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# **Genomic epidemiology of Carbapenemase-Producing Gram-Negative Bacteria at the human-animal-environment interface in Djibouti city, Djibouti**

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## Abstract

The emergence of carbapenem resistance is a major public health threat in sub-Saharan Africa but remains poorly understood, particularly at the human-animal-environment interface. This study provides the first One Health-based study on the epidemiology of Carbapenemase-Producing Gram-Negative Bacteria (CP-GNB) in Djibouti City, Djibouti, East Africa. In total, 800 community urine samples and 500 hospital specimens from humans, 270 livestock fecal samples, 60 fish samples, and 20 water samples were collected and tested for carbapenem resistance. The overall estimated CP-GNB prevalence was 1.9% (32/1650 samples) and specifically concerned 0.3% of community urine samples, 2.8% of clinical specimens, 2.6% of livestock fecal samples, 11.7% of fish samples, and 10% of water samples. The 32 CP-GNB included 19 *Escherichia coli*, seven *Acinetobacter baumannii*, five *Klebsiella pneumoniae*, and one *Proteus mirabilis* isolate. Short-read (Illumina) and long-read (Nanopore) genome sequencing revealed that carbapenem resistance was mainly associated with chromosomal carriage of *bla*<sub>NDM-1</sub>, *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>OXA-66</sub>, and *bla*<sub>OXA-69</sub> in *A. baumannii*, and with plasmid carriage in Enterobacterales (*bla*<sub>NDM-1</sub> and *bla*<sub>OXA-181</sub> in *E. coli*, *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-5</sub> and *bla*<sub>OXA-48</sub> in *K. pneumoniae*, and *bla*<sub>NDM-1</sub> in *P. mirabilis*). Moreover, 17/32 CP-GNB isolates belonged to three epidemic clones: (1) *A. baumannii* sequence type (ST) 1697,2535 that showed a distribution pattern consistent with intra- and inter-hospital dissemination; (2) *E. coli* ST10 that circulated at the human-animal-environment interface; and (3) *K. pneumoniae* ST147 that circulated at the human-environment interface. Horizontal exchanges probably contributed to carbapenem resistance dissemination in the city, especially the *bla*<sub>OXA-181</sub>-carrying ColKP3-IncX3 hybrid plasmid that was found in *E. coli* isolates belonging to different STs. Our study highlights that despite a relatively low CP-GNB prevalence in Djibouti City, plasmids harboring carbapenem resistance circulate in humans, animals and environment. Our findings

stress the need to implement preventive and control measures for reducing the circulation of this potentially emerging public health threat.

**Key words:** emergence; antibiotic resistance; carbapenemase; One Health; Africa

## 1. INTRODUCTION

Antimicrobial resistance (AMR) is one of the most significant global threats to human and animal health (Wall et al., 2016; WHO, 2022). The emergence and dissemination of Carbapenemase-Producing Gram-Negative Bacteria (CP-GNB) pose a major challenge to public health worldwide because carbapenems are regarded as last-resort antibiotics against multidrug-resistant (MDR) Gram-negative pathogens, and because effective therapeutic alternatives (e.g. colistin and ceftiderocol) are not widely available in low-income countries (Gupta et al., 2011; Logan and Weinstein, 2017).

Recently, CP-GNB have also been detected in domestic and wild animals worldwide (Köck et al., 2018; Ahlstrom et al., 2022; Torres et al., 2022), as well as in water and soil (Kizny Gordon et al., 2017; Mahmoud et al., 2020). Thus, global surveillance of CP-GNB using a One Health approach is required, although the surveillance capacity is much lower in low and middle-income countries (Djenadi et al., 2018; Zhao et al., 2021; Muloi et al., 2022; WHO, 2022).

CP-GNB have spread to sub-Saharan Africa, the region with the highest death rate attributable to and associated with AMR (Murray et al., 2022). Despite this worrying situation, little is known about AMR and specifically carbapenem-resistance in most African countries, which often lack effective surveillance programs to monitor the emergence and spread of

resistant bacteria (Mahamat et al., 2021). Most of the available studies on carbapenem resistance in sub-Saharan African countries have been conducted in hospital settings (Mitgang et al., 2018; Ouchar Mahamat et al., 2019; Dikoumba et al., 2022; Manyahi et al., 2022). Conversely, fewer studies have been performed in animals and ecosystems (Subbiah et al., 2020; Mahamat et al., 2021; Muloi et al., 2022; Ramsamy et al., 2022), limiting our understanding of the emergence, circulation and drivers of carbapenem-resistance genes in sub-Saharan Africa.

In Djibouti, few studies on CP-GNB are available (Mohamed et al., 2022), and currently no AMR surveillance network or action plan has been set up to limit the risk for humans, animals and ecosystems. Here, we studied CP-GNB genomic epidemiology in Djibouti City, Djibouti, East Africa. We used a One Health approach to detect and characterize CP-GNB in human (nosocomial and community-acquired infections), livestock, wildlife (fish), and water samples. We combined Illumina and Oxford Nanopore whole genome sequencing (WGS) technologies to assess CP-GNB circulation in these different compartments and to characterize the resistance-encoding determinants in their genetic environment.

## **2. MATERIALS AND METHODS**

**2.1. Ethics statement.** This study was approved by the Djiboutian Ministry of Public Health ethics board (N° 137/DG/INSPD/2023).

### **2.2. Study site and sample collection.**

This study was performed in Djibouti City, the capital of Djibouti, East Africa. Djibouti City has a population of 620,000 inhabitants (approximately 62% of the total population of this country).

Human clinical samples: This study concerned i) all consecutive urine samples from non-hospitalized patients with urinary tract infections sent to the Mer Rouge Medical Biology Laboratory, the main private medical microbiology analysis laboratory in Djibouti City, from 1 January to 31 July 2019 (7 months) (n=800 samples); and ii) all consecutive samples from inpatients with nosocomial infections (i.e. hospitalized for more than 48 hours) sent to the microbiology laboratories of the four main hospitals (Hospitals 1, 2, 3 and 4) of Djibouti City from 1 January to 31 March 2021 (3 months) (n=500 samples; urine, surgical wound, pus, upper and deep respiratory tract, blood, and cerebrospinal fluid samples). These four hospitals have 916 beds (600, 130, 106 and 80 beds in hospital 1, 2, 3 and 4, respectively) distributed in different acute care units (medicine, surgery, gynecology, obstetrics, pediatrics, intensive care unit, and emergency department). From these 1300 clinical samples (800 community urine samples and 500 hospital specimens), 477 non-duplicate and clinically significant bacterial isolates were identified: 327 isolates (41% of 800) from community samples and 150 isolates from hospital samples (30% of 500). Among these 477 bacterial strains, 318 (66.6%) were Gram-negative bacilli and 159 (33.3%) were Gram-positive cocci (*Enterococcus* spp., *Streptococcus* spp. and *Staphylococcus* spp.) (Supplementary table 1).

Animal (livestock and wild fish) and community septic tank samples: from 1 January to 31 March 2021, fresh fecal samples were collected from 270 livestock individuals (180 cows, 30 goats, 30 sheep, and 30 dromedaries) in the main livestock market of Djibouti City. In addition, 60 fish individuals from ten different fishmongers were collected at the main fish market of Djibouti City. All fish were caught at approximately 100 meters off the coast from the fish market. Twenty water samples (100 ml/each) from 20 community septic tanks (one sample per site over the 3-month study period) were also collected.

### **2.3. Sample processing.**

Briefly, 0.5g of each fresh fecal sample (livestock) was suspended in 5ml of sterile saline solution (0.9%), and 100 µl aliquots were plated on ChromID® CARBA SMART selective chromogenic medium bi-plates (bioMérieux, Marcy-l'Etoile, France), a medium used for CP-BGN screening. Fishes were kept in sterile sealed bags, transported to the laboratory in an ice box, and analyzed immediately. From each fish, a fragment of liver, kidney and spleen was homogenized in 225 mL of alkaline peptone water with 2% w/v NaCl, pH 8.5 for 60 s using a laboratory blender (BagMixer 400W, Interscience, Saint-Nom-la-Bretèche, France) in sterile conditions. Each homogenate was placed in a tube containing 10 ml of Luria-Bertani (LB) broth and incubated at 37° C overnight. Water samples were collected aseptically in sterile bottles and transported in an icebox at 4° C to the laboratory where they were processed within 3 h after collection. Water samples were filtered through 0.45-µm pore size nitrocellulose filters that were then deposited in 10 ml of LB broth and incubated at 37°C overnight. Then, 50 µl of each LB culture (filter and fish samples) was plated on ChromID® CARBA SMART selective chromogenic medium bi-plates (bioMérieux, Marcy-l'Etoile, France).

### **2.4. Bacterial identification, antimicrobial susceptibility testing, carbapenemase detection and prevalence estimations.**

Bacterial species identification and drug susceptibility testing were performed using all clinically significant bacterial isolates from community and hospital human samples, as well as all bacterial isolates identified on ChromID® CARBA SMART selective chromogenic medium bi-plates (bioMérieux, Marcy-l'Etoile, France) for each livestock, fish and water sample. Bacterial species were identified by Matrix-Assisted Laser Desorption Ionization–Time Of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany). Antimicrobial susceptibility was tested with the disk diffusion method on Müller-Hinton agar. The following

antibiotics were tested: amoxicillin (20 µg), amoxicillin-clavulanic acid (20/10 µg), aztreonam (30 µg), cefepime (30 µg), cefotaxime (5 µg), cefpodoxime (10 µg), ceftazidime (10 µg), piperacillin (30 µg), piperacillin-tazobactam (30/6 µg), ticarcillin (75 µg), ticarcillin-clavulanic acid (75/10 µg), imipenem (10 µg), ertapenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg), netilmicin (10 µg), tobramycin (10 µg), fosfomycin (200 µg), chloramphenicol (30 mg), and trimethoprim-sulfamethoxazole (30 µg). Results were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines and clinical breakpoints ([http://www.eucast.org/clinical\\_breakpoints](http://www.eucast.org/clinical_breakpoints)). Potential CP-GNB isolates were confirmed using the E-test method (bioMérieux, Marcy-l'Etoile, France) and the minimum inhibitory concentrations (MIC) for imipenem, meropenem, doripenem (for *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacterales) and ertapenem (for Enterobacterales) were determined. Carbapenemases were identified using the KPC/MBL and OXA-48 Confirm kit (Roso Diagnostica A/S, Taastrup, Danmark) and the NG Test Carba 5 immunoassay (NG Biotech, Guipry, France). In addition, extended spectrum beta-lactamase production, which might be associated with CP-GNB, was detected with the combined double-disk synergy method, and the MIC of colistin was determined in liquid medium using the UMIC Colistine<sup>®</sup> kit (Biocentric, Bandol, France).

CP-BGN prevalence among clinical human samples was estimated as the number of non-redundant and clinically significant samples harboring at least one CP-BNG isolate among all clinical samples/patients tested. CP-BGN prevalence among livestock, fish and water samples was estimated as the total number of individuals/water samples harboring at least one CP-BNG among all individuals/water samples collected.



## **2.5. Whole genome sequencing, *de novo* assembly, sequence type, and resistance gene identification.**

Short-read (Illumina) and long-read (Nanopore) Illumina (short-reads) WGS was performed by MicrobesNG (Birmingham, AL) for all confirmed CP-GNB isolates following a standardized protocol described elsewhere ([https://microbesng.com/documents/24/MicrobesNG\\_Sequencing\\_Service\\_Methods\\_v20210419.pdf](https://microbesng.com/documents/24/MicrobesNG_Sequencing_Service_Methods_v20210419.pdf)). A hybrid assembly was obtained using both the short- and long-read sequencing data for all samples and Unicycler (v0.5.0) (Wick et al., 2017) with default parameters. The genome assembly completeness was evaluated with BUSCO v5.3.2 (Simão et al., 2015; Manni et al., 2021). Sequence types (ST) were identified using the MLST software (<https://github.com/tseemann/mlst>). Plasmids were described using PlasmidFinder 2.1 (<https://cge.food.dtu.dk/services/PlasmidFinder>) to identify incompatibility groups (Inc). For plasmids without known Inc groups the description was completed with Platon 1.6 (Schwengers et al., 2020). Antibiotic resistance genes were identified using NCBI Antimicrobial Resistance Gene Finder (AMRFinderPlus) (<https://github.com/ncbi/amr/wiki>). The raw sequence data and whole genome assemblies (only one representative genome assembly per epidemic clone) generated in the current study are available in the European Nucleotide Archive (ENA) under Project ID PRJEB58617.

## **2.6. Variant calling**

For isolates belonging to the same bacterial species and ST, variant calling was performed. A *de novo* assembled genome was randomly selected from our dataset to serve as reference genome. Illumina reads of bacterial isolates with the same ST were aligned to the selected reference genome using the Burrows-Wheeler Aligner (BWA) 0.7.17 (Li and Durbin, 2009). Reads were sorted using samtools 1.15.1 (Li, 2013) and duplicate reads were tagged

with picard 2.26.10 (<https://broadinstitute.github.io/picard/>). Freebayes 1.3.6 (Garrison and Marth, 2012) was used for variant calling with the following parameters: alignments with a quality *--min-mapping-quality* <30, a depth *--min-coverage* <4, and alleles with a quality *--min-base-quality* <30 were excluded from the analysis. Genotypes with a conflicting base fraction >10% also were excluded. For each ST, the identified variants were grouped into a Variant Call Format (VCF) type file and were used to generate an unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree with the poppr 2.9.3 R package. PLINK v1.07 (Purcell et al., 2007) was used to calculate the number of Single Nucleotide Variants (SNVs) that separated all the pairs of genomes of CP-GNB with the same ST.

## **2.7. Exchanges of plasmids and other mobile genetic elements (MGE)**

Plasmid replicons belonging to the same Inc group were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (<https://github.com/ncbi/pgap>) and then aligned using BLAST Ring Image Generator (BRIG) 0.95 (Alikhan et al., 2011) to assess their degree of genetic identity. Replicons (chromosomes or plasmids) carrying identical carbapenem-resistance genes were aligned in pairs using Blastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Replicons sharing identical genomic portions (>99%) were annotated with PGAP. The shared genomic portions were plotted using the R package genoPlotR 0.8.11 (Guy et al., 2010).

## **3. RESULTS**

### **3.1. CP-GNB prevalence and antibiotic-resistance phenotypes**

Over the 10-month study period (January to July 2019 and January to March 2021), 1650 samples (1300 human, 270 livestock, 60 wild fish, and 20 community septic tank samples) were

analyzed. Among the 477 bacterial strains isolated from the 1300 human samples, 318 (66.6% of 477) were Gram-negative bacilli [*Enterobacteriaceae* (n = 266; 83.6%) and Gram-negative non-fermenting bacilli (n = 52; 16.4%)] and 159 (33.3%) were Gram-positive cocci (*Enterococcus* spp., *Streptococcus* spp. and *Staphylococcus* spp.).

From the 350 livestock/fish/septic tank samples cultured on ChromID<sup>®</sup> CARBA SMART selective chromogenic medium (bioMérieux, Marcy-l'Etoile, France), 134 (38.3% of 350) non-redundant and mono-microbial (only one isolate type present and identified per cultured sample) cultures were positive (Supplementary table 1). All these 134 bacterial strains were Gram-negative bacilli: *Enterobacteriaceae* (n = 126; 94%) and Gram-negative non-fermenting bacilli (n = 8; 16%) (Supplementary table 1).

Among the 452 Gram-negative bacteria (27.4% of 1650 samples) isolated (318 from human and 134 from livestock, fish and septic tank samples), the overall CP-GNB prevalence was 1.9% (32/1650; 95% confidence interval [CI]: 1.3–2.6) based on the EUCAST guidelines, clinical breakpoints, and phenotypic and molecular criteria. The highest CP-GNB prevalence was estimated in wild fish samples (11.7%; 7/60; 95% CI: 3.5–19.8), followed by community septic tank samples (10.0%; 2/20; 95% CI: 0.00–23.15). CP-GNB prevalence was lower in livestock (2.6%; 7/270; 95% CI: 0.7–4.5) and human (1.2%; 16/1300; 95% CI: 0.6–1.8) samples. Among human samples, a significant difference was observed (Chi-square 16.4588; p <0.001) between community urine (0.3%; 2/800; 95% CI: 0.0–0.6) and hospital clinical specimens (2.8%; 14/500; 95% CI: 1.4–4.3).

CP-GNB species were mainly identified as *Escherichia coli* (59.4%; 19/32), isolated from community urine and hospital clinical, livestock, wild fish and community septic tank samples (Fig. 1), followed by *A. baumannii* (21.9%; 7/32), isolated only from hospital clinical specimens, *Klebsiella pneumoniae* (15.6%; 5/32), isolated from hospital clinical specimens and

wild fish, and *Proteus mirabilis* (3.1%; 1/32), isolated from a hospital clinical specimen. All drug susceptibility test results for *A. baumannii* and *Enterobacteriaceae* are summarized in Supplementary table 2, Fig. S1.

### 3.2. Genome assemblies

High quality *de novo* genomic assemblies were recovered for 31 isolates. One isolate was excluded due to carriage of chromosomal contigs of two distinct species (*E. coli* and *A. baumannii*). A single circular chromosomal contig was obtained for most bacterial genomes (25/31), while chromosomes of other genomes were fragmented in two (5/31) or six (1/31) contigs. Assembly completeness ranged from 98.7 to 100% according to the BUSCO score.

### 3.3. AMR genetic determinants in CP-GNB

In total, 82 acquired genes conferring resistance to 12 antibiotic families were identified (4 to 33 different genes in each isolate) (Supplementary table 2). Moreover, 13 chromosomal point mutations conferring resistance to beta-lactams, quinolones, fosfomycin and colistin were identified in 22 isolates. All CP-GNB isolates carried at least one carbapenemase-encoding gene (Fig. 2). Two genes encoding New Delhi metallo- $\beta$ -lactamases (NDM) were identified: *bla*<sub>NDM-1</sub> (n=10) and *bla*<sub>NDM-5</sub> (n=4). Five genes encoding oxacillinases (OXA) known to hydrolyze carbapenems also were detected: *bla*<sub>OXA-181</sub> (n=17), *bla*<sub>OXA-23</sub> (n=7), *bla*<sub>OXA-66</sub> (n=6), *bla*<sub>OXA-48</sub> (n=1), and *bla*<sub>OXA-69</sub> (n=1). Two chromosomal point mutations conferring resistance to carbapenems were identified: *ftsI\_N337NYRIN* (n=2) and *ompK36\_D135DGD* (n=3). In addition to carbapenem-resistance genetic determinants, all isolates carried other resistance genes or chromosomal point mutations that confer resistance to at least another beta-lactam and at least two other antibiotic families (Fig. 2; Supplementary table 2).

### 3.3. Sequence Types of CP-GNB isolates

Nine different STs were identified in *E. coli* isolates (n=19) (Fig. 1 and 2), including a new ST (ST13464) that displays a novel allele on the *mph* marker (1291). ST10 was the most prevalent ST in *E. coli* (n=8). *K. pneumoniae* isolates (n=5) belonged to ST147 (n=4) or ST383 (n=1). *A. baumannii* isolates (n=7) belonged to ST1697,2535 (n=6) or ST231,1604 (n=1). These two ST had duplicated *gdhB* genes with two distinct alleles: *gdhB*-3,189 on ST1697,2535 and *gdhB*-4,182 on ST231,1604. No ST was defined for *P. mirabilis* (n=1) due to the absence of a MLST scheme for this species.

### **3.4. Identification of epidemic clones**

To provide evidence for potential clonal expansion, the genetic distances between isolates of the same ST were calculated by variant calling for each of the most common ST: *E. coli* ST10 (n=8), *K. pneumoniae* ST147 (n=4), and *A. baumannii* ST1697,2535 (n=6). The eight *E. coli* ST10 isolates, were obtained from a patient with a nosocomial infection in hospital 3 (n=1), one home septic tank (n=1), three livestock samples (n=3), and three fish samples (n=3) (Figs 1 and 2). They were separated by 18 SNVs in total (Fig. 2), with median and maximum pairwise distances of 7 and 12 SNVs, respectively. These very low genetic distances (3.8 SNVs/Mbp) are consistent with a recent spread of this epidemic clone in these three compartments. Similarly, three of the four *K. pneumoniae* ST147 isolates (one nosocomial infection sample and two fish samples) were separated by only four SNVs (7.4 SNVs/Mbp) and constituted the second epidemic clone (Fig. 2). The fourth *K. pneumoniae* isolate (60535-630H) exhibited much higher genetic distance (409 SNVs) from the other three isolates. The six *A. baumannii* ST1697,2535 isolates were separated by 27 SNVs in total (6.7 SNVs/Mbp) and displayed median and maximum pairwise distances between isolates of 9 and 14 SNVs, respectively. CP-GNB isolates belonging to the same local epidemic clone carried identical AMR genetic determinants.

### 3.6. Genetic environment of drug resistance genes in CP-GNB isolates

On average, CP-GNB isolates carried three distinct plasmids [range: 1-6]. In *A. baumannii* isolates, all acquired AMR genes, including carbapenemase-encoding genes, were carried by chromosomes, except the single *A. baumannii* ST231,1604 (60540-48h) isolate that carried a *bla*<sub>OXA-23</sub> copy on its chromosome and another on the plasmid replicon p60540-48h-OXA-23 (Fig. 2). In Enterobacterales, all carbapenemase-encoding genes and most acquired AMR genes (77%) were carried by plasmids, many of which were multi-replicon plasmids (Fig. 2; Supplementary table 2). Most *E. coli* isolates (16/18), including the *E. coli* ST10 local epidemic clone, carried the ColKP3-IncX3 hybrid replicon that was always associated with the carbapenemase-encoding gene *bla*<sub>OXA-181</sub> and the quinolone resistance gene *qnrS1* (Fig. 3a and 3b). One *E. coli* isolate (60562-22cv) harbored a similar plasmid backbone and AMR genes, but lacked the ColKP3 replicon. In one *E. coli* isolate (60533-321H), all five *K. pneumoniae* (60535-630H, 60545-306h, 60546-50h, 60550-6p and 60552-5p) and the single *P. mirabilis* isolate (60538-27h), carbapenemase-encoding genes were mostly carried by MDR plasmids. Each of these plasmids carried a single carbapenemase-encoding gene and 13 to 17 genes conferring resistance to seven or eight antibiotic families. *K. pneumoniae* 60546-50h (ST383) harbored a MDR IncFIB(pNDM-Mar)-IncHI1B(pNDM-Mar) hybrid plasmid that carried *bla*<sub>NDM-5</sub> and also a IncL plasmid (63.5-kb) that carried *bla*<sub>OXA-48</sub> without other resistance genes.

### 3.7. Horizontal spread of plasmids

CP-GNB belonging to different species or to different ST within the same species carried identical carbapenemase-encoding genes, either on the same plasmid replicon (Inc type) or on different replicon types (Fig. 2), suggesting the possible dissemination of these genes by horizontal transfer. To investigate this hypothesis, the sequences of plasmids with identical Inc groups and carrying the same carbapenemase-encoding gene were compared. The ColKP3-

IncX3 hybrid plasmids (including the similar IncX3 plasmid lacking the ColKP3 replicon) were widespread among *E. coli* isolates because they were identified in eight distinct *E. coli* STs in this study (including the ST10 clone) (Fig. 3a and 3b). BRIG analyses using the *E. coli* 60551-10p\_ColKP3-IncX3 plasmid as reference sequence revealed ~100% of DNA identity among these plasmids (Fig. 3a). Eleven SNVs (213.7 SNVs/Mbp) were identified by comparing these plasmids through variant calling using the same reference sequence (Fig. 3b). BRIG analyses using again the *E. coli* 60551-10p\_ColKP3-IncX3 plasmid as reference sequence revealed ~100% of nucleotide identity with other ColKP3-IncX3 plasmids from human (KP400525.1, MG893567.1, OK558605.1, KX523903.1, AP018554.1, MN792919.1, MK412915.1, CP024806.1), livestock (KX894452.1), pet (MK416154.1) and food samples (KT005457.1) collected in Asia and Europe (Supplementary table 2, Fig. S2).

### 3.8. Exchanges of other mobile genetic elements

Horizontal spread of other MGE was investigated by pairwise BLAST comparison of the *de novo* assembled contigs (chromosome or plasmid sequences) that carried identical carbapenemase-encoding genes. The *E. coli* isolate 60533-321H (ST38) from hospital 1 and the *K. pneumoniae* isolate 60535-630H (ST147) from hospital 2 harbored the p60533-321H\_NDM-1, a 127.3-kb IncFIA(HI1)-IncR hybrid plasmid, and the p60535-630H\_NDM-1, a 81.5-kb IncFIB(pQil) plasmid, respectively. Both plasmids carried the carbapenemase-encoding gene *bla*<sub>NDM-1</sub>. Extensive sequence identity (100.0%) was observed between these plasmids over 25.3-kb (which included *bla*<sub>NDM-1</sub>), although gene synteny was different (Fig 3c).

The *bla*<sub>NDM-5</sub> gene was detected in the three *K. pneumoniae* ST147 isolates belonging to an epidemic clone (including the *K. pneumoniae* isolate 60545-306h from hospital 3) and also in the single *K. pneumoniae* ST383 isolate (hospital 3) (Fig. 3c). In the 60545-306h isolate, *bla*<sub>NDM-5</sub> was carried by the p60545-306h\_NDM-5, a 298.7-kb multi-replicon plasmid that

harbored IncFIB(pNDM-Mar), IncHI1B(pNDM-Mar) and IncR replicons. In the *K. pneumoniae* isolate 60546-50h, *bla*<sub>NDM-5</sub> was carried by the p60546-50h\_NDM-5, a 374.5-kb IncFIB(pNDM-Mar)-IncHI1B(pNDM-Mar) hybrid plasmid. The two plasmids (Fig. 3d) showed 99.99% of identity over 265.9 kb (i.e. most of their length).

#### 4. DISCUSSION

CP-GNB circulation across the human-animal-environment interface remains poorly understood in the sub-Saharan African region. This study provides the first data on CP-GNB epidemiology in Djibouti City, Djibouti. CP-GNB were identified in human samples (community urine and hospital clinical specimens) and also in livestock, wild fish and water samples, indicating their wide distribution in Djibouti City despite relatively low levels of prevalence.

Using high-resolution WGS, this study provides evidence on the circulation of at least three CP-GNB epidemic clones in Djibouti City that belong to three different species: *A. baumannii* ST1697,2535, *E. coli* ST10, and *K pneumoniae* ST147. *A. baumannii* ST1697,2535 was previously detected only once in a blood sample from a hospitalized patient in Bengaluru, India (isolate G20250459; <https://pubmlst.org/organisms/acinetobacter-baumannii>), and carried the same carbapenemase-encoding genes as the epidemic clone from Djibouti City on its chromosome: *bla*<sub>NDM-1</sub>, *bla*<sub>OXA-23</sub> and *bla*<sub>OXA-66</sub>. The *A. baumannii* ST1697,2535 epidemic clone from Djibouti City found in this study was involved in intra-hospital and inter-hospital transmission in three of the four investigated hospitals, highlighting its dissemination among different hospitals. *E. coli* ST10 has been reported worldwide (Zong et al., 2018), and has been frequently isolated in East Africa from different sources (Büdel et al., 2020; Muloi et al., 2022; Milenkov et al., 2023). However, *E. coli* ST10 isolates displaying carbapenemases were seldom



reported and were mainly displaying NDM-encoding genes rather than OXA-encoding genes (Zhang et al., 2017; Paveenkittiporn et al., 2021). In the present study, the MDR *E. coli* ST10 displaying ColKP3-IncX3-bla<sub>OXA-181</sub> was the most widespread clone because it was detected in all three One Health compartments investigated (humans, environment and animals, including fish). To our knowledge, this is the first report showing the concomitant circulation of a carbapenem-resistant clone among humans, animals and water in Africa. *K. pneumoniae* ST147 is considered as an emerging high-risk clone worldwide that has acquired plasmids with various carbapenemases during the late 2000s (Peirano et al., 2020). It is endemic in India, Italy, Greece and North African countries where it is an important AMR pathogen. *K. pneumoniae* ST147 has been isolated from a variety of human clinical specimens and animal and environmental samples (Ovejero et al., 2017; Rojas et al., 2017; Suzuki et al., 2020; Zhang et al., 2019), highlighting its ubiquitous circulation. *K. pneumoniae* ST147 isolates displaying NDM-encoding genes and particularly bla<sub>NDM-5</sub> have also been isolated in different countries worldwide (Shin et al., 2016; Rojas et al., 2017; Sonnevend et al., 2017; Takeuchi et al., 2018; Turton et al., 2019). In our study, this clone was isolated from a hospital clinical specimen and from fish samples collected at the local market. The concomitant circulation of the same carbapenem-resistant clone in hospitals, community and fish (*E. coli* ST10) or in hospitals and fish (*K. pneumoniae* ST147) suggests that both hospitals and community wastewater might contaminate surface water sources with CP-GNB because fish is considered a water contamination sentinel (Mills and Lee, 2019). In Djibouti City, untreated or insufficiently treated wastewater (hospitals and community) is discharged into the sea (Ahmed et al., 2017) and this could facilitate the surface water contamination by CP-GNB or MGE that harbor resistance genes.

Comparison of the high-quality replicon sequences obtained by combining short and long reads showed that carbapenemase-encoding genes are also spreading through intra- and

inter-species horizontal transfers of MGE. Besides clonal propagation, these horizontal exchanges might contribute to carbapenem-resistance dissemination within and among hospitals and at the human-animal-environment interface in the city. First, the ColKP3-IncX3-*bla*<sub>OXA-181</sub> plasmid detected in most of our *E. coli* isolates showed very little genetic variability also when compared with published sequences worldwide. This limited our ability to distinguish between local horizontal transmission and multiple introductions from abroad. The ColKP3-IncX3-*bla*<sub>OXA-181</sub> plasmid has been repeatedly detected in Africa, Asia and Europe and has been isolated from human, animal and environment samples, pinpointing its ubiquitous circulation (Zurfluh et al., 2015; Ouédraogo et al., 2016; Pulss et al., 2017; Nigg et al., 2019). Future studies should determine the fitness patterns that drive the dissemination of this emergent, highly successful plasmid. Second, our analyses of intraspecies and interspecies exchanges of MGE carrying carbapenemase-encoding genes suggested the transfer of large genetic segments between some plasmids in Enterobacterales isolated from hospital samples. Genetic exchanges between the *K. pneumoniae* plasmids p60545-306h\_NDM-5 and p60546-50h\_NDM-5 from two patients in hospital 3 might have contributed to *bla*<sub>NDM-5</sub> dissemination in this hospital. Likewise, the genetic homology observed between *E. coli* p60535-630H\_NDM-1 from hospital 1 and *K. pneumoniae* p60535-630H\_NDM-1 from hospital 2 might be the result of MGE transfers between these plasmids that then mediated *bla*<sub>NDM-1</sub> transfer. These two isolates were from different hospitals, suggesting the inter-hospital spread of the donor bacterium before MGE exchanges or of the recipient bacterium after MGE exchanges. The previously described important genomic plasticity among these plasmids could play an important role in carbapenem resistance epidemiology in Enterobacterales (Stoesser et al., 2020).

## 5. CONCLUSIONS

CP-GNB were isolated from a variety of sources and locations in Djibouti City. Genomic analyses revealed the absence of efficient barriers to CP-GNB circulation within and between hospitals, and at the human-animal-environment interface in the city. CP-GNB circulation at the human-animal-environment interface occurred not only at the community level, but also in hospital settings that might have played an important role in CP-GNB spread. Genomic analyses revealed the complex epidemiology of CP-GNB in Djibouti City where carbapenem-resistance genes are spreading through clonal propagation, horizontal transfer of plasmids and other MGE. These findings highlight the challenge of controlling the dissemination of these clinically relevant bacteria. Our findings suggest that several strategies are required to AMR circulation, such as improving hand hygiene and other infection-control practices, extensive environmental remediation, antibiotic stewardship and AMR awareness campaigns. As inter-hospital dissemination is mainly attributed to transfer from colonized patients (Vila et al., 1999; Naas et al., 2006), preventive measures should be reinforced and the receiving hospitals should be informed about patients who are colonized or infected with AMR bacteria. Hospital and community wastewater should be properly managed. The available water safety plans also can contribute to reduce the risk of water contamination by AMR bacteria (Bartram, 2009; Organization, 2015). Future studies should determine whether animal and environment act as CP-GNB reservoirs or are just contaminated compartments.

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**Declaration of competing interest**

The authors declare they do not have known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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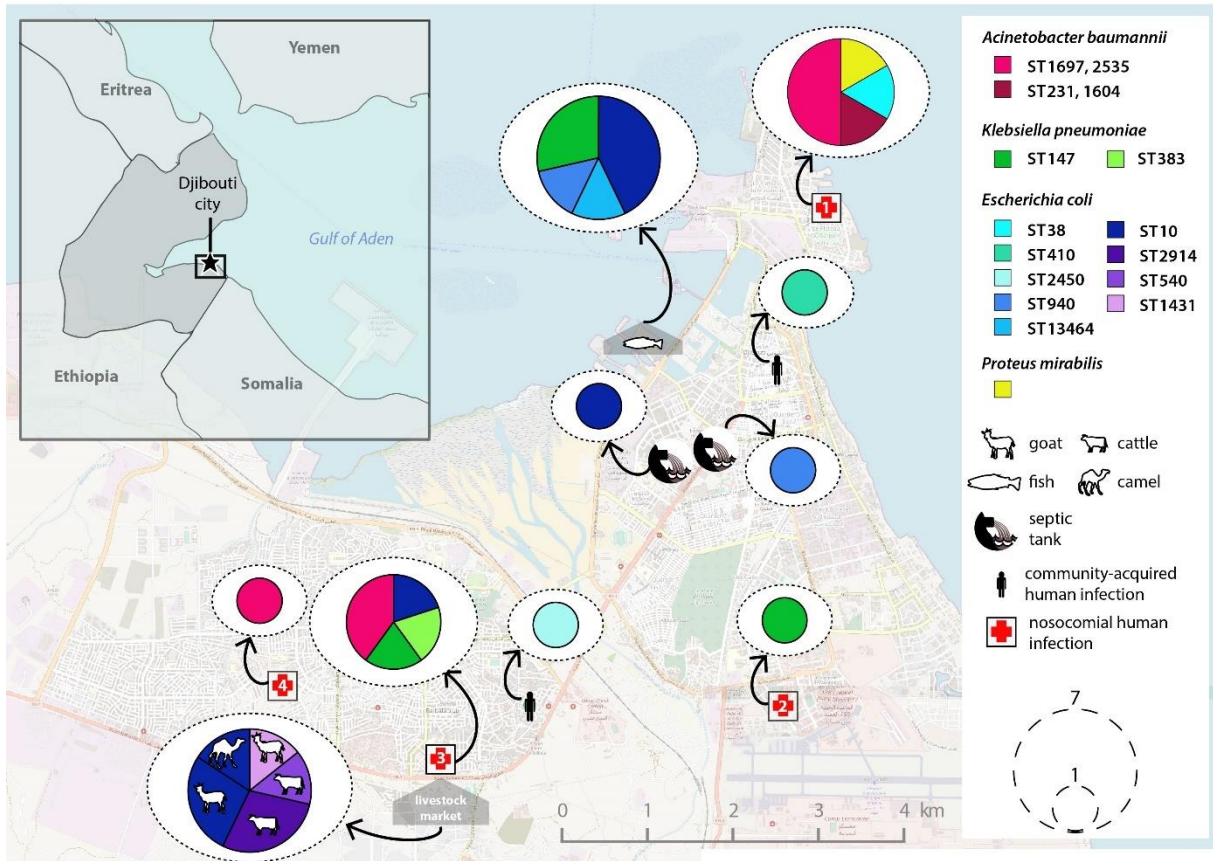
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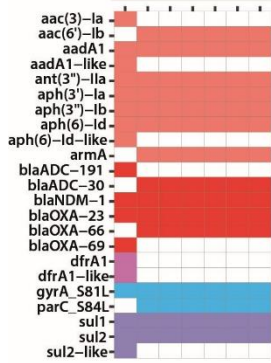
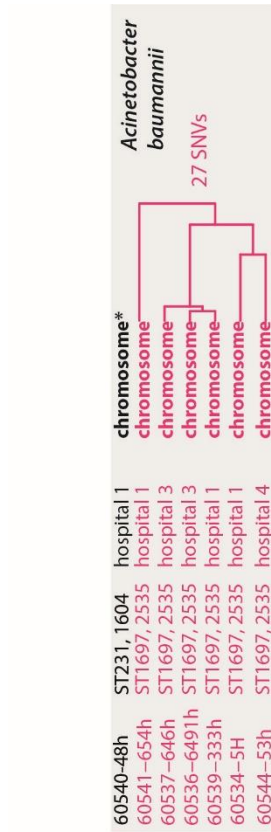
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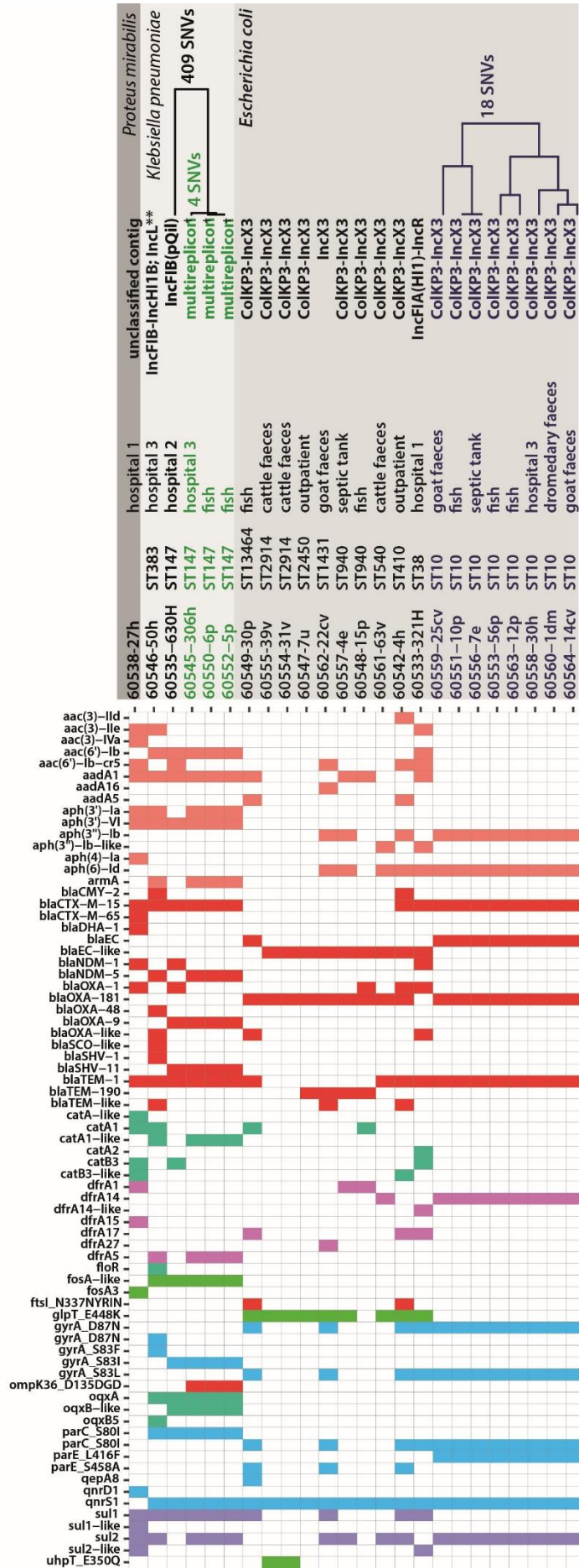
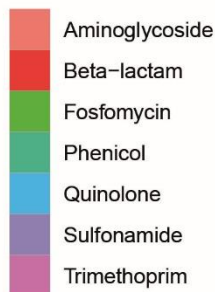


**Fig. 1. Map of the isolates identified as carbapenem-producing Gram-negative bacteria (CP-GNB) in Djibouti City.** The pie chart size reflects the total number of CP-GNB isolates at each location (hospitals, community, or market), and colors indicate the different species and sequence types (ST).





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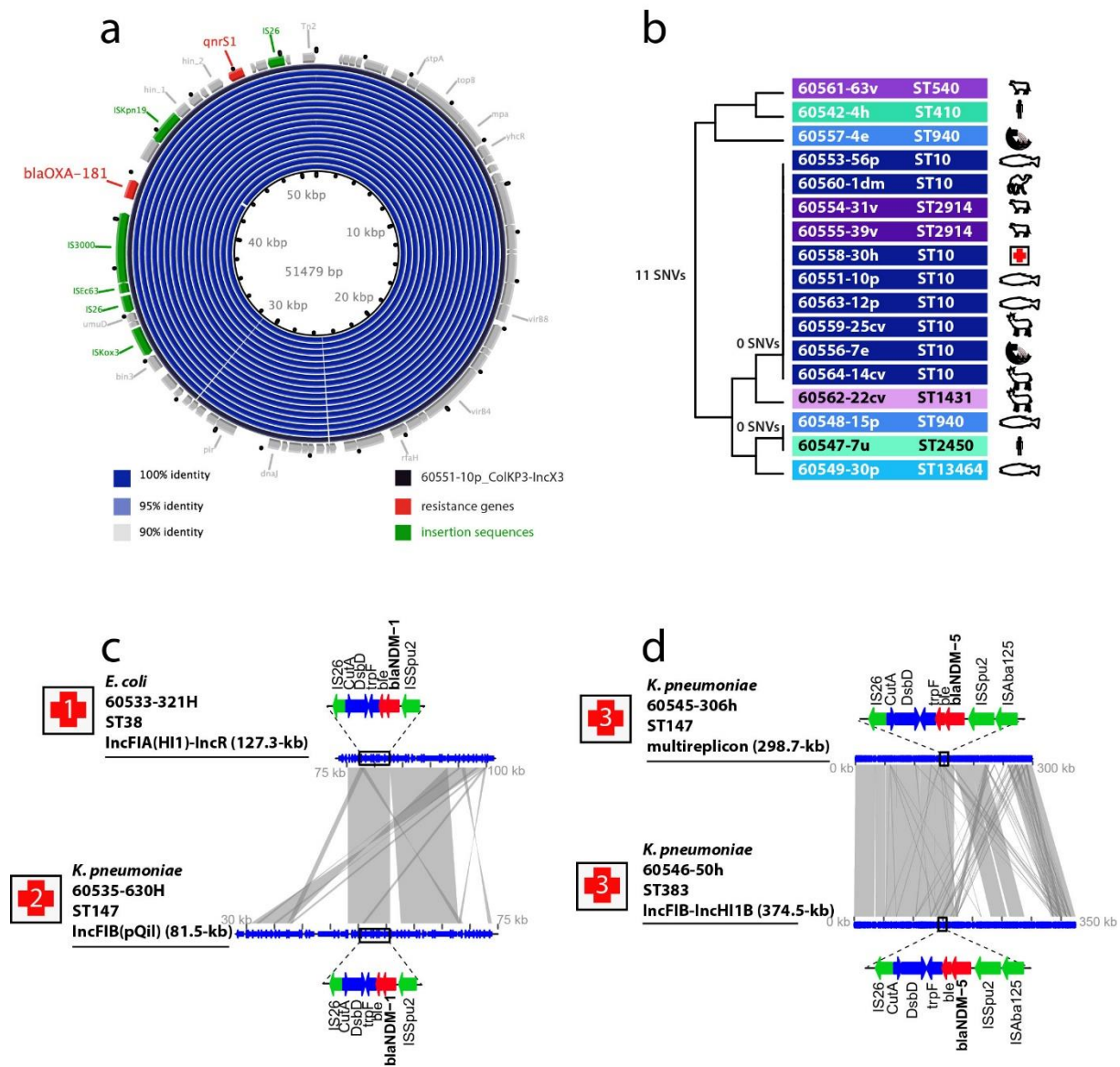


**Fig. 2. Carbapenem-producing Gram-negative bacterial (CP-GNB) species, sampling sources, sequence types, clonality, carbapenemase-encoding genes, and other antibiotic resistance determinants in their genomic context.** CP-GNP genomes are grouped by species. The sampling source, sequence type, and type of replicon carrying the carbapenemase-encoding gene(s) are indicated for each CP-GNP genome. The heat map illustrates the presence/absence of antibiotic resistance genes and chromosomal point mutations conferring resistance to aminoglycosides, beta-lactams, fosfomycin, amphenicol, quinolones, sulfonamides, trimethoprim and tetracycline. For each of the most frequent sequence types, single-nucleotide variants (SNVs) were detected by variant calling and were used to generate a unweighted pair method with arithmetic mean (UPGMA) tree.

\*60540-48h harbors the *bla*<sub>OXA-23</sub> gene on its chromosome and a 8.2-kb circular contig without resistance genes.

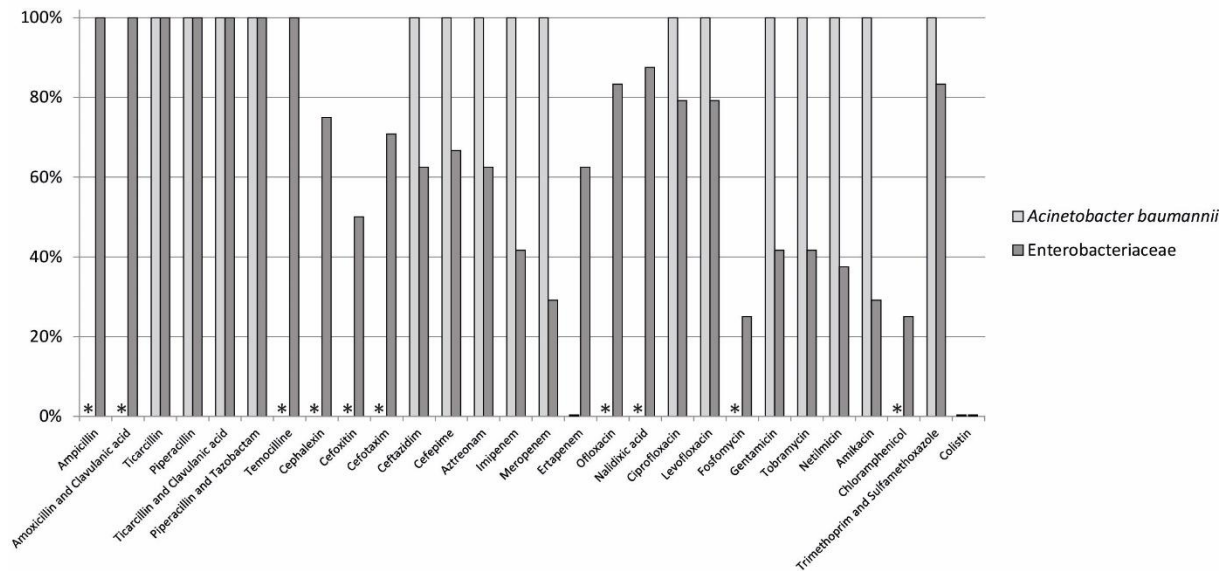
\*\*60546-50h harbors *bla*<sub>NDM-5</sub> on the IncFIB-IncHI1B hybrid plasmid and *bla*<sub>OXA-48</sub> on a IncL.

†298.7-kb multi-replicon plasmid that carries the IncFIB(pNDM-Mar), IncHI1B(pNDM-Mar), and IncR replicons.

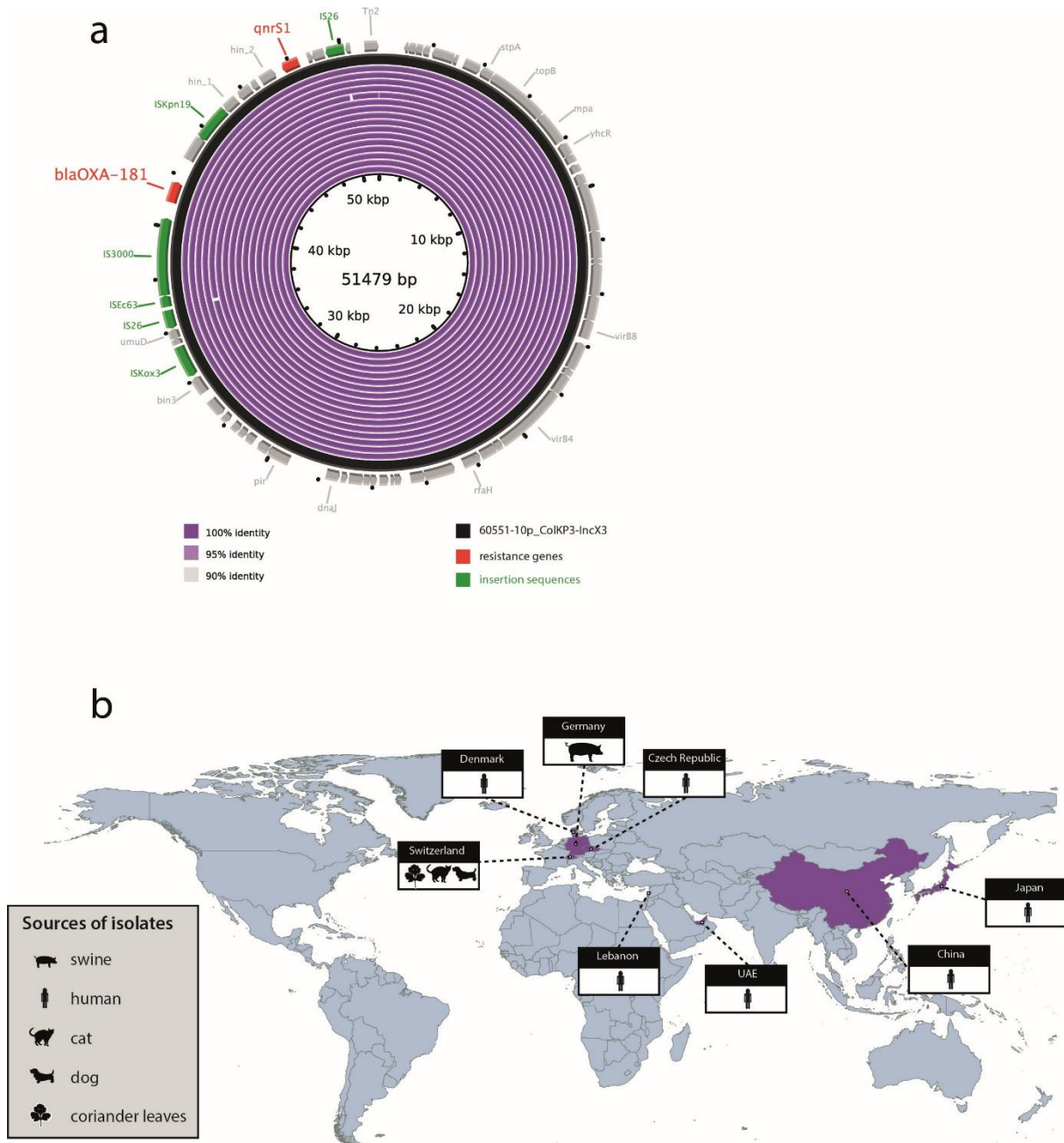


**Fig. 3. Intra- and inter-species horizontal exchanges of mobile genetic elements (MGE) that carry carbapenemase-encoding genes in Djibouti City.** (a-b) Putative plasmid transfers between carbapenem-resistant *Escherichia coli* isolates in Djibouti City. (a) From the outside to the inside, the rings represent the backbone/variable region of the reference ColKP3-IncX3 plasmid (60551-10p\_ColKP3-IncX3; black ring) with genetic annotations (arrows are colored according to the functional groups indicated in the legend) and the compared IncX3 or ColKP3-IncX3 plasmids (blue rings). (b) Reads produced from the Illumina sequencing of *E. coli* isolates harboring the IncX3 or ColKP3-IncX3 plasmids were aligned to the reference ColKP3-IncX3 plasmid (60551-10p\_ColKP3-IncX3). Single-nucleotide variants (SNVs) were detected by variant calling and were used to generate a unweighted pair group method with arithmetic mean (UPGMA) tree. (c-d) Putative horizontal transfers of other MGE that harbor carbapenem-resistance genes. Genetic maps highlighting shared genomic regions between pairs of isolates that display identical carbapenemase-encoding genes. Regions with BLAST identity of  $\geq 99\%$  are shown in gray. Genetic annotations are represented by arrows. The genomic environment

of the carbapenemase-encoding gene (black box) is magnified and genes are colored according to functional groups (antibiotic resistance genes in red, insertion sequences in green, and other genes in blue).



**Fig. S1. Antimicrobial resistance phenotypic profiles of carbapenemase-producing Gram-negative bacterial isolates.** Prevalence of resistance to each of the 29 antibiotics or antibiotic combinations tested. Untested antibiotics are indicated by stars.



**Fig. S2. Putative international sources of introduction of the ColKP3-IncX3 plasmid in Djibouti.** (a) From the outside to the inside, the rings represent the backbone/variable region of the reference ColKP3-IncX3 plasmid (60551-10p\_ColKP3-IncX3; black ring) with genetic annotations (arrows are colored according to the functional groups indicated in the legend) and the compared plasmids (violet rings). (b) Global map highlighting previous detections of ColKP3-IncX3 plasmids.