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Corresponding author(s): Cheryl P. Andam

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

No software was used for data collection.

Bacterial isolates from unique pediatric and adult patients with bloodstream infection were obtained from clinical blood cultures submitted to the Department of Pathology and Laboratory Medicine at DHMC from January 2017 to January 2022. Species identification was initially performed using either the FilmArray Blood Culture Identification (BCID) panel for samples collected between January 2017 and May 2021 or the FilmArray Blood Culture Identification 2 (BCID2) panel for samples from June 2021 onwards. In total, 59 isolates were identified as belonging to the E. cloacae species complex. All isolates were stored at −80°C in dimethyl sulfoxide solution.

Antimicrobial susceptibility testing (AST) was carried out using the automated MicroScan Walkaway 96 Plus (Beckman-Couter, La Brea, California, USA). Two FDA (Food and Drug Administration)-cleared AST panels were used: the NUC62 Panel from January 2017 to May 2019, followed by the Neg MIC Panel 46 from June 2019 onwards. Each panel underwent verification study before use. For the NUC62 Panel, manufacturer breakpoints approved at FDA clearance were applied. When switching to the Neg MIC Panel 46, off-label breakpoints for carbapenems and certain cephalosporins were validated to match the Clinical Laboratory Standards Institute M100 S28 guidelines 29. We tested the following antimicrobial compounds: amikacin, gentamicin, sulfamethoxazole/trimethoprim, ertapenem, meropenem, cefoxitin, cefazolin, cefepime, cefotaxime, cefotetan, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, levofloxacin, aztreonam, ampicillin, ampicillin-sulbactam, amoxicillin-clavulanic acid, piperacillin/tazobactam. AST results are presented in Supplementary Data 1.

Data analysis

DNA extraction, library preparation, and whole genome sequencing
Subcultured isolates were grown onto commercially prepared tryptic soy agar with 10% sheep red blood cells (Remel, Lenexa, Kansas, USA) and in brain heart infusion broth (BD Difco, Franklin Lakes, New Jersey, USA) at 37°C for 24 h. For short-read sequencing, we extracted and

purified DNA from broth cultures using the QuickDNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, California, USA) in accordance with the manufacturer's procedure. DNA libraries were prepared with the Illumina DNA Prep Kit and IDT 10 bp UDI indices following the manufacturer's procedure. An Illumina NextSeq 2000 sequencer was used to sequence DNA samples as multiplexed libraries following the manufacturer's guidelines. Illumina bcl-convert (v.3.9.3) was employed for demultiplexing, sequence quality control, and adapter trimming of the Illumina short reads. Sequencing resulted in 151-nt paired end reads.

For long-read sequencing of the nine isolates that harbored mcr genes, DNA was extracted using the Quick-DNA high molecular weight MagBead Kit (Zymo Research, Irvine, California, USA) and prepared using the Oxford Nanopore Technologies (ONT) SQK-LSK114 native barcoding kit following the manufacturer's procedure. Sequencing was performed on the ONT GridION platform with a FLOW-MIN114 Spot-ON Flow Cell, R10 version and employing a translocation speed of 400bps. Base calling was done using Guppy v.7.0.9 with the option super-accurate mode. DNA concentrations were measured with the Qubit fluorometer (Invitrogen, Grand Island, New York, USA). Sequencing was carried out at the SeqCenter (Pittsburgh, Pennsylvania, USA).

Genome assembly, sequence quality check, and species confirmation

Illumina short reads were de novo-assembled into adjoining sequences using the SPAdes v.3.14.1 assembler implemented in Shovill v.1.1.0 (https://github.com/tseemann/shovill) (Supplementary Data 2). Adapter sequences were trimmed by enabling the -trim option. Adapter sequences on the ONT long reads were deleted using porechop v.0.2.4 (https://github.com/rrwick/Porechop). Further filtering of high quality reads was carried out with Filtlong v.0.2.1 (https://github.com/rrwick/Filtlong) by discarding reads < 1kb and 10% of the worst reads. The Illumina short reads and ONT long reads were then combined and hybrid genome assemblies were generated from them using Unicycler v.0.5.0 31. Genome sequence quality was determined using CheckM v.1.1.3 and QUAST v.5.0.2. Genome completeness ranged between 98.40 – 99.61% (mean = 99.29%) and contamination ranged between 0.41 – 2.02% (mean = 0.99%) (Supplementary Data 1). All hybrid genome assemblies were of high quality comprising of <=7 contigs and mean N50 of 4.75 Mbp (Supplementary Data 3).

Bactinspector (https://gitlab.com/antunderwood/bactinspector) was used to assign species identity of the assembled genomes. Bactinspector searches NCBI's RefSeq database using mash to find best matching genomes. Furthermore, we uploaded our genome assemblies through the Type (strain) Genome Server to confirm their taxonomy and results were similar to the Bactinspector outcomes. We also calculated the genome-wide average nucleotide identity (ANI) for every possible pair of genomes using fastANI v.1.32. ANI refers to the mean nucleotide identity of all orthologous pair of genes that are shared between a pair of microbial genomes. We used the >=95% ANI threshold to delineate species boundaries.

Phylogenetic tree reconstruction

The draft genomes were annotated using Prokka v.1.14.6 and the pan-genome characterized using Panaroo v.1.2.7. Sequence alignments of the 3,022 core genes (i.e., gene families present in 99% of the genomes) were concatenated to create the core genome alignment, and from which single nucleotide polymorphisms (SNPs) were obtained using SNP-sites v.2.5.1. The core SNP alignment was then used to reconstruct a maximum likelihood phylogeny in RAxML v.8.2.12, implementing a generalized time reversible 41 model of nucleotide substitution and Gamma distribution of rate heterogeneity. We used figtree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and Interactive Tree of Life (iTOL) v.6.8.1 for phylogeny visualization and annotation.

In silico sequence typing and identification of AMR genes in genome and plasmid assemblies

We used MLST v.2.19.0 (https://github.com/tseemann/mlst) to identify STs in our dataset. Multi-locus sequence typing is done by comparing query alleles with previously deposited allele combinations of the E. cloacae species complex compiled in the PubMLST database (https://pubmlst.org/organisms/enterobacter-cloacae). ST designation is based on allelic variation in seven single-copy housekeeping genes (dnaA, fusA, gyrB, leuS, pyrG, rplB, rpoB). Genome assemblies with novel ST configurations were curated on PubMLST. ABRicate v1.0.1 (https://github.com/tseemann/abricate) was used to screen the hybrid genome assemblies to identify plasmid replicons using PlasmidFinder 45 (Supplementary Data 4). The presence of clinically relevant AMR genes was determined using the NCBI AMRFinderPlus v.3.10.23 and its accompanying NCBI-compiled database. The genetic environment of mcr-9.1 on plasmid and chromosomes was manually inspected using the Geneious Prime software v.2022.2.1. We further ran the chromosomal sequences of genomes EXB47 and EXB59 through the MobileElementFinder database (https://cge.food.dtu.dk/services/MobileElementFinder/) to determine if there were ICE (Integrative and Conjugative Elements) and IME (Integrative and Mobilizable Elements) associated with chromosomal mcr-9.1. Clinker v.0.0.25 (https://github.com/gamcil/clinker) was used to determine the sequence similarity of mcr-9.1 detected in chromosomes and plasmid genome assemblies. Plasmid genomes were visualized using Proksee 47. Within Proksee, default settings were applied, using mobileOD-db v.1.1.3 to identify coding sequences related to replication/recombination/repair, transfer, integration/excision, and phages elements. CARD-RGI v.1.3.0 was used to annotate AMR genes (https://card.mcmaster.ca/analyze/rgi).

In silico analysis of globally distributed plasmids carrying mcr-9 and mcr-10

All publicly available sequences in the PLSDB database of complete bacterial plasmids were screened for the presence of mcr-9 and mcr-10 genes using AMRFinderplus v.3.10.23 as described above. The PLSDB contains an extensive set of complete bacterial plasmids from the NCBI's RefSeq and international nucleotide sequence database collaboration (which includes DDBJ, EMBL-EBI and GenBank). Associated metadata of the global plasmid sequences were also retrieved (Supplementary Data 5) and used for further downstream analysis. Plasmid incompatibility types of the mcr-9 and mcr-10 harboring plasmids were determined using the PlasmidFinder database as described above. Plasmid mobility was predicted and categorized as mobilizable, non-mobilizable or conjugative using MOB-suite v.3.1.9.

Resistance gene co-occurrence in plasmids carrying mcr-9 and mcr-10

We used ReGAIN v.1.0.5 to elucidate on probabilistic patterns of AMR gene co-occurrence. We selected resistance genes that occur in 5 – 95% of the global plasmid dataset to exclude ubiquitously occurring genes and reduce noise in creating the Bayesian network. For every pair of AMR genes, we calculated the conditional probability, relative risk, and bidirectional probability score. We carried out 500 bootstrap replicates. A 0.5 significance threshold was used to filter out weakly supported gene pairs. We also used the multivariate analysis (MVA) module of ReGAIN to visualize the structure of underlying resistance gene data. MVA was constructed by applying Jaccard distances on the gene-presence absence matrix and principal component analysis. A k-means clustering algorithm was used to incorporate ellipses at 95% confidence around clusters.

Statistical analysis

All statistical analyses were carried out using the stat_compare_means on ggpubr v.0.4.0 package (https://rpkgs.datanovia.com/ggpubr/) in RStudio v.2022.02.1 + 461. Kruskal-Wallis test was used to determine statistical differences between the distribution of plasmids grouped by their plasmid replicons and their AMR gene content and genome sizes. Wilcoxon signed rank test was used to compare the number of AMR genes in multi-replicon and single-replicon plasmids. We calculated Jaccard distances using the vegdist function on the vegan 2.6-6.1 package

on RStudio 2024.04.2+764. Results were considered significant when $p < 0.05$.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The dataset supporting the conclusions of this article is included within the article and its supplementary files. Short- and long-reads data of the nine Enterobacter isolates carrying mcr genes and that sequenced in this study have been deposited in the NCBI Short Reads Archive (SRA) under BioProject PRJNA1168907 and their genome accession numbers are listed in Supplementary Data 3. Plasmid genome assemblies containing mcr-9.1 or mcr-10.1 sequenced in this study have been deposited in NCBI's BankIT database (Supplementary Data 4). Accession numbers of the global plasmid sequences are presented in Supplementary Data 5 and are available in NCBI and PLSDB database. The source data for Figure 3 is in Supplementary Data 5.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	Samples used in the study were subcultured bacterial isolates that had been previously archived as routine part of clinical laboratory operations from January 2017 to January 2022.
Reporting on race, ethnicity, or other socially relevant groupings	Samples used in the study were subcultured bacterial isolates that had been previously archived as routine part of clinical laboratory operations from January 2017 to January 2022.
Population characteristics	Samples used in the study were subcultured bacterial isolates that had been previously archived as routine part of clinical laboratory operations from January 2017 to January 2022.
Recruitment	Samples used in the study were subcultured bacterial isolates that had been previously archived as routine part of clinical laboratory operations from January 2017 to January 2022.
Ethics oversight	The Committee for the Protection of Human Subjects of DHMC and Dartmouth College granted ethical approval (CPHS # STUDY00031572). Samples used in the study were subcultured bacterial isolates that had been previously archived as routine part of clinical laboratory operations. No patient blood or other specimens were used in this study. Patient protected health information was not collected nor used in this study. Hence, the study protocol was deemed not to be human subjects research, and therefore informed consent was not required for this study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculation was performed. Sample size is based on the number of bacterial isolates from clinical specimens received by the Dartmouth-Hitchcock Medical Center in the routine course of clinical laboratory operations. The number of genomes analyzed is based on the number of high quality sequences obtained.
Data exclusions	No data exclusion was performed. Bacterial isolates were included in all analyses.
Replication	No replication was performed. Bacterial isolates were included in all analyses.
Randomization	No randomization was performed. Bacterial isolates were included in all analyses.
Blinding	No blinding was performed. Bacterial isolates were included in all analyses.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Plants

Seed stocks	No plants were used in this study.
Novel plant genotypes	No plants were used in this study.
Authentication	No plants were used in this study.