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The global distribution and spread of the mobilized colistin resistance gene \textit{mcr-1}

Ruobing Wang\textsuperscript{1}, Lucy van Dorp\textsuperscript{2}, Liam P. Shaw\textsuperscript{2}, Phelim Bradley\textsuperscript{3}, Qi Wang\textsuperscript{1}, Xiaojuan Wang\textsuperscript{1}, Longyang Jin\textsuperscript{1}, Qing Zhang\textsuperscript{4}, Yuqing Liu\textsuperscript{4}, Adrien Rieux\textsuperscript{5}, Thamarai Dorai-Schneiders\textsuperscript{6}, Lucy Anne Weinert\textsuperscript{7}, Zamin Iqbal\textsuperscript{3,8}, Xavier Didelot\textsuperscript{9}, Hui Wang\textsuperscript{1} & Francois Balloux\textsuperscript{2}

Colistin represents one of the few available drugs for treating infections caused by carbapenem-resistant \textit{Enterobacteriaceae}. As such, the recent plasmid-mediated spread of the colistin resistance gene \textit{mcr-1} poses a significant public health threat, requiring global monitoring and surveillance. Here, we characterize the global distribution of \textit{mcr-1} using a data set of 457 \textit{mcr-1}-positive sequenced isolates. We find \textit{mcr-1} in various plasmid types but identify an immediate background common to all \textit{mcr-1} sequences. Our analyses establish that all \textit{mcr-1} elements in circulation descend from the same initial mobilization of \textit{mcr-1} by an \textit{IS}A\textsubscript{pl1} transposon in the mid 2000s (2002–2008; 95\% highest posterior density), followed by a marked demographic expansion, which led to its current global distribution. Our results provide the first systematic phylogenetic analysis of the origin and spread of \textit{mcr-1}, and emphasize the importance of understanding the movement of antibiotic resistance genes across multiple levels of genomic organization.

\textsuperscript{1}Department of Clinical Laboratory, Peking University People’s Hospital, Beijing 100044, China. \textsuperscript{2}UCL Genetics Institute, University College London, Gower Street, London WC1E 6BT, UK. \textsuperscript{3}Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK. \textsuperscript{4}Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Sciences, Shandong Province, Jinan 250100, China. \textsuperscript{5}UMR PVBMT, CIRAD, 97410 St Pierre, Reunion, France. \textsuperscript{6}Division of Infection and Pathway Medicine, 49 Little France Crescent, Edinburgh EH16 4SB, UK. \textsuperscript{7}Department of Veterinary Medicine, Cambridge CB3 0ES, UK. \textsuperscript{8}European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), Wellcome Genome Campus, Cambridge CB10 1SD, UK. \textsuperscript{9}Department of Infectious Disease Epidemiology, Imperial College London, Norfolk Place 21, London W2 1PG, UK. These authors contributed equally: Ruobing Wang, Lucy van Dorp, Liam P. Shaw. Correspondence and requests for materials should be addressed to H.W. (email: wanghui@pkuph.edu.cn) or to F.B. (email: f.balloux@ucl.ac.uk)
Colistin was largely abandoned as a treatment for bacterial infections in the 1970s owing to its toxicity and low renal clearance, but has been reintroduced in recent years as an antibiotic of ‘last resort’ against multi-drug-resistant infections. It is therefore alarming that the prevalence of resistance to colistin has become a significant concern, following the identification of plasmid-mediated colistin resistance conferred by the mcr-1 gene in late 2015. Colistin resistance is emblematic of the growing problems of antimicrobial resistance worldwide.

Up until 2015, resistance to colistin had only been linked to mutational and regulatory changes mediated by chromosomal genes. The mobilized colistin gene mcr-1 was first described in a plasmid carried by an Escherichia coli isolated in China in April 2011. The presence of colistin resistance on mobile genetic elements poses a significant public health risk, as these can spread rapidly by horizontal transfer, and may entail a lower fitness cost. At the time of writing, mcr-1 has been identified in numerous countries across five continents. Significantly, mcr-1 has also been observed on plasmids containing other antimicrobial resistance genes such as carbapenemases and extended-spectrum β-lactamases and extended-spectrum β-lactamases.

The mcr-1 element has been characterized in a variety of genomic backgrounds, consistent with the gene being mobilized by a transposon. To date, mcr-1 has been observed on a wide variety of plasmid types, including IncI2, IncFII, and IncX4. Intensive screening efforts for mcr-1 have found it to be highly prevalent in a number of environmental settings, including the Haihe River in China, recreational water at public urban beaches in Brazil, and fecal samples from otherwise healthy individuals. Although both Brazil and China have now banned the use of colistin in agriculture, the evidence that mcr-1 can spread within hospital environments even in the absence of colistin use raises the possibility that the spread of mcr-1 will not be contained by these bans. The global distribution of mcr-1 over at least five continents is well documented, but little is known about its origin, acquisition, emergence, and spread. In this study, we aim to shed light on these fundamental issues using whole-genome sequencing (WGS) data from 110 novel mcr-1-positive isolates from China in conjunction with an extensive collection of publicly available sequence data sourced from the NCBI repository as well as the Short Read Archive (SRA).

Our data and analyses support an initial single mobilization event of mcr-1 by an ISAAsmcr-1-orfISAAsmcr-1 transposon around 2006. The transposon was immobilized on several plasmid backgrounds following the loss of the flanking ISAAsmcr-1 elements, and spread through plasmid transfer. The current distribution of mcr-1 points to a possible origin in Chinese livestock. Our results illustrate the complex dynamics of antibiotic resistance genes across multiple embedded genetic levels (transposons, plasmids, bacterial lineages and bacterial species), previously described as a nested ‘Russian doll’ model of genetic mobility.

Evolutionary model. It has been proposed that mcr-1 is mobilized by a composite transposon formed of a ~2600 bp region containing mcr-1 (1626 bp) and a putative open reading frame encoding a PAP2 superfamily protein (765 bp), flanked by two ISAAsmcr-1 insertion sequences. ISAAsmcr-1 is a member of the IS30 family of insertion sequences, which utilize a ‘copy-out, paste-in’ mechanism with a targeted transposition pathway requiring the formation of a synaptic complex between an inverted repeat (IR) in the transposon circle and an IR-like sequence in the target. Snesrud and colleagues hypothesized that after the initial formation of such a composite transposon, these insertion sequences would have been lost over time, leading to the stabilization of mcr-1 in a diverse range of plasmid backgrounds. In the following, we sought to test this model by performing an explicit phylogenetic analysis of the region surrounding mcr-1 using our comprehensive global data set.

Immediate genomic background of mcr-1. If there had been a unique formation event for the composite transposon, followed by progressive transposition and loss of insertion sequences, we would expect to be able to identify a common immediate background region for mcr-1 in all samples. Indeed, we were able to identify and align a shared region or remnants of it in all 457 sequences surrounding mcr-1 (see Methods), supporting a single common origin for all mcr-1 elements sequenced to date. The majority of the sequences contained no trace of ISAAsmcr-1 (n = 260) indicating that the mcr-1 transposon had been completely stabilized in their genomic background. Forty-two sequences contained indication of the presence of ISAAsmcr-1 both upstream and downstream, either in full copies (n = 16), a full copy upstream and a partial copy downstream (n = 7), a partial copy upstream and a full copy downstream (n = 1), or partial copies upstream and downstream (n = 18). Some sequences only had ISAAsmcr-1 present upstream as a complete (n = 55) or partial (n = 99) sequence, and one sequence had only a partial downstream ISAAsmcr-1 element. The downstream copy of ISAAsmcr-1 was inverted in some sequences (n = 3) and some sequences had full assemblies. Accession numbers and metadata for the 457 isolates are provided in supplementary data 1.
copies of ISApl1 present elsewhere on the same contig \((n = 7)\), consistent with its high observed activity in transposition\(^{24}\).

Further inspection of the transposon alignment revealed that the 186 bp region between the 3'-end of the upstream ISApl1 and mcr-1 contained IR-like sequences similar to the IRR and IRL of ISApl1 (respectively: 93–142 bp, 23/50 identity; and 125–175 bp, 21/50 identity). The most variable positions in this 186 bp region were at 177 bp and 142 bp, approximately coinciding with the end of the alignment with the IRs and were more variable in sequences lacking ISApl1, suggesting possible loss of function of the transposition pathway associated with ISApl1 (Fig. 3d). Some of these SNPs occurred in a stretch previously identified as the promoter region for mcr-1\(^{25}\), and this region showed strong signals of recombination. A small number of sequences (3%) had SNPs present in mcr-1 itself. These tended to be at the upstream/5'-end of the sequence, particularly in the first three positions. A subset of the sequences from Vietnam \((n = 28)\) included a secondary 1.7 kb insertion downstream of mcr-1 containing a putative transposase, indicating subsequent rearrangements involving this region after initial mobilization of the transposon (Fig. 3e).

To reconstruct the phylogenetic history of the composite mcr-1 transposon, we created a sequence alignment for 457 sequences (Fig. 3c) after removal of recombinant regions identified with ClonalFrameML, including the region immediately upstream of mcr-1 between positions 1212–1247 (Fig. 3d). A midpoint-rooted maximum parsimony phylogeny showed that there was a dominant sequence type with subsequent diversification, likely indicating the ancestral form of the composite transposon (Fig. 4). There was no discernible clustering of isolates by bacterial species.
(Supplementary Fig. 1) or sample source (Supplementary Fig. 2), suggesting the composite transposon does not evolve differently in these different backgrounds.

A Bayesian dating approach (BEAST) was applied to infer a timed phylogeny of the maximal alignable region of the mcr-1 carrying transposon (see Methods). Based on this 3522 site alignment we infer a common ancestor for 364 dated isolates in 2006 (Supplementary Fig. 3; 2002–2008 95% highest poster density (HPD) with a strict clock and coalescent model) with a mutation rate around $7.51 \times 10^{-3}$ substitutions per site per year (Supplementary Table 1). There was no clear overall geographic clustering in the Maximum Clade Credibility (MCC) tree (Supplementary Figure 4).

Wider genomic background of mcr-1. Next, we explored the wider genomic background upstream and downstream of the conserved transposon sequences. We had sufficiently long assembled contigs for 182 isolates to identify plasmid types based on co-occurrence with plasmid replicons (see Methods) and identified mcr-1 in 13 different plasmid backgrounds. IncI2 and IncX4 were the dominant plasmid types, accounting for 51 and 38% of the isolates, respectively (Fig. 5) similar to the proportions observed by Matamoros et al. One isolate in our data set was highly heterogeneous across these plasmid types. For example, sequences with one or two copies of ISApl1 were found on six and four types, respectively, which supports their mobility compared with those without ISApl1, which were found in five plasmid types. Of the contigs carrying one copy of ISApl1, 61% were found in IncI2 plasmids, and 50% of contigs carrying two copies of ISApl1 belonged to IncHI2 plasmids. Conversely, the common IncX4 plasmids carried only two transposons with two copies of ISApl1 and none with a single copy of the element.

We identified two extended plasmid backbone sequences that could be aligned. The first such alignment encompasses a shared sequence of 7161 bp between 108 plasmid backgrounds and has been previously referred to as 'Type A'. These sequences contain 54 sequences co-occurring with an IncI2 replicon, with 54 of unknown plasmid type, and encompass a large fraction of the genetic diversity found in the mcr-1 transposon, although a large proportion (9/108) belonged to the dominant sequence type (i.e., B07_1_WFA61 in Fig. 4). The second alignment is 34,761 bp long and is common to nine IncX4 plasmids and partly overlaps with a background previously defined as 'Type D'.

We applied BEAST to infer a timed phylogeny for each of these alignable regions after removal of SNPs showing evidence of recombination. For the IncI2 background we infer that a common ancestor to all 108 isolates existed in 2006 (1998–2010 95% CI relaxed exponential clock model) assuming a constant population size model (Supplementary Fig. 5). For the IncX4 backgrounds we dated the common ancestor of the eight isolates to 2011 (2010–2013 95% CI relaxed exponential clock model) assuming a constant population size model (Supplementary Fig. 6). Posterior density distributions of root dating under different population models are shown in Supplementary Figs. 7–8. The difference in dating inferred for these two plasmid backgrounds and the recent date obtained for IncX4 highlight the dynamic nature of the integration of the mcr-1 carrying transposon, even if in the IncX4 phylogeny isolates from East Asia and Europe and the Americas cluster together. The inferred mutation rates obtained for the IncI2 and IncX4 backgrounds consistently lie around $5-10 \times 10^{-3}$ substitutions per site per year (Supplementary Table 1).

Environmental distribution of the composite mcr-1 transposon. It has been suggested that agricultural use of colistin, as has been widespread in China since the early 1980s, caused the initial emergence and spread of mcr-1. According to the evolutionary model in Fig. 2, the ancestral mobilizable state is represented by the transposon carrying both its ISApl1 elements. The transposon is thought to lose its capability for mobilization after the loss of both ISApl1 elements, although a single copy is reported to keep some ability to mobilize, with the upstream copy being functionally more important. Comparing human (n = 108) and non-human (n = 252) isolates, there were significantly more sequences with some trace of the insertion sequence ISApl1 both upstream and downstream in non-human isolates (32/220 vs. 5/108, $\chi^2$-test, p = 0.033). This comparison held when only comparing agricultural isolates to human isolates (n = 213) (28/213 vs. 5/108, $\chi^2$-test, p = 0.029). Furthermore, of the 42 isolates that have ISApl1 fragments both upstream and downstream, the majority were from Asia (n = 30) with only a quarter from Europe (n = 10) ($\chi^2$ test, p = 0.12). This result is not driven by an over representation of agricultural isolates from Asia in our data set ($\chi^2$ test, p = 0.38).

Discussion

We assembled a global data set of 457 mcr-1-positive sequenced isolates and could show that there was a single integration event of mcr-1 into an ISApl1 composite transposon, followed by its subsequent spread between multiple genomic backgrounds. Our phylogenetic analyses point to a date for the insertion of mcr-1 into the gene transposon shared across our isolates in the mid 2000s (2002–2008 95% HPD). We could identify the likely sequence of the ancestral transposon type and show the pattern of
diversity supports a single mobilization with subsequent diversification during global spread.

Despite the limited number of WGSs for samples before 2012, with the oldest sequence available from 2008 (Fig. 1c), our estimate is consistent with the majority of available evidence from retrospective surveillance data,26 which has found the presence of mcr-1 in samples dating back to 2005 in Europe.28 One retrospective study of Chinese isolates from 1970–2014 reported three mcr-1-positive E. coli dating from the 1980s,29 although mcr-1 then did not reappear until 2004. This observation seems surprising in light of our results, which clearly exclude such an early spread of mcr-1 at least on this ISAPl1 transposon background.

Our estimates of the age of spread of the representative IncI2 and IncX4 plasmid backgrounds are more recent, dating to ~2008 and 2013, respectively, but are both consistent with the age of the transposon mobilization event. We did not constrain the evolutionary rates in any of our phylogenetic analyses. It is thus encouraging that the different rates are highly consistent between the mcr-1 transposon and the two plasmid backgrounds. Although this points to high internal consistency between our estimates, we were surprisingly unable to find any previously published estimates for the evolutionary rate of bacterial plasmids.

The current distribution and observed genetic patterns are in line with a center of origin in China. This is the place where we observe the highest proportion of isolates carrying intact or partial copies of the ISAPl1 flanking elements. Transposon sequences carrying ISAPl1 elements were also overrepresented in environmental and agriculture isolates, relative to those collected from humans. This pattern is in line with agricultural settings acting as the source of mcr-1 within bacteria isolated from humans.21 The current global distribution has been achieved through multiple translocations, and is illustrated by the interspersed geographic origins in our phylogenetic reconstructions. A likely driver for the global spread is trade, in particular food animals and meat, although direct global movement by colonized or infected humans is also likely to have played a role in the current distribution.

The origin of mcr-1 prior to its mobilization remains elusive. Despite an exhaustive search of sequence repositories, including

Fig. 3 The genetic element carrying mcr-1 is a composite transposon and is alignable across our global data set. a Length distribution of the alignment across sequences. b Length distribution subset by plasmid type. c The composite transposon, consisting of ISAPl1, mcr-1, a PAP2 orf, and ISAPl1. The region indicated by the red arrow was used in phylogenetic analyses, after the removal of recombination. d The 186 bp region upstream of mcr-1 showed strong signals of recombination (gray box) that coincided with the promoter regions of mcr-1 (red box), and this diverse region was removed from the subsequent alignment. e Twenty-eight sequences from Vietnam had a 1.7 kb insertion containing a putative transpose, suggesting subsequent rearrangement after initial mobilization.
the short-read archive, we found not a single mcr-1 sequence outside the ISAp1 transposon background. ISAp1 was first identified in the pig pathogen *Actinobacillus pleuropneumoniae* suggesting that it may also have been an ancestral host for mcr-1, although to our knowledge no mcr-1-positive *A. pleuropneumoniae* isolates have been described. The phosphoethanolamine transferase from *Paenibacillus sophorae* has also been proposed as a possible candidate. However, this seems most unlikely as *Paenibacilli* are Gram-positive and are thus intrinsically resistant to polymixins. Moreover, although the two sequences share functional similarities, this should be interpreted as a case of possible parallel evolution rather than direct filiation. *Moraxella* has also been suggested as being the source of mcr-1, following the identification of genes in *Moraxella* with
limited homology to mcr-1 (~60% nucleotide sequence identity). However, this sequence identity is too low for Moraxella to be considered as viable candidates for the origin of mcr-1. The search for the initial source of mcr-1 remains open until a mcr-1 sequence is identified outside of the ISApl1 sequence background. We note that there are an increasing number of mobilized genes that can confer colistin resistance, with mcr-2 reported less than a year after mcr-1 was initially described\(^\text{35}\) and more recent descriptions of the phylogenetically distant mcr-3, mcr-4, and mcr-5\(^\text{36,38}\). There appear to be commonalities between the mechanisms of the mcr genes, despite their different sequences and locations near to different insertion sequences. For example, mcr-2 has 76.7% nucleotide identity to mcr-1 and was found in colistin-resistant isolates that did not contain mcr-1, and appeared to be mobilized on an IS1595 transposon\(^\text{35}\). Despite the genetic sequence data will become part of the routine toolbox in sophisticated computational tools and even more extensive at a global scale. We hope that future efforts relying on more phylogenetic reconstruction of antimicrobial resistance elements Nevertheless, we believe our results highlight the potential for mcr-1-positive sequenced isolates through our own sequencing efforts plasmids, and bacterial host limits our ability to reach strong available, the complex communities. Equally, although we took advantage of the most sophisticated bioinformatics and phylogenetic tools currently available, the complex 'Russian doll' dynamics of the transposon, plasmids, and bacterial host limits our ability to reach strong inferences on some important aspects of the spread of mcr-1. Nevertheless, we believe our results highlight the potential for phylogenetic reconstruction of antimicrobial resistance elements at a global scale. We hope that future efforts relying on more sophisticated computational tools and even more extensive genetic sequence data will become part of the routine toolbox in infectious disease surveillance.

### Methods

**Compilation of genomic data set.** We blasted for mcr-1 in all NCBI GenBank assemblies (as of 16th March 2017, n = 90,759) using a 98% identity cutoff. 195 recirculated multiple assemblies, 73 complete plasmids, 231 contigs, 73 complete chromosome assemblies) contained at least one contig with a full-length hit to mcr-1 (1626 bases). We only included samples with a single copy of mcr-1. The only isolate with multiple copies was a previously published isolate with three chromosomal copies of mcr-1 and seven copies of ISApl1\(^\text{35}\). We searched a snapshot of all WGS bacterial raw read data sets in the SRA (December 2016), looking for samples containing mcr-1 by using a k-mer index (k = 31), which we had previously constructed\(^\text{35}\); software available at: https://github.com/phemlib/bigkit. A total of 184 data sets were found to contain at least 70% of the 31-mers in mcr-1. After removing duplicates (i.e., those with a draft assembly available) we could assemble contigs with mcr-1 for 152 of these.

Our final data set comprised 457 isolates from six genera across 31 different countries, ranging in date from 2008 to 2017. Where only a year was provided as the date of isolate collection the date was set to the midpoint of that year. Whenever identified isolates did not comprise previously assembled genomes or complete plasmids, the raw fastq files were first inspected using FastQC and trimmed and filtered on a case-by-case basis. De novo assembly was then conducted using Plasmid SPADES 3.10.0 using the ‘careful’ switch and otherwise default parameters\(^\text{44}\). For those isolates sequenced using PacBio a different pipeline was employed. Correction, trimming, and assembly of raw reads was performed using Canu\(^\text{45}\) and assembled reads were corrected and trimmed using the tool Circulator\(^\text{46}\). The quality of resultant assemblies was assessed using infoso. In both cases the mcr-1 carrying contigs in this final data set were identified using blastn v2.2.31\(^\text{47}\).

We ran Plasmid Finder 1.348 with 95% identity to identify plasmid replications on the mcr-1 carrying contigs. In total, 182 unique contigs could be assigned a plasmid type using this method.

### Novel samples from China

We selected 110 mcr-1-positive isolates from China for WGS from a larger survey effort of both clinical and livestock isolates. A total of 2824 non-repetitive clinical isolates, including 1867 E. coli and 1187 K. pneumoniae were collected from 15 provinces of mainland China from 2011 to 2016. Seventy-two isolates were resistant to polymyxin B, comprising 40 E. coli and four K. pneumoniae carrying mcr-1. Livestock samples were collected from four provinces of China in 2013 and 2016. One broiler farm of the Shandong province provided chicken anal swabs, liver, heart, and wastewater isolated in 2013. In 2016, samples including feces, wastewater, anal swabs, and internal organs of sick livestock were collected from swine farms, cattle farms, and broiler farms in four provinces (Jilin, Shandong, Henan, and Guangdong). A total of 601 E. coli and 126 K. pneumoniae were isolated, of which 167 (137 E. coli and 30 K. pneumoniae) were resistant to polymyxin B. We detected mcr-1 in 135 E. coli and two K. pneumoniae, as well as in eight E. coli isolated from environmental samples, which were collected from influents and effluents of four tertiary care teaching hospitals.

Of all the isolates were sent to the microbiology laboratory of Peking University People’s Hospital and confirmed by routine biochemical tests, the Vitek system (bioMérieux, Hazelwood, MO, USA) and/or MALDI-TOF (Bruker Daltonics, Bremen, Germany). The minimum inhibitory concentrations (MICs) of polymyxin B was determined using the broth dilution method. The breakpoints of polymyxin B for Enterobacteriaceae were interpreted with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/clinical_breakpoints) guidelines. Colistin-resistant isolates (MIC of ≥2 μg/ml) were screened for mcr-1 by PCR and sequencing as described previously\(^\text{48}\).

### Identification and alignment of mcr-1 transposon

We searched for the mcr-1 carrying transposon region across isolates by blasting for its major components: ISApl1 (Actinobacillus pleuropneumoniae reference sequence: EF407820), mcr-1 (from E. coli plasmid pHNSHP45: KP347127.1), and short sequences representing the sequences immediately upstream and downstream of mcr-1 (from KP347127.1) using blastn-short. Contiguous sequences containing mcr-1 were aligned using Clustal Omega\(^\text{49}\) and then manually curated and amended using Jalview\(^\text{50}\), resulting in a 3679 bp alignment containing the common ~2600 bp identified by Snedrub and colleagues\(^\text{12}\). The downstream copy of ISApl1 was more often fragment or inverted. Twenty-eight isolates, which were all assemblies from the same study in Vietnam had a ~1.7 kb insertion downstream of mcr-1 (Fig. 3e) before the downstream ISApl1 element.

### Phylogenetic analyses

For constructing the transposon phylogeny, we excluded the downstream ISApl1 and the insertion sequence observed in a small number of samples, as well as regions identified as having signals of recombination by ClonalFrameML\(^\text{52}\), resulting in a 3532 bp alignment. We removed two homoplastic sites (requiring >1 change on the phylogeny), before constructing a maximum likelihood neighbor-joining tree based on the Harmoning distance between sequences. We calculated branch lengths using non-negative least squares with mns.phly in phangorn v2.2.0\(^\text{53}\). Phylogenies were visualized with ggtree v1.8.1\(^\text{54}\).

### Phylogenetic dating

Given recombination can conceal the clonal phylogenetic signal we also applied ClonalFrameML\(^\text{52}\) to identify regions of high recombination in a subset of IncI2 and IncX4 plasmid background alignments. Where recombination hotspots were identified, they were removed from the alignment. In the IncI2 alignment, two regions containing complete plasmid sequence were identified, one at the origin of transfer, one in the middle of the plasmid sequence. An alignment of IncI2 plasmids (7161 sites, 110 isolates) and IncX4 plasmids (34,761 sites, 8 isolates). Sequences were annotated using their known sampling times expressed in years. For both plasmid alignments, the HKY substitution model was selected based on evaluation of all possible substitution models in bModelTest. BEAST analyses were then applied under both a coalescent population model (coalcoastal Bayesian skyline implementation) and a divergent growth model (Coalcoastal Exponential population implementation). In addition, a strict clock, with a lognormal prior, and a relaxed clock (both lognormal and exponential) were tested. MCMC was run for 50,000,000 iterations sampling every 1000 steps and convergence was checked by inspecting the effective sample sizes and parameter value traces in the software Tracer v1.6.0. Analyses were repeated three times to ensure consistency between the obtained posterior distributions. Posterior trees for the best fitting model were combined in TreeAnnotator after a...
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Author contributions
H.W. and F.B. conceived the project and designed the experiments. R.W., Q.W., X.W., L. J. Q.Z., Y.L., and H.W. collected samples. R.W., Q.W., X.W., L.J., Q.Z., and Y.L. performed microbial identification, antimicrobial susceptibility testing, screening for mcr-1, and DNA extraction for WGS. R.W. Lv.D., L.P.S. assembled the new sequence data. Lv. D. and L.P.S. curated the global data set and performed the computational analyses. T.D.-S. advised on functional aspects of colistin resistance. A.R., L.A.W., and X.D. helped with the phylogenetic reconstructions. P.B. and Z.I. performed the search for mcr-1-positive samples on the Short Read Archive. H.W. takes responsibility for the accuracy and availability of the epidemiological and raw sequence data, and F.B. for all bioinformatics and computational methods and results. Lv.D., L.P.S., and F.B. wrote the paper with contributions from X.D. and H.W. All authors read and commented on successive drafts and all approved the content of the final version.

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