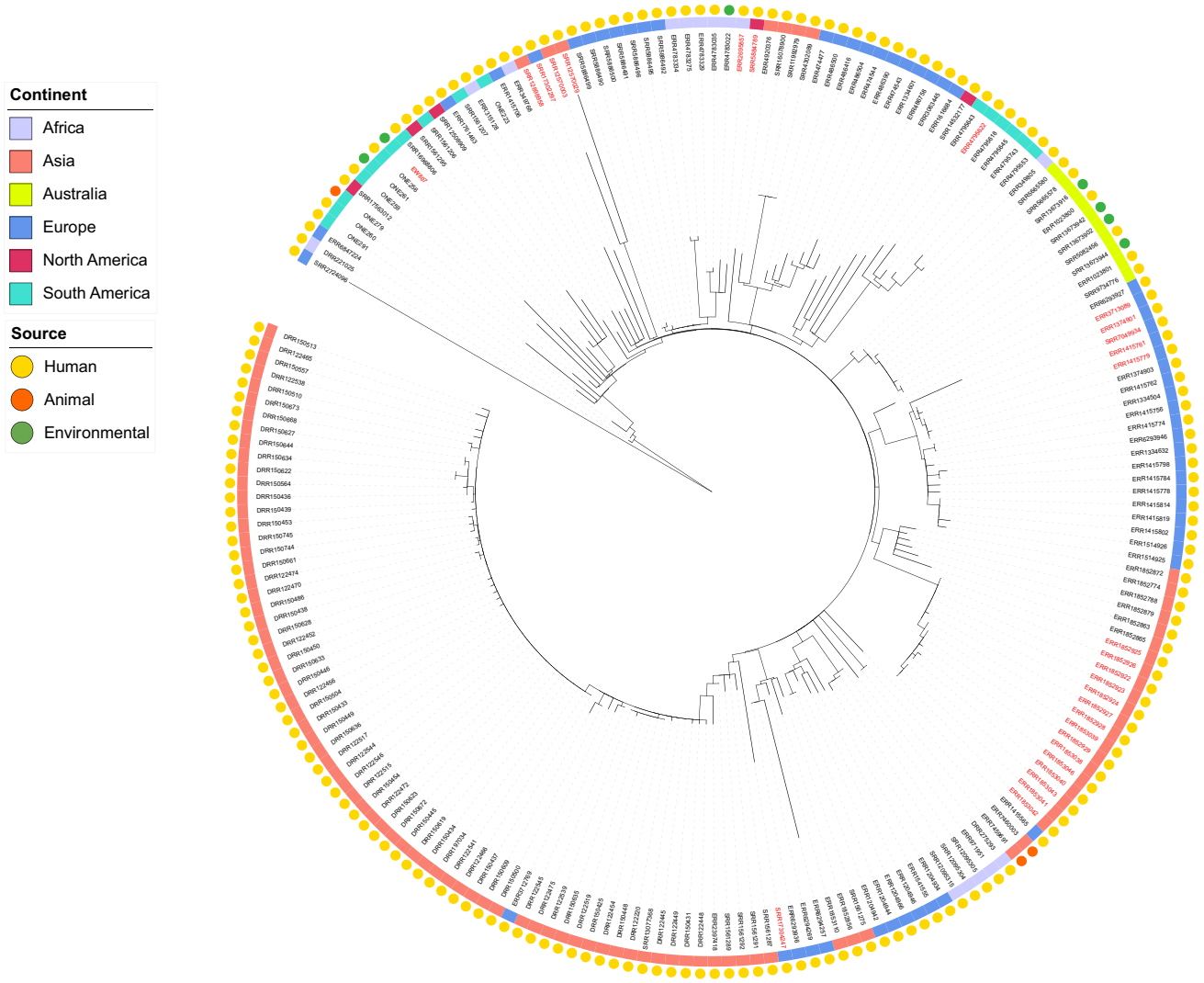

Fig. 1 Phylogenetic tree based on cgMLST of 204 worldwide distributed *K. pneumoniae* strains belonging to ST340 O4:KL15. NDM-1-producing strains are highlighted in red. Strain EW807 (this study; GenBank accession number JAQFWR010000000) is highlighted in bold. Countries of continents: Africa (Kenya, Malawi, Myanmar, Nepal, Nigeria); Asia (Bangladesh, Philippines, Qatar, Singapore, Thailand, United Arab Emirates); Australia (Australia); Europe (Croatia, Czech Republic, Germany, Greece, Ireland, Netherlands, Norway, Serbia, Slovakia. Spain, UK); North America (USA, Canada); South America (Argentina, Brazil, Colombia)

(Fig. 1). Therefore, to the best of our knowledge, this is the first report of $bla_{\text{NDM-1}}$-producing *K. pneumoniae* ST340 O4:KL15 strain in the environment.

Recently, reports showed an increase in coproducing of carbapenemases in Latin America due to the increased use of broad-spectrum antimicrobials during the COVID-19 pandemic (García-Betancur et al. 2021; PAHO 2021; Vásquez-Ponce et al. 2022). In this regard, $bla_{\text{NDM-1}}$-producing strains can donate and/or capture carbapenemase-encoding genes and spread to the environment, which has optimal conditions for long-term harboring and dissemination to multiple sources, raising concerns (Singer et al. 2016). It is important to highlight that the water sample used in this study was collected in the initial period of COVID-19 cases, and therefore, these data may be underestimated.

In summary, our results highlight an unusual association of $bla_{\text{NDM-1}}$ and ST340 O4:KL15 in an XDR environmental strain, evidencing the plasmid transmission of $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ in CG258 and interspecies. Furthermore, the genomic findings provided might be useful for comparative One Health genomic studies of *K. pneumoniae* ST340 O4:KL15, which have emerged from human clinical settings. Therefore, critical priority $bla_{\text{NDM-1}}$-producing pathogen appears to be starting to spread in the environment of Brazil, supporting and alerting for a critical challenge to a post-pandemic scenario.

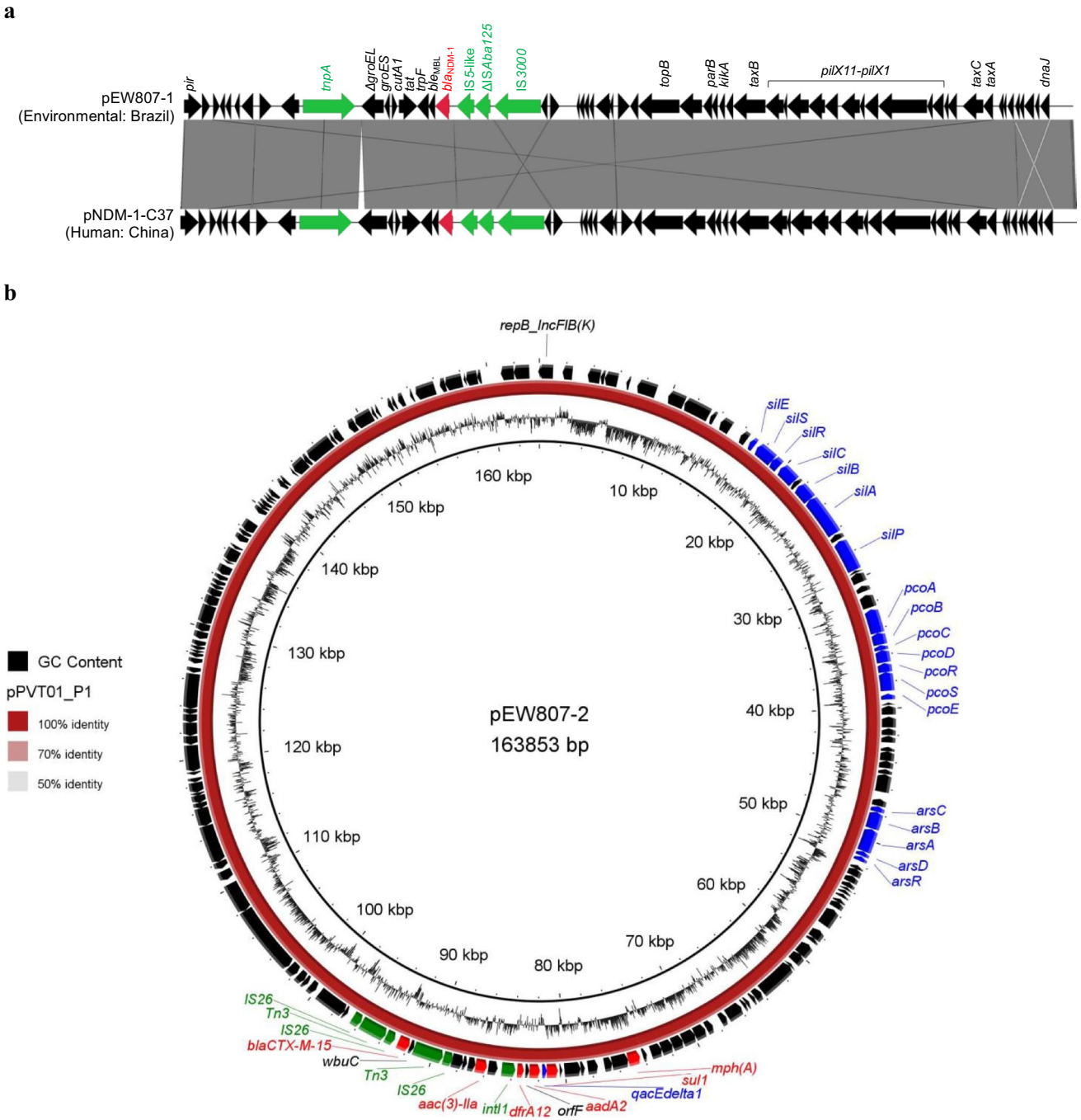

Fig. 2 Plasmids carrying bla_{NDM-1} and $bla_{CTX-M-15}$ in *K. pneumoniae* ST340 O4:KL15. **a** Comparison between IncX3 plasmids carrying the bla_{NDM-1} gene in *K. pneumoniae* from surface water (pEW807-1, GenBank accession number JAQFWR010000001) and *Enterobacter hormaechei* subsp. *hoffmannii* from a human sample (pNDM-1-C37, GenBank accession number MZ667211). Genetic environment of bla_{NDM-1} is highlighted as follows: green, red, and black arrows indicate insertion elements, ARGs, and other genes, respectively. The gray shading represents shared regions of homology. **b** Comparison between IncFIB(K) plasmids harboring multidrug resistance and multimetal tolerance regions in *K. pneumoniae* from surface water (pEW807-2, GenBank accession number JAQFWR010000002) and *Klebsiella pneumoniae* from infected dog (pPVT01_P1, GenBank accession number JABSUB010000001) in Brazil. Multidrug resistance and multimetal tolerance regions are highlighted as follows: green, red, blue, and black arrows indicate genes related to insertion elements, antimicrobial resistance, metal tolerance/biocide resistance, and others, respectively. IS*26* and Tn*3* elements are truncated (i.e., ΔIS*26* and ΔTn*3*)

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Author contribution All authors contributed to the study conception and design. Material preparation, sample collection, and analysis were performed by J. P. R. F., R. d. S. R., M. S. R., L. D. R. d. S., and E. A. S.. The manuscript was written by J. P. R. F. and E. G. S. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

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Competing interests The authors declare no competing interests.

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