Characterization of the global distribution and diversified plasmid reservoirs of the colistin resistance gene \textit{mcr-9}

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The emergence and spread of mobilized colistin resistance \textit{(mcr)} genes have triggered extensive concerns worldwide. Here, we characterized the global distribution of \textit{mcr}-9, a newly-identified variant of \textit{mcr}, by assembling the data set of \textit{mcr}-9-positive isolates from GenBank database and the literature available. Genetic features of all the \textit{mcr}-9-harboring plasmids were determined by bioinformatic analysis. We showed that \textit{mcr}-9 is globally distributed in 21 countries across six continents, with a wide dissemination among various species of \textit{Enterobacteriaceae} strains from human, animal, food and environment. IncHI2-ST1 plasmids were found to be the predominant replicon type carrying \textit{mcr}-9. Comparative genomics highlighted that IncHI2-type plasmids may also serve as a critical reservoir of \textit{mcr}-9, from which different types of circulating plasmids acquired the \textit{mcr}-9. Results revealed that the \textit{rcnR-rcnA-pcoE-pcoS-IS\textsuperscript{903}-mcr-9-wbuC} structure was consistent in most \textit{mcr}-9 cassettes, suggesting a relatively unitary model involved in the mobilization of \textit{mcr}-9. It is most likely that the spread of \textit{mcr}-9 was mainly attributed to the conjugation and recombination events of \textit{mcr-9}-carrying plasmids. In summary, our results provide a comprehensive picture of the distribution and genetic environment of \textit{mcr}-9, and demonstrate the central roles played by IncHI2 plasmids in the worldwide dissemination of \textit{mcr}-9.

Antibiotic resistance poses a great threat to global public health and carbapenem-resistant \textit{Enterobacteriaceae} is triggering a health crisis worldwide\textsuperscript{1,2}. Colistin, a cationic cyclic-peptide, is one of the last-resort antibiotics to defend against severe infections caused by carbapenem-resistant \textit{Enterobacteriaceae}\textsuperscript{3}. However, since the initial discovery of a plasmid-mediated mobilized colistin resistance gene \textit{(mcr-1)} in China in late 2015\textsuperscript{4}, a number of diversified bacterial strains carrying \textit{mcr-1} have been detected across over 50 countries covering six continents\textsuperscript{5}. The prevalent plasmid-borne MCR enzyme can catalyze chemical addition of phosphoethanolamine to lipid A moiety of bacterial lipopolysaccharides, the target of colistin, which consequently promotes colistin resistance\textsuperscript{6}.

In recent years, a growing number of \textit{mcr}-like genes (namely, from \textit{mcr-2} to \textit{mcr-10}) have been identified\textsuperscript{7–14}. These ongoing discoveries indicate a rapid evolution of MCR family under selective pressures, which raise global health concerns. \textit{mcr}-9 is a newly emerging variant of the mobilized colistin resistance determinants, which was first identified in a clinical \textit{Salmonella enterica} isolate in the USA in May, 2019\textsuperscript{13}. Since its initial identification, \textit{mcr}-9 has been reported in several other countries, such as China\textsuperscript{13}, Sweden\textsuperscript{14}, and France\textsuperscript{15}. Not only that, \textit{in silico} analysis using the GenBank database indicated that \textit{mcr-9} had already been presented in a number of \textit{Enterobacteriaceae} isolates recovered worldwide\textsuperscript{13,17}. The high prevalence of \textit{mcr-9} suggests one more threat to public health. However, little information is available about the global epidemiology and dissemination patterns of \textit{mcr-9}.

To explore these issues, here we studied the geographic and host distribution of \textit{mcr-9}-carrying strains, and investigated the genomic features of various types of \textit{mcr-9}-harboring plasmids from an extensive collection of publicly available sequence data sourced from the NCBI repository by bioinformatics analysis. Our findings may contribute to a better understanding of the prevalence and dissemination of the newly identified \textit{mcr-9} gene, and be helpful for developing better strategies to manage its spread.

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Results and discussion

Geographic and isolation source of mcr-9. To obtain a comprehensive picture of the distribution of mcr-9, we blasted for mcr-9 in the NCBI GenBank database (as of 12th February 2020, n = 78) using a 99% identity cutoff and retrieved 71 complete plasmids and 6 chromosomes containing a full-length hit to mcr-9. A systematic review of the literature on mcr-9 published until 12 February 2020 resulted in the inclusion of 6 articles\textsuperscript{13,15–19}. As a result, a total of 138 mcr-9-harboring strains were included for statistics. The statistical results showed that isolates carrying mcr-9 were identified in 21 countries across six continents (Fig. 1a), including Europe (n = 72; 52.2%), Asia (n = 27; 19.6%), North-America (n = 27; 19.6%), Oceania (n = 10; 7.2%), South-America (n = 1; 0.7%) and Africa (n = 1; 0.7%). The large number of mcr-9-positive isolates from Europe was notable, which can be largely ascribed to the inclusion of 30 isolates from horses in Sweden\textsuperscript{16} and 28 from human in Europe by a previous clinical surveillance program\textsuperscript{18}. Anyway, the high incidence of mcr-9 in Europe is alarming, which demands more attention. In addition, the prevalence of mcr-9 around the world might be underestimated, due to the limited availability of its epidemiological investigations. Large-scale surveillance and molecular epidemiological studies are urgently required to better understand the global distribution and dissemination of mcr-9, thereby facilitating establishment of effective treatments to control its spread.

According to the current data, various kinds of Enterobacteriaceae isolates were found to disseminate mcr-9, among which Enterobacter spp. were the most common ones (n = 51, 37.0%), followed by Klebsiella spp. (n = 41, 30.0%), Salmonella spp. (n = 14, 10.1%), Escherichia spp. (n = 13, 9.4%), Citrobacter spp. (n = 9, 6.5%), Leclercia spp. (n = 6, 4.3%), Cronobacter spp. (n = 2, 1.4%), Raoultella spp. (n = 1, 0.7%), and Phytobacter spp. (n = 1, 0.7%) (Fig. 1b). Of these mcr-9 isolates, 77 were of human origin (55.8%), 40 of animal origin (29.0%), 5 of environment origin (3.6%) and 4 strains (2.9%) were isolated from food. The source for 12 isolates (8.7%) could not be obtained for an incomplete strain information (Fig. 1c). These data point towards a widespread dissemination of mcr-9 among clinically important pathogens, which has serious implications on clinical therapies and public health.

Figure 1. Overview of the distribution of mcr-9. (a) Worldwide distribution of mcr-9-positive isolates. The map was generated using the online software dituhui (https://g.dituhui.com/). (b) Distribution of host species harboring mcr-9. (c) Distribution of the isolation source of mcr-9-positive strains.
Active surveillance for clinical discovery of mcr-9-harborin pathogens is needed to provide a clue to defend against infections by superbugs with colistin resistance.

**Overall landscape of the mcr-9-carrying plasmids.** Seventy-one mcr-9-carrying plasmids with complete sequences were retrieved from the GenBank database (accessed on 12th February 2020, Table S1). Among them, IncHI2 was the dominant replicon type carrying mcr-9, accounting for 90.1% (64/71) of the plasmids. Of the 64 IncHI2 mcr-9-carrying plasmids, 60 plasmids had the IncHI2 replicon alone, 3 had IncHI2 plus IncR, and 1 had IncHI2 plus IncA/C2, with plasmid size varying from 222 kb to 477 kb. We found that mcr-9 plasmids with IncHI2 replicon alone had a worldwide distribution, while plasmids containing both IncHI2 and IncR replicons had only been recovered from China, suggesting that IncHI2-IncR hybrid plasmids may represent another important vehicle in mediating the dissemination of mcr-9 in China. Phylogenetic analysis showed that three IncHI2-IncR hybrid plasmids, pMCR-SCNJ07 (accession no. MK933279), pT5282-mphA (KY270852) and pN1863-HI2 (MF344583) were clustered with several IncHI2 plasmids (Fig. 2), implying that they may have diverged from a common ancestor and a subsequent genetic recombination event leading to the formation of hybrid plasmids. And, the IncHI2-IncA/C2 hybrid plasmid pGM14-002_1 (accession no. CP028197), which was recovered from a Salmonella enterica isolate in Czech Republic in 2018, followed a similar pattern. From the phylogenetic tree, we also found that mcr-9-harborin plasmids recovered from human isolates were interspersed with plasmids from animals and the environment (Fig. 2), indicating that plasmid-borne mcr-9 might have circulated in the entire ecosystem.

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IncHI2-type plasmids have a broad host range, which represent one of the most commonly encountered plasmid groups in the Enterobacteriaceae family. Among them, IncHI2-ST1 plasmids are known to disseminate clinically important antimicrobial resistance genes and are most frequently reported to play a critical role in the evolution of complex resistance phenotypes within disease-causing strains of *Enterobacteriaceae*. Our presentation here highlighted that IncHI2-ST1 plasmids are a major vehicle carrying *mcr*-9 around the world using the pMLST tool. In addition to IncHI2 plasmids, there was one IncFII (FIA-FIB) plasmid p1_045523 (accession no. CP032893) carrying *mcr*-9. And, the replicon type for 6 *mcr*-9-bearing plasmids could not be determined, with plasmid size ranging from 56 kb to 133 kb. These findings revealed that multiple types of plasmids are involved in the spread of *mcr*-9.

IncHI2 plasmids are known carriers of determinants for resistance not only to antibiotics such as β-lactams, quinolones and aminoglycosides, but also to heavy metals such as copper, silver ions, and mercuric ions. Analysis of the resistance genes revealed that IncHI2 *mcr*-9-carrying plasmids contained between 0 and 5 additional ESBL or carbapenemase genes (Table S1). Among these resistance genes, *bla*TEM1 was the most common one (32/71, 45.1%) that co-existed with *mcr*-9, followed by *bla*TEM2 (29/71, 40.8%). In particular, 3 out of the 64 (35.9%) IncHI2 *mcr*-9-carrying plasmids harbored one of the carbapenemase genes, namely *bla*TEM, *bla*SHV, *bla*CARB, and *bla*OXA-48. By contrast, transfer of the colistin resistance and carbapenemase genes by a single plasmid could raise the risk of dissemination of these resistance genes, which is of great concern for clinical therapies.

### Genetic characterization of plasmids carrying *mcr*-9

Comparative analysis was performed to obtain a comprehensive view of the genetic features of 60 IncHI2-type *mcr*-9-bearing plasmids (hybrid plasmids were excluded). Results showed that these plasmids were diverse in terms of genetic structure, except that *tra1* and *tra2* regions as well as the region between *mcr*-9 and the nearest region of *tra1* were conserved among all the IncHI2 plasmids. Searches for IncHI2 *mcr*-9-bearing plasmids deposited in GenBank suggested that IncHI2 plasmids are the most prevalent and are spread widely around the world using the pMLST tool. In addition, our present analysis highlighted a leading role of IncHI2-type plasmid in the dissemination of *mcr*-9. Analysis on the plasmid reservoirs of *mcr*-9 highlighted a leading role of IncHI2-type plasmid in the dissemination of *mcr*-9. Searches for IncHI2 *mcr*-harboring plasmids deposited in GenBank suggested that IncHI2 plasmids also served as an important vehicle in carrying *mcr*-1 and *mcr*-3. Unlike the scenario seen with the *mcr*-1 or *mcr*-3, which was inserted into different genetic loci in IncHI2 plasmids, *mcr*-9 was consistently located in the sil-cop region. This finding also suggests that *mcr*-9 was inactive during dynamic gene transposition.
**Figure 3.** Genetic characterization of IncH12-type mcr-9-harboring plasmids (hybrid plasmids are excluded). (a) Sequence alignment of 60 IncH12 mcr-9 plasmids. pRH-R27(accession no. LN555650) was used as a reference to compare with other plasmids. Gaps indicate regions that were missing in the respective plasmid compared to the reference plasmid. The outer circle with dark blue arrows denotes annotation of reference plasmid. mcr-9 gene is highlighted by red arrows. Backbone (tra1 and tra2 region) and four accessory resistance regions (ter region, the sil–cop region, MDR region 1 and MDR region 2) are indicated by red curves. The mcr-9-harboring region is indicated by the dotted box. Information about the IncH12 plasmids tested is provided in Table S1. (b) Linear comparison of the mcr-9-harboring region in pRH-R27, p17277A_477 (CP043927) and pMRVIM0813 (KP975077). The corresponding region on non-mcr-9-carrying plasmid R478 (BX664015, top) is shown for comparison. Grey shading denotes regions of shared homology among different plasmids ranging from 80% to 100%. Colored arrows represent open reading frames, with brown, green, and red arrows representing heavy metal resistance genes, mobile elements, and the mcr-9 gene, respectively. Other plasmid backbone genes and hypothetical genes are colored gray.
events, suggesting that transposon elements of mcr-9 could be lost soon after the mobilization of mcr-9 onto the IncH? backbone.

To further characterize the genetic contexts of mcr-9, representative mcr-9-harboring regions from different types of plasmids and chromosomes were subjected to comparison analysis. mcr-9 was found in a variety of genetic contexts, which were differed by the truncation status of neighboring genes (from 

\[ \text{rcnR} \] to \[ \text{qseB} \]) encompassing mcr-9. Results also revealed that the core structure of all known mcr-9 cassettes was \[ \text{rcnR-rcnA-pcoE-pcoS-IS903-mcr-9-wbuC} \] (Fig. 6), and it was identical in most (86.0%, 61/71) of the study plasmids, indicating that these core elements encompassing mcr-9 are most likely to be required for the evolution of mcr-9 cassettes. We failed to identify potential transposon elements (intact or truncated) mediating the transfer of mcr-9, posing the high possibility that conjugation and recombination events of mcr-9-carrying plasmids are mainly responsible for the spread of mcr-9. The lacking of the downstream two-component regulatory genes \[ qseC \] and \[ qseB \], which were proposed to be involved in the induction of colistin resistance mediated by mcr-9,
was commonly observed among the *mcr-9*-harboring plasmids (Figs. 3–6). More research should be performed to confirm the essential role of *qseC*-*qseB* module or the involvement of other genes in *mcr-9* induction.

**Conclusion**

In conclusion, we assembled the data set to date of *mcr-9*-positive sequenced plasmids and chromosomes through an exhaustive search of publicly available databases. This study allowed us to obtain a clear picture of the global distribution of *mcr-9*-harboring isolates, which covered 21 countries across six continents and were distributed in at least 9 *Enterobacteriaceae* species of diverse origins. The co-transfer of *mcr-9* and carbapenemase genes in clinically important pathogens posed a high risk to the public health and clinical treatment. IncHII-ST1 plasmid was found to be the predominant replicon type carrying *mcr-9*, implying that IncHII-ST1 plasmids may be a major

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**Figure 5.** Genetic characterization of IncFII-type *mcr-9*-carrying plasmid p1_045523 and six untyped *mcr-9*-carrying plasmids. (a) Comparison of the p1_045523 with the IncHI2 plasmid pRH-R27 and IncFII plasmid pOZ172. p1_045523 was used as a reference to compare with other plasmids. (b) Linear comparison of the *mcr-9*-harboring region in pRH-R27 and p1_045523. (c) Comparative genome maps of pCAV1099-114 (accession no. CP011596), pCAV1135-115 (CP011617), pCAV1015-114 (CP017930), pSHV12-1301491 (CP031568), pCFSAN002050 (CP006057), pLEC-b38d (CP026168) and the IncHII plasmid pRH-R27. pCAV1099-114 was used as a reference to compare with other plasmids. (d) Linear comparison of the *mcr-9*-harboring region in six untyped *mcr-9*-carrying plasmids and pRH-R27. The outer circle with dark blue arrows denotes annotation of reference plasmid. Gaps in the circular maps refer to plasmid regions that were missing in the respective plasmid compared to the reference plasmid. *mcr-9* gene is highlighted by red arrows. The *mcr-9*-harboring region is indicated by the dotted box. Colored arrows represent open reading frames, with brown, green, and red arrows representing heavy metal resistance genes, mobile elements, and the *mcr-9* gene, respectively. The remaining genes are shown in gray. Grey shading in the linear maps denotes regions of shared homology among different plasmids ranging from 80% to 100%.
vehicle in mediating the dissemination \( mcr-9 \). Besides, our results extended significantly our understanding of genetic features of different types of \( mcr-9 \)-bearing plasmids, and highlighted that IncHI2-type plasmids serve as a critical reservoir of \( mcr-9 \). In addition, the highly similarity of \( mcr-9 \) cassette in different plasmids posed the possibility that the dissemination of \( mcr-9 \) across bacterial populations was mainly mediated by conjugation and recombination events of \( mcr-9 \)-carrying plasmids. A limitation of this study lies in that the phenotypes of the \( mcr-9 \)-harboring strains are not available, which makes it difficult to correlate phenotypic with genotypic features. Whole-genome sequencing based surveillance and epidemiological studies are needed to achieve better insights into the genetic features of \( mcr-9 \)-positive strains, which might eventually allow us to develop effective strategies to manage their spread.

**Materials and Methods**

**Retrieval of genomic data and literature search.** To obtain the \( mcr-9 \)-harboring genome sequences, a nucleotide BLAST with standard options was performed in the NCBI GenBank database with the nucleotide base sequence of \( mcr-9 \) as a query (GenBank accession no. MK791138). Models and uncultured environmental samples were excluded. Complete plasmids and chromosomes containing at least one contig with a full-length hit to \( mcr-9 \) with 100% query coverage and at least 99% identity were selected and exported. Information about
geographic distribution and isolation source of the mcr-9-positive strains were investigated. Additionally, relevant papers that published on mcr-9 were identified in PubMed and Google Scholar using the query ‘mcr-9 AND colistin, and non-repetitive information of the geographic and isolation source of mcr-9 strains were included in the statistics.

Phylogenetic analysis. The complete sequences of all available mcr-9-carrying plasmids as of 12th February 2020 were retrieved from the GenBank. The plasmid replicon type and MLST were determined using the PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and pMLST (https://cge.cbs.dtu.dk/services/pMLST/). ResFinder (http://genomiccepidemiology.org/) was employed for the identification of antimicrobial resistance genes. All the IncHI2 mcr-9-carrying plasmids were used for phylogenetic analysis using Parsnp in the Harvest package, and the phylogenetic tree was visualized by iTOL (https://itol.embl.de/).

Comparative analysis of mcr-9-harboring genome sequences. The annotations of mcr-9-carrying plasmids were performed using Prokka combined with BLASTp/BLASTn searches against the NCBI database. Alignments among highly homologous complete plasmid sequences were performed using BLAST and visualized with EasyFig v 2.2.3. Sequence alignments among mcr-9-carrying plasmids or chromosomes were performed using BLAST and visualized with Easyfig v 2.2.3.

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Author contributions
L.Z. designed the study. X.D. and J.Z. collected the data. Y.G. and Z.Z. analyzed and interpreted the data. Y.L. wrote the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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