First Report of $bla_{VIM-4^-}$ and $mcr-9$-Coharboring Enterobacter Species Isolated from a Pediatric Patient

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ABSTRACT An Enterobacter hormaechei isolate harboring $bla_{VIM-4}$ and $mcr-9$ was recovered from a pediatric patient in a U.S. hospital. The $bla_{VIM-4}$ and $mcr-9$ genes are carried on the same IncH12 plasmid, pME-1a. The isolate tested susceptible to colistin, without observed induction of colistin resistance. The $mcr-9$ gene is located between two insertion elements, IS903 and IS1, but lacks the downstream regulatory genes ($qseC$ and $qseB$) found in other isolates that harbor $mcr-9$.

IMPORTANCE We describe the complete genome assembly and sequence of a clinical Enterobacter isolate harboring both $bla_{VIM-4}$ and $mcr-9$ recovered from a pediatric patient in the United States with a history of travel to Egypt. Moreover, to the best of our knowledge, this is the first report of an Enterobacter isolate harboring both $bla_{VIM-4}$ and $mcr-9$ from the United States. The $bla_{VIM-4}$ and $mcr-9$ genes are carried on the same IncH12 plasmid, pME-1a. The isolate tested susceptible to colistin, without observed induction of colistin resistance. The $mcr-9$ gene is located between two insertion elements, IS903 and IS1, but lacks the downstream regulatory genes ($qseC$ and $qseB$) found in other isolates that harbor $mcr-9$.

KEYWORDS carbapenem, carbapenemase, colistin, Enterobacter, mcr-9, VIM, VIM-4

The rapid spread of carbapenemase-producing Enterobacteriaceae poses a significant global public health threat as therapeutic options are limited (1, 2). Carbapenemases are $\beta$-lactamases capable of hydrolyzing carbapenems and other $\beta$-lactams, leading to resistance to one of the most efficacious classes of antibiotics. These enzymes are grouped into molecular classes A, B, and D (3). Class B metallo-$\beta$-lactamases (MBLs) are capable of hydrolyzing penicillins, cephalosporins, and carbapenems but lack the ability to hydrolyze aztreonam (3). A member of this class, Verona integron-encoded MBL (VIM), has been reported in Pseudomonas aeruginosa, Acinetobacter baumannii, and members of the Enterobacteriaceae family (4). VIM-producing Enterobacteriaceae have been reported in several countries, especially in the Mediterranean region (4), including isolates from Kuwait (5) and the United Arab Emirates (4), but have rarely been described in the northeastern United States, where Klebsiella pneumoniae carbapenemase (KPC) is the predominate carbapenemase (1). As of 28 May 2019, 62 variants of $bla_{VIM}$ have been reported in the National Database of Antibiotic-Resistant Organisms (NDARO) (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/). Here, we report the comprehensive characterization of a clinical Enterobacter hormaechei isolate harboring $bla_{VIM-4}$ from a New York City (NYC) patient using both the Illumina and Oxford Nanopore DNA sequencing platforms. Surprisingly, the
Genomic analysis identified the presence of the \textit{mcr-9} gene on the same plasmid harboring \textit{bla\textsubscript{VIM-4}}.

The \textit{E. hormaechei} ME-1 isolate was recovered from a 3-year-old boy with the \(\beta\)-thalassemia trait who had a throat infection in Egypt 1 month prior to presentation at an NYC hospital in 2018. He was treated with an intramuscular injection of penicillin, but over the next 2 weeks he developed erythema and induration around the injection site. Two weeks later, he received 1 week of cephalaxin, given the concern for cellulitis. He then presented to an emergency room in NYC with fever and a poorly healing wound with a central 1-cm eschar, and a surrounding rim of erythema was observed at the site of the prior intramuscular injection. He was found to have leukocytosis and an increasing percentage of bands on his blood count differential. A skin biopsy of the wound was performed. The Gram stain from the biopsy specimen demonstrated Gram-negative rods, and culture yielded an organism that was identified as a member of the \textit{Enterobacter cloacae} complex by matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI Biotyper; Bruker Daltonics, Inc., Billerica, MA, USA), later identified as \textit{E. hormaechei} by whole-genome sequencing (WGS). Using broth microdilution antibiotic susceptibility testing (6), the isolate tested resistant to ceftazidime, ceftriaxone, cefuroxime, ertapenem, imipenem, meropenem, nitrofurantoin, piperacillin-tazobactam, ceftazidime-avibactam, ceftolozane-tazobactam, and trimethoprim-sulfamethoxazole; intermediate to tobramycin; and susceptible to amikacin, aztreonam, ciprofloxacin, doxycycline, levofloxacin, minocycline, tigecycline, tetracycline, and colistin. He was discharged prior to the availability of these culture results and was treated empirically with 2 weeks of oral clindamycin. The medical team was unable to reach his family after discharge to adjust his antimicrobial therapy. However, he was seen 3 months later, where his follow-up exam revealed that his rash had fully healed with a scar despite having never received treatment for \textit{E. hormaechei}, suggesting that the organism was colonizing the wound but was not a pathogen.

The isolate was assayed on a research-use-only FilmArray antimicrobial resistance (AMR) panel (BioFire Diagnostics, LLC, Salt Lake City, UT, USA) utilizing the sample-to-answer BioFire system, and tested positive for both the \textit{bla\textsubscript{CTX-M}} and \textit{bla\textsubscript{VIM}} genes. To better understand the genetic structure of the \textit{bla\textsubscript{VIM}}-harboring element and to confidently assemble all the plasmids contained in the strain, WGS was performed using a combination of the Illumina NextSeq (Illumina, San Diego, CA, USA) and MinION (Oxford Nanopore Technologies, Oxford, United Kingdom) platforms. Together, the sequence analysis generated high-quality assemblies and completely closed genomes of the isolate and all the plasmids harbored by the isolate.

Briefly, DNA was isolated from overnight cultures using a MasterPure Gram-positive DNA purification kit as recommended by the manufacturer (Epicentre, Madison, WI, USA). Libraries were prepared for sequencing using Illumina Nextera XT kits and sequenced on an Illumina NextSeq platform with paired 150-base sequence reads. In addition, a library for MinION sequencing was prepared using the Rapid Barcoding Sequencing kit (SQK-RBK004) and loaded onto an R9.4 flow cell. The run was performed on a MinION Mk1B device, and the sequencing reads were assembled using Unicycler (7).

The isolate contained a chromosome of 4,804,295 bp (Table 1) and 4 plasmids ranging in size from 2,317 bp to 276,502 bp. The average G+C content of the chromosome was 55.6\%, and the chromosome harbored 5 prophages. The chromosome of ME-1 was compared to the genomes of the type strains of various \textit{Enterobacter} species and found to belong to \textit{Enterobacter hormaechei} subsp. \textit{steigerwaltii} clade B Hoffmann VIII (8). \textit{In silico} multilocus sequence typing (MLST) analysis assigned ME-1 to sequence type (ST) ST542 (allelic profile 178-4-4-6-37-4-6). \textit{In silico} mining of antibiotic resistance genes revealed that the chromosome of ME-1 harbored \textit{bla\textsubscript{ACT-7}}, an intrinsic AmpC enzyme, and \textit{fosA}, which confer resistance to some \(\beta\)-lactams and fosfomycin, respectively.

The isolate harbored a total of 4 plasmids (pME-1a, pME-1b, pME-1c, and pME-1d) belonging to incompatibility IncHI2 and CoIE groups (Table 1). Analyses of acquired
antibiotic resistance genes were performed using ResFinder 3.1 (9). Plasmid pME-1a harbored 12 antibiotic resistance genes conferring resistance to β-lactams (bla\textsuperscript{VIM-4} and bla\textsuperscript{CTX-M-9}), aminoglycosides (aadA2 and aadA24), sulfonamide (sul1), trimethoprim (dfrA1 and dfrA16), and quinolones (qnrA1) (Fig. 1). Surprisingly, and with biological significance, a Blast analysis revealed that pME-1a also harbors the newly reported colistin resistance gene mcr-9, identical to the mcr-9 gene identified in a Salmonella isolate (10). The other plasmids (pME-1b, pME-1c, and pME-1d) were not found to harbor any antibiotic resistance genes.

Plasmid pME-1a is 276,520 bp in length with an average G+C content of 46.3% and encodes 356 predicted open reading frames (Fig. 1). Full plasmid sequence queries of pME-1a against the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) showed that it has high similarity to other IncHI2 plasmids, e.g., pMRVIM0813 (98% query coverage and 99.99% sequence identity; GenBank accession number KP975077), pC45-VIM4 (98% query coverage and 99.98% sequence identity; GenBank accession number LT991958), and IncHI2 prototype plasmid R478 (79% query coverage and 99.97% sequence identity; GenBank accession number BX664015) (11). Similarly to plasmid R478, the conjugative transfer genes in pME-1a are also present in two separate regions, Tra1 and Tra2, presumably facilitating the dissemination of this plasmid between various members of the Enterobacteriaceae (Fig. 1) (11). Like plasmid R478, the Tra2 region in pME-1a encodes the majority of the mating pair formation (Mpf) complex, required for intracellular DNA transfer during bacterial conjugation, but there is an insertion of the IS5 family transposase (IS182) in the parR gene (11). The Tra1 region was similar to plasmid R478, harbored few Mpf complex genes, and encoded fewer OriT and relaxosome components (11).

The bla\textsuperscript{VIM-4} gene was identified on an In416 integron, with the cassette structure of bla\textsuperscript{VIM-4}-aacA7-dfrA1-ΔssdA1-smr-ISPa21. The same integron has been found in an IncA/C plasmid containing bla\textsuperscript{VIM-4} in Klebsiella pneumoniae, Escherichia coli, and Enterobacter isolates from an outbreak in Kuwait (5). In416 appears to be a common integron associated with the spread of bla\textsuperscript{VIM} (5). In addition, we found another novel integron with the cassette array of dfrA16-aadA2-dfrA16-aadA2-smr. Further examination of the plasmid sequences of pME-1a revealed the presence of a recently identified mobilized colistin resistance (mcr) gene, mcr-9 (10). The gene mcr-9 was initially recognized by in silico screening of sequenced Salmonella enterica subsp. enterica serotype Typhimurium (S. Typhimurium) genomes which revealed its sequence similarity to the mcr-3 gene (10). The S. Typhimurium isolate harboring mcr-9 had a colistin MIC value of 2 µg/ml, which is consistent with MICs observed for strains harboring other mcr alleles (12).

In contrast, using the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (6), the isolate described here had a much lower colistin MIC value (0.12 µg/ml) (13). In pME-1a, mcr-9 is located downstream of the bla\textsuperscript{CTX-M-9} gene (Fig. 1). Inspection of the regions surrounding pME-1 mcr-9 reveal that it is located between two insertion elements, IS903 (upstream) and IS1 (downstream) (Fig. 2). Sequence query of the region surrounding mcr-9 using NCBI GenBank showed

**TABLE 1** Key features of chromosome and plasmids harbored by ME-1

| Sample name | Size (bp) | MLST    | Plasmid incompatibility type | Antibiotic resistance gene(s) |
|-------------|-----------|---------|-------------------------------|------------------------------|
| Chromosome  | 4,804,295 | ST542   | NA                           | fasa, bla\textsuperscript{ACT-7} |
| pME-1a      | 276,520   | NA      | IncHI2                       | aadA24, aadA24, sul1, dfrA1, dfrA16, mcr-9, bla\textsuperscript{VIM-4}, bla\textsuperscript{CTX-M-9}, qnrA1 |
| pME-1b      | 6,237     | NA      | ColRNAI                      | NA                           |
| pME-1c      | 2,496     | NA      | ColRNAI                      | NA                           |
| pME-1d      | 2,317     | NA      | ColRNAI                      | NA                           |

\textsuperscript{a}Multiple copies on the plasmid.

\textsuperscript{b}NA, not applicable.
similarity to other sequenced IncH12 plasmids from Enterobacter: pCTXM9_020038 (GenBank accession number CP031724) and pMRVIM0813 (GenBank accession number KP975077) (Fig. 2). Analysis of the gene organization surrounding mcr-9 in the S. Typhimurium HUM_TYPH_WA_10_R9_3274 isolate (10) revealed a unique cupin fold metalloprotein, WbuC, downstream of mcr-9, and interestingly, the upstream flanking region showed sequence homology to the inverted repeat region right (IRR) associated with IS903 (GenBank accession number NZ_NAAN01000063) (Fig. 2). We are unable to determine whether a complete IS903 element is upstream because the mcr-9-harboring plasmid associated with the S. Typhimurium isolate was not completely sequenced and
only a short \textit{mcr-9}-bearing contig (2,661 bp) is available for comparison (GenBank accession number \textit{NZ_NAAN01000063}) (Fig. 2). This limitation highlights the importance of using long-read nanopore sequencing coupled with hybrid assembly to identify and monitor the transfer and rapid evolution of antibiotic resistance genes among bacteria.

A recent study from France reported a colistin-resistant isolate, \textit{E. coli} isolate 68A, harboring \textit{mcr-9} on an IncH12 plasmid (14). Unlike \textit{E. hormaechei} ME-1, the sequenced plasmid contig did not appear to harbor other antibiotic resistance genes (14). Interestingly, the analysis of the region surrounding \textit{mcr-9} revealed the presence of insertion element IS903 upstream of \textit{mcr-9}, similar to the organization of pME-1a, pCTXM9\_020038, and pMRVIM0813 (Fig. 2). However, unlike pME-1a, the \textit{mcr-9}-harboring plasmid from \textit{E. coli} isolate 68A has a different downstream region bearing \textit{wbuC}, \textit{qseC}, \textit{qseB}, and an ATPase gene (Fig. 2). In this study, the investigators were able to achieve clinical levels of colistin resistance by inducing the two-component system encoded by \textit{qseB} and \textit{qseC} using subinhibitory concentrations of colistin (14). In contrast, plasmids pME-1a, pCTXM9\_020038, and pMRVIM0813 lack the \textit{qseB} and \textit{qseC} regulatory genes (Fig. 2). To further evaluate whether this two-component system is necessary for \textit{mcr-9} induction in ME-1, we performed an induction assay and evaluated colistin resistance as described previously (14). Using subinhibitory concentrations of colistin at 0.03 and 0.06 \(\mu \text{g/ml}\), the colistin MIC values of ME-1 did not change from 0.12 \(\mu \text{g/ml}\), and gene expression of \textit{mcr-9} was not elevated compared to expression in the absence of colistin (data not shown). The chromosomally carried \textit{dnaJ} gene was used to normalize gene expression. These results confirm the importance of the \textit{qseB/qseC} two-component system in the induction of colistin resistance mediated by \textit{mcr-9}.

Consistent with the ability of \textit{mcr-9} to confer colistin resistance, Carroll and colleagues placed \textit{mcr-9} under the control of an exogenous and inducible promoter and demonstrated colistin resistance in \textit{E. coli} NEB5\textit{a} (10). Together, this finding suggests that the low-level expression of \textit{mcr-9} in strain ME-1 is likely due to the absence of the \textit{qseB/qseC} two-component regulators. However, we cannot rule out the possibility that the two insertion elements (IS903 and IS1) that flank the gene in the pME-1a plasmid influence the expression of this resistance gene.
In summary, we describe the complete genome assembly and sequence of a clinical Enterobacter isolate harboring both bla\textsuperscript{VIM-4} and mcr-9 recovered from a pediatric patient in the United States with a history of travel to Egypt. Moreover, to the best of our knowledge, this is the first report of an Enterobacter isolate harboring both bla\textsuperscript{VIM-4} and mcr-9 from the United States. Studies are under way to better understand the role of the mcr-9 gene and the contribution of the upstream IS903 insertion in strain ME-1.

**Accession number(s).** The complete nucleotide sequences of the chromosome of ME-1, pME-1a, pME-1b, pME-1c, and pME-1d were deposited as GenBank accession numbers CP041733 to CP041737, respectively.

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