Detection of Five mcr-9-Carrying Enterobacterales Isolates in Four Czech Hospitals

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ABSTRACT The aim of this study was to report the characterization of the first mcr-positive Enterobacterales isolated from Czech hospitals. In 2019, one Citrobacter freundii and four Enterobacter isolates were recovered from Czech hospitals. The production of carbapenemases was examined by a matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay. Additionally, bacteria were screened for the presence of carbapenemase-encoding genes and plasmid-mediated colistin resistance genes by PCR. To define the genetic units carrying mcr genes, the genomic DNAs of mcr-carrying clinical isolates were sequenced on the PacBio Sequel I platform. Results showed that all isolates carried blaVIM-1 and mcr-like genes. Analysis of whole-genome sequencing (WGS) data revealed that all isolates carried mcr-9-like alleles. Furthermore, the three sequence type 106 (ST106) Enterobacter hormaechei isolates harbored blaVIM-1 gene, while the ST764 E. hormaechei and ST95 C. freundii included blaVIM-4. Analysis of plasmid sequences showed that, in all isolates, mcr-9 was carried on IncHI2 plasmids. Additionally, at least one multidrug resistance (MDR) region was identified in each mcr-9-carrying IncHI2 plasmid. The blaVIM-4 gene was found in the MDR regions of p48880_MCR_VIM and p51929_MCR_VIM. In the three remaining isolates, blaVIM-1 was localized on plasmids (~55 kb) exhibiting repA-like sequences 99% identical to the respective gene of pKPC-CAV1193. In conclusion, to the best of our knowledge, these 5 isolates were the first mcr-9-positive bacteria of clinical origin identified in the Czech Republic. Additionally, the carriage of the blaVIM-1 on pKPC-CAV1193-like plasmids is described for the first time. Thus, our findings underline the ongoing evolution of mobile elements implicated in the dissemination of clinically important resistance determinants.

IMPORTANCE Infections caused by carbapenemase-producing bacteria have led to the revival of polymyxins as the “last-resort” antibiotic. Since 2016, several reports describing the presence of plasmid-mediated colistin resistance genes, mcr, in different host species and geographic areas were published. Here, we report the first detection of Enterobacterales carrying mcr-9-like alleles isolated from Czech hospitals in 2019. Furthermore, the three ST106 Enterobacter hormaechei isolates harbored blaVIM-1, while the ST764 E. hormaechei and ST95 Citrobacter freundii isolates included blaVIM-4. Analysis of WGS data showed that, in all isolates, mcr-9 was carried on IncHI2 plasmids. blaVIM-4 was found in the MDR regions of IncHI2 plasmids, while blaVIM-1 was localized on pKPC-CAV1193-like plasmids, described here for the first time. These findings underline the ongoing evolution of mobile elements implicated in dissemination of clinically important resistance determinants. Thus, WGS characterization of MDR bacteria is crucial to unravel the mechanisms involved in dissemination of resistance mechanisms.

KEYWORDS VIM-4, MCR-9, IncHI2, Enterobacter cloacae, Citrobacter freundii

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A significant increase in infections caused by carbapenemase-producing bacteria (1), coupled with the lack of novel antibiotics (2), has led to the revival of polymyxins as the “last-resort” antibiotic (3). Consequently, higher prevalence of colistin resistance among carbapenemase-producing *Klebsiella pneumoniae* strains has been reported worldwide (4). In *K. pneumoniae*, resistance to colistin is mainly mediated via chromosomal mutations in genes involved in lipopolysaccharide synthesis (5). However, in 2016, the first plasmid-mediated colistin resistance gene, *mcr-1*, was identified among Chinese *Escherichia coli* isolates (6). Following the first description, several reports describing the presence of *mcr-1* in different host species and geographic areas were published (7, 8). Thus far, the *mcr* gene family comprises *mcr-1* to *mcr-10* (9). These genes encode phosphoethanolamine transferases that catalyze the addition of phosphoethanolamine to the phosphate group of lipid A, reducing the negative charge of the bacterial outer membrane and attenuating its affinity for colistin, resulting in antibiotic resistance.

Among the *mcr*-like genes, *mcr-1* and *mcr-9* are the most widely disseminated. The *mcr-9* gene has been identified from 40 countries across six continents. However, half of *mcr*-9-positive isolates (1,035/1,682 strains) were recovered in the United States, among which *Salmonella enterica* was the most common host species, especially in turkeys and chickens (9).

Here, we report the first detection of *mcr-9*-positive members of the *Enterobacterales* isolated from Czech hospitals.

In 2019, 4 isolates belonging to *Enterobacter cloacae* complex and one isolate belonging to *Citrobacter freundii* species were recovered from five patients admitted to Czech hospitals (see Table S1 in the supplemental material). In all isolates, which exhibited a meropenem MIC of >0.125 μg/ml (10), carbapenemase production was detected by a positive result in the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay (11). Screening for carbapenemase-encoding genes by PCR showed that all isolates carried *bla*<sub>VIM</sub>-like genes (12, 13). Additionally, bacteria were positive for the presence of plasmid-mediated colistin resistance genes by PCR, as described previously (14). All VIM-producing isolates exhibited resistance to piperacillin, piperacillin-tazobactam, and cephalosporins, while the variations in the MICs of carbapenems that were observed (Table S2) might reflect the presence of additional resistance mechanisms in some of the isolates. Variations were also observed in the MICs of non-β-lactam antibiotics. However, all isolates were susceptible to colistin, according to data obtained by the broth dilution method (15) and interpreted according to EUCAST criteria (https://www.eucast.org/clinical_breakpoints/).

To define the genetic units carrying *mcr* genes, the genomic DNAs of *mcr*-carrying clinical isolates were extracted using a NucleoSpin microbial DNA kit (Macherey-Nagel, Düren, Germany) and were sequenced using long-read sequencing technology on the PacBio Sequel I platform (Pacific Biosciences, Menlo Park, CA, USA). Library preparation was done following the manufacturer’s recommendation for microbial multiplexing for the Express kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA). DNA was sheared using Hydrodrome-long on a Megaruptor 2 device (Diagenode), and no size selection was performed during library preparation. The microbial assembly pipeline offered by SMRT Link v8.0 (Pacific Biosciences) was used to perform genome assembly with a minimum seed coverage of 30. For sequence analysis and annotation, BLAST (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database, and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were used. Comparative genome alignment was performed using Mauve v.2.3.1 (16). Figures were generated from sequence data using BRIG v.0.95 (17).

Analysis of whole-genome sequencing (WGS) data by PubMLST databases (https://pubmlst.org/) revealed that the *C. freundii* isolate belonged to sequence type 95 (ST95). Additionally, 3 of 4 isolates belonging to *E. cloacae* complex were ST106, while the remaining isolate was assigned to ST764. In silico *hsp60* typing of the genome
sequences showed that four *Enterobacter* isolates belonged to the species *Enterobacter hormaechei* (18).

Analysis of WGS data using ResFinder 3.2 revealed that all isolates carried plasmid-mediated colistin resistance *mcr*-9-like alleles. Furthermore, the three ST106 *E. hormaechei* isolates harbored the carbapenemase-encoding gene, *bla* _VIM-1_, while the ST764 *E. hormaechei* and ST95 *C. freundii* isolates included the *bla* _VIM-4_ allele. Also, all isolates included additional genes for resistance to aminoglycosides, tetracyclines, trimethoprim, chloramphenicol, sulfonamides, quinolones, and/or macrolides (Table 1). The presence of the resistance genes was confirmed by the antimicrobial resistance phenotypes (Table S1) of the isolates harboring those genes.

Analysis of plasmid sequences showed that, in all isolates, the *mcr*-9 allele was carried on IncHII plasmids (Table 1) (p48212_MCR, p48880_MCR_VIM, p48946_MCR, p49790_MCR, and p51929_MCR_VIM). Plasmids p48212_MCR, p48946_MCR, and p49790_MCR showed high degrees of similarity to each other (99% coverage and 99% identity), while lower diversity was observed in plasmids p48880_MCR_VIM (90% coverage and 99% identity) and p51929_MCR_VIM (77% coverage and 99% identity) compared to p48212_MCR. All plasmids exhibited sequences closely related to other *mcr*-9-carrying IncHII plasmids, like pC45-VIM4 from *E. cloacae* complex isolate C45 (GenBank accession no. LT991958) and pC45-001 from *E. hormaechei* strain C45, recovered from a clinical sample (GenBank accession no. CP042552) in Australia (Fig. 1), and typed as sequence type 1 (ST1) following the IncHII pDLST scheme (19). IncHII plasmid backbones were composed of regions for replication (*reHII*), conjugative transfer (*trh* genes), and plasmid maintenance (*par* gene). Additionally, IncHII plasmids carried tellurium resistance genes (*terZABCDEF*), commonly associated with this plasmid family, in addition to *terY1*, *terY2*, and *terW* (20). Also, genes conferring arsenic resistance (*arsCBRH*) were found in IncHII plasmids. In all IncHII plasmids, the *mcr*-9 allele was inserted upstream the _pcoS_ gene, as observed in other IncHII plasmids like pC45-001 (GenBank accession no. CP042552). In all IncHII plasmids except p48880_MCR, the *mcr*-9 gene was bounded by an IS9038 element (upstream) and an ORF (downstream), encoding a cupin fold metalloprotein, followed by IS26. However, in plasmid p48880_MCR, carrying the *mcr*-9.2 allele, an IS1R insertion sequence was found downstream of the *mcr*-9.2 gene. In all isolates, _qseC _and _qseB _regulatory genes were not found in association with the *mcr*-9 gene. Based on previous studies (21, 22), in the presence of subinhibitory concentrations of colistin, _qseC _and _qseB _gen genes can induce the expression of the *mcr*-9 gene, leading to increased MICs. The data mentioned above may explain the susceptibility to colistin.

### Table 1: WGS data of *mcr*-carrying Enterobacterales isolates recovered from Czech hospitals

| Isolate                        | ST | Replicon of MCR-9- and VIM-encoding plasmids | Plasmid size (bp) | Resistance genes* | Additional replicons |
|-------------------------------|----|---------------------------------------------|-------------------|------------------|---------------------|
| *E. cloacae* complex ENCL48212 | 106| IncHII (ST1)                               | 302,551           | *mcr*-9, *aac(6’)-IIC, aadA2b, aph(6)-Id, dfrA19, catA2, sul1, sul2, tetD, *aac(6’)-Ib-cr*, qnrA1, ereA, *bla_*SHV-12, *bla_*VIM-1b, *qnrS1*, *bla_*TEM-1b, *sul1*, *aadA2b*, *aac(6’)-Ib3* | Col(pHAD28), IncFIB(pECLA) |
| *E. cloacae* complex ENCL48946 | 106| pKPC-CAV1193-like IncHII (ST1)             | 55,220            | *mcr*-9, *aac(6’)-IIC, *aadA2b*, *aph(3’)-Ib*, *aph(6)-Id*, *dfrA19*, *catA2*, *sul1*, *sul2*, *tetD*, *ereA*, *bla_*SHV-12, *bla_*VIM-1b, *qnrS1* | Col(pHAD28), IncFIB(pECLA) |
| *E. cloacae* complex ENCL49790 | 106| pKPC-CAV1193-like IncHII (ST1)             | 302,836           | *mcr*-9, *aac(6’)-IIC, *aadA2b*, *aph(3’)-Ib*, *aph(6)-Id*, *dfrA19*, *catA2*, *sul1*, *sul2*, *tetD*, *aac(6’)-Ib-cr*, *qnrA1*, *ereA*, *bla_*SHV-12, *bla_*VIM-1b, *sul1*, *qnrS1*, *aadA2b*, *qnrS1* | Col(pHAD28), IncFIB(pECLA) |
| *E. cloacae* complex ENCL48880 | 764| pKPC-CAV1193-like IncHII (ST1)             | 55,220            | *mcr*-9, *aac(6’)-IIC, *aadA2b*, *aph(3’)-Ib*, *aph(6)-Id*, *dfrA19*, *catA2*, *sul1*, *sul2*, *tetD*, *aac(6’)-Ib-cr*, *qnrA1*, *ereA*, *bla_*SHV-12, *bla_*VIM-1b, *sul1*, *qnrS1*, *aadA2b*, *qnrS1* | Col(pHAD28), IncFIB(pECLA), IncFII(pECLA), IncR |
| *C. freundii* CIFS31929       | 95 | IncHII (ST1)/IncM1                          | 369,945           | *mcr*-9, *aac(6’)-II, *aad(3)-I, *aac(6’)-Ib3*, *ant(2’)-Ia*, *aadA1*, *aadA2b*, *aph(3’)-Ia*, *dfrA19*, *catA2*, *cmlA1*, *sul1*, *tetA*, *aac(6’)-Ib-cr*, *qnrA1*, *bla_*VIM-1b | Col(pHAD28), IncFIB(pECLA), IncFII(pECLA), IncR |

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*bla_*SHV-12 and *mcr*-like genes are underlined.

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**Note:** Additional replicons mentioned include replicons for plasmid maintenance (*par* gene) and conjugative transfer (*trh* genes).
The mcr-9 genes from all clinical strains were transferred to the azide-resistant laboratory strain E. coli A15 by conjugation, confirming the ability of IncHI2 plasmids to further disseminate mcr-9 in other clones or species.

Moreover, at least one multidrug resistance (MDR) region was identified in each mcr-9-carrying IncHI2 plasmids. Differences in MDR regions were observed among mcr-9-carrying IncHI2 plasmids. Interestingly, the carbapenemase-encoding gene blaVIM-4 was found in the MDR regions of IncHI2 plasmids p48880_MCR_VIM and p51929_MCR_VIM, as previously described for plasmid pME-1a, which was characterized from an Enterobacter hormaechei isolate harboring blaVIM-4 and mcr-9, recovered from a pediatric patient in a U.S. hospital (21). In plasmid p48880_MCR_VIM, the blaVIM-4 gene was part of the class 1 integron In416, comprising the blaVIM-4, aacA7, dfrA1, DaadA1, and smr2 cassettes, while the class 1 integron In1174, which includes an array of aacA4 and blaVIM-4 gene cassettes, was identified in plasmid p51929_MCR_VIM.

On the other hand, in isolates ENCL48212, ENCL48946, and ENCL49790, the blaVIM-1 gene was localized on plasmids (p48212_VIM, p48946_VIM, and p59790_VIM) of approximately 55 kb. The blaVIM-1-carrying plasmids shared extensive similarity with
plasmid p16005813B from *Leclercia adecarboxylata* strain 16005813 (72% coverage and 99% identity; GenBank accession no. MK036884) (Fig. 2), encoding IMP-8 carbapenemase. The *bla* <i>VIM</i>-1-carrying plasmids could not be typed by the PCR-based replicon typing (PBRT) method (23). However, in the plasmid sequences, *repA*-like sequences of 612 bp exhibiting 99% identity with the *repA* gene of pKPC-CAV1193 (GenBank accession no. CP013325) from *Klebsiella pneumoniae* strain CAV1193 were identified. Additionally, a complete transfer region was not found in pKPC-CAV1193-like plasmids, explaining the failure of *bla*<i>VIM</i>-1-positive plasmids to be transferred, by conjugation experiments, to the azide-resistant laboratory strain *E. coli* A15, which was used as a recipient.

The MDR regions of *VIM*-1-encoding plasmids included the class 1 integron In110,
whose variable region comprised \( \text{bla}_{\text{VIM-1}} \), \( \text{aac}A4 \), and \( \text{aad}A1 \) (24). In all three VIM-1-encoding plasmids, \( \text{In}110 \) was localized in a \( \text{Tn}1696 \)-like sequence (nucleotides 13689 to 30156 in \( \text{p48212}_{\text{VIM}} \)). The \( \text{IIR} \) of \( \text{In}110 \) was located between the \( \text{resI} \) and \( \text{resII} \) sites of the \( \text{Tn}1696 \) module in precisely the same position as \( \text{In}4 \) in \( \text{Tn}1696 \). The 3’ conserved segment of the integron was bounded with a \( \text{Tn}1696 \) fragment, consisting of \( \text{IS}6100 \), the \( \text{resI} \) site, and the \( \text{mer} \) operon. The \( \text{Tn}1696 \)-like transposons were flanked by \( \text{IRtnp} \) and \( \text{IRmer} \) of \( \text{Tn}1696 \), with \( \text{IRtnp} \) being disrupted by \( \text{IS}4321 \) while \( \text{IRmer} \) remained intact. Target site duplications of 6 bp (CAGCAG) were identified at the boundaries of \( \text{IIRs} \) of the \( \text{Tn}1696 \)-like sequence, indicating its transposition within \( \text{pKPC-CAV1193} \)-like plasmids. Interestingly, resistance islands composed of the class 1 integron \( \text{In}110 \) associated with a \( \text{Tn}1696 \)-like sequence have been previously identified in plasmids \( \text{pKpn-431cz} \) and \( \text{pLec-476cz} \), characterized from VIM-1-producing \( \text{Enterobacterales} \) isolates of Czech origin (25). Additionally, in \( \text{p48212}_{\text{VIM}}, \text{p48946}_{\text{VIM}}, \) and \( \text{p59790}_{\text{VIM}} \) plasmids, the resistance genes \( \text{bla}_{\text{TEM-1}} \), as part of the \( \text{Tn}3 \) transposon, and \( \text{qnrS1} \) were found.

In conclusion, to the best of our knowledge, these 5 isolates were the first \( \text{mcr}-9 \)-positive bacteria of clinical origin identified in the Czech Republic (Fig. S1). Previous reports from the Czech Republic described the emergence of the \( \text{mcr}-1.1 \) allele in \( \text{Enterobacterales} \) recovered from retail meat and the \( \text{mcr}-4.3 \) allele in an \( \text{Acinetobacter baumannii} \) strain isolated from a clinical sample (26, 27). Despite the fact that all 5 \( \text{mcr}-9 \)-carrying isolates were colistin susceptible, the identification of these isolates highlights the risk for the hidden spread of important resistance determinants such as plasmid-mediated colistin resistance genes. Additionally, these 5 isolates cocarried the carbapenemase-encoding gene \( \text{bla}_{\text{VIM}} \) and several other resistance genes that conferred resistance to aminoglycosides, tetracyclines, trimethoprim, chloramphenicol, sulfonamides, quinolones, and/or macrolides (Table 1), limiting therapeutic choices.

Based on epidemiological data, the 5 \( \text{mcr}-9 \)-carrying isolates were recovered from three different hospitals, with two of them belonging to the same territory, suggesting three independent insertion events of MCR resistance mechanisms in Czech hospitals. In agreement with epidemiological data, the genomic data confirmed this suggestion. \( \text{E. hormaechei} \) isolates \( \text{ENCL48212}, \text{ENCL48946}, \) and \( \text{ENCL49790} \) belonged to the same sequence type (\( \text{ST}106 \)) and harbored similar \( \text{IncHI2} \) plasmids carrying \( \text{mcr}-9.1 \) and similar \( \text{pKPC-CAV1193} \)-like plasmids carrying \( \text{bla}_{\text{VIM-1}} \). On the other hand, the \( \text{C. freundii} \) \( \text{CIFR51929} \) isolate included an \( \text{IncHI2} \) plasmid cocarrying \( \text{mcr}-9.1 \) and \( \text{bla}_{\text{VIM-4}} \) resistance genes. In plasmid \( \text{p51929}_{\text{MCR-}_{\text{VIM}}} \), the \( \text{bla}_{\text{VIM-4}} \) gene was part of the class 1 integron \( \text{In}1174 \). Finally, the \( \text{E. hormaechei} \) isolate \( \text{ENCL48880} \), which belonged to \( \text{ST}764 \), harbored the \( \text{mcr}-9.2 \) and \( \text{bla}_{\text{VIM-4}} \) genes localized on \( \text{IncHI2} \) plasmid, \( \text{p48880}_{\text{MCR-}_{\text{VIM}}} \). In \( \text{p48880}_{\text{MCR-}_{\text{VIM}}} \), the \( \text{mcr}-9.2 \) allele was found in a slightly different genetic environment than the \( \text{mcr}-9.1 \) allele in \( \text{p48212}_{\text{MCR}}, \text{p48946}_{\text{MCR}}, \) and \( \text{p49790}_{\text{MCR}} \), and \( \text{p51929}_{\text{MCR-}_{\text{VIM}}} \). Unlike \( \text{p51929}_{\text{MCR-}_{\text{VIM}}} \), the \( \text{bla}_{\text{VIM-4}} \) gene was part of the class 1 integron \( \text{In}416 \) in \( \text{p48880}_{\text{MCR-}_{\text{VIM}}} \).

The association of the \( \text{IncHI2} \) plasmid group with \( \text{mcr}-1 \) or \( \text{mcr}-9 \) genes has been frequently reported (21, 28). However, the carriage of the \( \text{bla}_{\text{VIM-1}} \) gene on \( \text{pKPC-CAV1193} \)-like plasmids was described for the first time. Interestingly, MDR regions of the \( \text{bla}_{\text{VIM-1}} \)-carrying plasmids \( \text{p48212}_{\text{VIM}}, \text{p48946}_{\text{VIM}}, \) and \( \text{p49790}_{\text{VIM}} \) exhibited extensive similarity to the respective regions of plasmid \( \text{pKpn-431cz} \) (Fig. 2), previously described from \( \text{Enterobacterales} \) recovered from Czech hospitals (25). Thus, the acquisition of the \( \text{bla}_{\text{VIM-1}} \)-carrying MDR region from \( \text{pKpn-431cz} \) by a \( \text{pKPC-CAV1193} \)-like plasmid is a plausible hypothesis regarding the formation of \( \text{p48212}_{\text{VIM}}, \text{p48946}_{\text{VIM}}, \) and \( \text{p49790}_{\text{VIM}} \) plasmids. These findings highlight the ongoing evolution of mobile elements implicated in the dissemination of clinically important resistance determinants.

Data availability. The genomes and plasmids of \( \text{ENCL48212}, \text{ENCL46946}, \text{ENCL49790}, \) \( \text{ENCL48880}, \) and \( \text{CIFR51929} \) have been deposited in GenBank under accession no. \( \text{CP059413} \) to \( \text{CP059417}, \text{JACEHD010000001} \) to \( \text{JACEHD010000006}, \text{CP059422} \) to \( \text{CP059426}, \text{CP059418} \) to \( \text{CP059421}, \) and \( \text{CP059427} \) to \( \text{CP059429} \), respectively.
SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

FIG S1, PDF file, 0.2 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.

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