Journal of Antimicrobial Chemotherapy

Genetic characterization of *mcr-1*-bearing plasmids to depict molecular mechanisms underlying dissemination of the colistin resistance determinant

Ruichao Li[1,2]†, Miaomiao Xie[1]†, Jinfei Zhang[1], Zhiqiang Yang[1], Lizhang Liu[1], Xiaobo Liu[1], Zhiwei Zheng[1], Edward Wai-Chi Chan[2] and Sheng Chen[1,2]*

[1]*Shenzhen Key Lab for Food Biological Safety Control, Food Safety and Technology Research Center, Hong Kong PolyU Shen Zhen Research Institute, Shenzhen, P. R. China;* [2]*The State Key Lab of Chirosciences, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR*

*Corresponding author. Tel: +852-34008795; E-mail: sheng.chen@polyu.edu.hk
†These authors contributed equally to the work.

Objectives: To analyse and compare *mcr-1*-bearing plasmids from animal *Escherichia coli* isolates, and to investigate potential mechanisms underlying dissemination of *mcr-1*.

Methods: Ninety-seven ESBL-producing *E. coli* strains isolated from pig farms in China were screened for the *mcr-1* gene. Fifteen *mcr-1*-positive strains were subjected to molecular characterization and bioinformatic analysis of the *mcr-1*-bearing plasmids that they harboured.

Results: Three major types of *mcr-1*-bearing plasmids were recovered: IncX4 (~33 kb), IncI2 (~60 kb) and IncHI2 (~216–280 kb), among which the IncX4 and IncI2 plasmids were found to harbour the *mcr-1* gene only, whereas multiple resistance elements including bla_{CTX-M}, bla_{CMY}, bla_{TEM}, *fosA*, *qnrS*, *floR* and *oqxAB* were detected, in various combinations, alongside *mcr-1* in the IncHI2 plasmids. The profiles of *mcr-1*-bearing plasmids in the test strains were highly variable, with coexistence of two *mcr-1*-bearing plasmids being common. However, the MIC of colistin was not affected by the number of *mcr-1*-carrying plasmids harboured. Comparative analysis of the plasmids showed that they contained an *mcr-1* gene cassette with varied structures (*mcr-1-orf*, IS*Apl1-mcr-1-orf* and Tn*6330*), with the IncHI2 type being the most active in acquiring foreign resistance genes. A novel transposon, Tn*6330*, with the structure IS*Apl1-mcr-1-orf*-IS*Apl1* was found to be the key element mediating translocation of *mcr-1* into various plasmid backbones through formation of a circular intermediate.

Conclusions: The *mcr-1* gene can be disseminated via multiple mobile elements including Tn*6330*, its circular intermediate and plasmids harbouring such elements. It is often co-transmitted with other resistance determinants through IncHI2 plasmids. The functional mechanism of Tn*6330*, a typical composite transposon harbouring *mcr-1*, should be further investigated.

Introduction

The continuous emergence of novel antibiotic resistance-encoding genetic elements among the major bacterial pathogens in recent years has undermined current efforts to devise new antimicrobial strategies, and poses an enormous threat to public health.[1,2] Polymyxins, including polymyxin B and colistin, are cationic antimicrobial peptides which act on Gram-negative bacteria by disrupting the outer and inner membranes.[3,4] Polymyxins were discovered in the late 1940s but deemed unsuitable for treatment of bacterial infections because of their neurotoxic effects; however, emergence of MDR Gram-negative bacteria has prompted a renewed interest in this old antibiotic, which is currently regarded

as a possible last-resort agent to eradicate MDR organisms.[5,6] Resistance to colistin has been reported among different bacterial species, the underlying mechanism of which is mainly intrinsic in nature. On the other hand, acquired resistance due to modifications of the bacterial outer membrane, efflux pumps and capsular polysaccharides have also been reported.[3,7] Recently, pioneering work performed by Liu *et al.*[8] described the recovery of a conjugative plasmid-mediated polymyxin resistance gene *mcr-1*, which encodes an enzyme belonging to the phosphoethanolamine transferase enzyme family, from both animals and human.[8] Following this discovery, a number of studies have reported the presence of *mcr-1* in different species of Enterobacteriaceae which exhibited MDR phenotypes recovered from farmed animals,

food samples and clinical settings around the world.[9,10] The emergence of *mcr-1* has been traced back to *Escherichia coli* strains isolated in the 1980s when colistin was first introduced into veterinary practice in China,[11] indicating that this gene has existed in food animals for a considerable period.

Plasmids harbouring *mcr-1* have been reported to include the IncX4, IncI2, IncP, IncFII and IncHI2 types.[9,12–15] The prevalence of *mcr-1* in plasmids harboured by different bacterial species highlights its potential of being transferred horizontally. Notably, *mcr-1* was often found to be located downstream of IS*Apl1*,[13] which is an IS belonging to the IS*30* family.[16] The close genetic association between IS*Apl1* and *mcr-1* indicates that IS*Apl1* may play a pivotal role in the dissemination of *mcr-1*. Data regarding the complete sequences of *mcr-1*-bearing plasmids and the role of IS*Apl1* in mediating transposition of the *mcr-1* gene are scarce. In this study, we aimed to investigate if the *mcr-1* gene was prevalent in farm settings, and the range of plasmids which harboured such a resistance gene. By characterizing the *mcr-1*-bearing plasmids in *E. coli* isolated from farmed animals in different parts of China, we discovered a *mcr-1*-bearing transposable element which can form a circular intermediate that plays a key role in genetic translocation, and hence transmission of the *mcr-1* element among a wide range of potential human pathogens.

Materials and methods

Bacterial strains and identification

Cefotaxime-resistant *E. coli* strains were isolated from faeces of healthy pigs on six farms located in six provinces in China, namely Guangdong, Fujian, Jiangsu, Shandong, Henan and Liaoning, during the period September 2014–March 2015, by using MacConkey agar plates supplemented with 4 mg/L cefotaxime. The strains were identified as *E. coli* by MALDI-TOF MS using a Bruker MicroFlex LT mass spectrometer (Bruker Daltonics) and confirmed using API20E strips (bioMérieux, Inc.).

Antimicrobial susceptibility tests

MICs of 14 antimicrobial agents as listed in Table S1 (available as Supplementary data at *JAC* Online) were determined using the agar dilution method; results were interpreted according to CLSI recommendations. *E. coli* strain ATCC 25922 was used as a quality control strain.

Prevalence of mcr-1-positive E. coli strains

All *E. coli* strains were screened for the presence of the *mcr-1* gene by PCR using primers as previously described.[8] The PCR products were purified and sequenced by Sanger sequencing to confirm the genetic identity.

XbaI-PFGE, S1-PFGE and Southern hybridization

E. coli strains that carried the *mcr-1* gene were subjected to further characterization. PFGE of XbaI-digested genomic fragments was performed to assess the genetic relatedness of isolates, using the CHEF-MAP-PER System (Bio-Rad); genomic DNA of the *Salmonella enterica* var. *Braenderup* H9812 strain restricted with XbaI was used as the reference standard. Cluster analysis of PFGE patterns was typically performed by the BioNumerics (Applied Maths) system. S1 nuclease-PFGE was performed to characterize the plasmid profiles; the location of *mcr-1* was identified by Southern hybridization with digoxigenin-labelled *mcr-1*

probe according to the manufacturer's instructions for the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics).

Plasmid sequencing and bioinformatics analyses

Plasmids extracted from the 15 bacterial strains using the QIAGEN Plasmid Midi Kit were used to prepare the sequencing libraries, which were constructed by the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB) and sequenced by the NextSeq 500 Illumina platform, with 2×150 bp paired-end reads. *De novo* assembly of the reads was conducted with SOAPdenovo2,[17] followed by the use of ResFinder[17] and PlasmidFinder[18] to identify resistance genes and plasmid types among the scaffolds. Construction of complete plasmid sequences was accomplished by using available plasmid reference sequences to align and assemble the contigs, and confirmed by mapping paired-end reads to the finished complete sequences. In an attempt to obtain the complete gene map of *mcr-1*-bearing plasmids that *in silico* analysis failed to produce, PacBio RSII single-molecule, real-time (SMRT) sequencing was performed to create libraries of 20 kb at the Wuhan Institute of Biotechnology, Wuhan, China. The library preparation work was conducted according to the instructions of the manufacturer, Pacific Biosciences. Illumina contigs were joined together by using the PacBio long contigs after assembly with HGAP 3.0. The annotations of the plasmid sequences were conducted by RAST[19] and edited manually. Alignments with highly homologous complete plasmid sequences available in NCBI for these three plasmid types were performed by using the BRIG tool.[20] Comparison of four complete *mcr-1* IncHI2 plasmids was performed and visualized with Easyfig.[21] The representative *mcr-1*-bearing plasmid sequences pECJS-59-244, pECJS-B60-267, pECJS-61-63 and pECGD-8-33 were submitted to NCBI with the accession numbers KX084394, KX254341, KX254342 and KX254343, respectively.

Detection of circular intermediates

Based on the knowledge that the IS*30* family ISs could form DNA circular intermediates[22] and that IS*Apl1* exhibits similarity with the IS*30* family members (39% identity with IS*30* transposase from *E. coli* NP_414790 in protein sequence), a set of reverse primers targeting *mcr-1* was designed to investigate the potential of the IS*Apl1*-*mcr-1* segment to circularize (Table S2). The PCR products were sequenced by Sanger sequencing to obtain a complete map of the circular intermediate.

Results

Characterization of mcr-1-positive, ESBL-producing E. coli

Among the 97 cefotaxime-resistant *E. coli* strains isolated from faecal samples of pigs from various farms, 35 isolates (36%) were found to harbour the *mcr-1* gene. These *mcr-1*-positive strains, which were isolated from pigs in farms located in five geographically diverse provinces of China, were shown to exhibit genetically divergent PFGE types (Figure S1). However, strains collected from the same province were generally genetically more related than those obtained from other provinces (data not shown). The MICs of colistin were either 4 or 8 mg/L for all the *mcr-1*-positive strains, with the majority exhibiting resistance to multiple antimicrobial agents except meropenem (Table S1). To obtain a comprehensive view of the genetic features of *mcr-1*-bearing plasmids in these isolates, 15 *mcr-1*-positive *E. coli* strains of different PFGE types were selected for further characterization. S1-PFGE and Southern hybridization analysis showed that each of these isolates carried multiple plasmids, among which some were found to harbour more than one *mcr-1*-bearing

plasmid. The size of plasmids observed in these isolates ranged from ~33 to ~280 kb, with three major categories being observable: ~33, ~60 and ~216–280 kb. Most of the strains were found to harbour one ~33 or 60 kb plasmid, with some, such as strains FJ-B42 and JS-B65, carrying both. Some strains, such as JS-B60, JS-64, JS-B73 and JS-59, were also found to carry one *mcr-1*-bearing plasmid of ~216–280 kb in size (Table 1).

Genetic characterization of plasmids harbouring the *mcr-1* gene

Plasmids extracted from the 15 *mcr-1*-positive strains were subjected to nucleotide sequencing using the Illumina platform. Raw reads were subjected to *de novo* assembly to obtain contigs for each sample. BLASTN analysis against the resistance gene database, using the Illumina contigs, showed that plasmids recovered from these 15 strains comprised multiple drug resistance determinants (Table S3); this finding was consistent with the MDR phenotypes observable among the test strains (Table S2). In addition to *mcr-1*, plasmids derived from most of the strains were found to harbour multiple resistance elements, including but not limited to bla_{CTX-M}, bla_{CMY}, bla_{TEM}, *fosA*, *qnrS*, *floR* and *oqxAB*. However, it should be noted that there is still a slight possibility that some of the resistance genes (Table S3) were not plasmid borne, and were presumably located in the chromosome since the plasmid DNA used for high-throughput sequencing could be contaminated by chromosomal DNA, a phenomenon confirmed by other sequencing projects in our laboratory.

Six IncX4, seven IncI2 and seven IncHI2 plasmids were confirmed to harbour the *mcr-1* gene among the 15 test strains, five of which were found to contain two *mcr-1*-bearing plasmids (Table 1). The complete plasmid sequences of ~33 and ~60 kb plasmids could be obtained by *in silico* analysis of the Illumina sequencing data using the corresponding reference sequences (pHNSHP45 and pECJS-B65-33). The ~33 kb plasmid, pECGD-8-33, obtained from sample GD-8, was shown to belong to the IncX4 type, comprising 55 coding sequences (CDSs) with a size of 33 307 bp, and an overall GC content of 41.84%. The complete plasmid sequence of the ~60 kb plasmid, pECJS-61-63, obtained from sample JS-61, was shown to belong to the IncI2 type and comprise 88 CDSs, with a size of 63 656 bp and a GC content of 42.64%. The complete plasmid sequences of the 216–280 kb plasmids could not be generated from the Illumina sequencing data. Two representative plasmids, from samples JS-59 and JS-B60, respectively, were subjected to PacBio sequencing to obtain the complete sequence. The complete sequence of a 216–280 kb category plasmid, namely pECJS-59-244, which was derived from sample JS-59, was obtained. It was shown that this plasmid belonged to the IncHI2 type, contained 321 CDSs, and had a size of 243 572 bp, of which the GC content was 46.10%. Another IncHI2 plasmid, pECJS-B60-267, which was obtained from sample JS-B60, was found to be 267 486 bp in size. These two IncHI2-type plasmids were compared with the contigs of IncHI2 plasmids in other samples (Figure 1). IncHI2-type plasmids have been reported as genetic elements mediating the transmission of MDR genes (Table S4). Results of comparative analysis showed that a wide range of resistance genes and genetic elements could be found in a mosaic MDR region of IncHI2-type plasmids, including integrons, ISs and various resistance gene cassettes. The map of *mcr-1*-bearing plasmids could be completed successfully by using references of similar types to assist assembly of contigs of different samples, with the exception of IncHI2-type plasmids,

Table 1. The sizes and profiles of *mcr-1*-bearing plasmids harboured by 15 ESBL-producing *E. coli* strains

Strain	*mcr-1* plasmids (kb)[a]	Other plasmids (kb)[a]	Plasmids with complete sequence[b]	Plasmids with scaffolds[b]	*mcr-1* locus[c]	Circular form
GD-8	33	104; 90; 60	pECGD-8-33(IncX4)	—	*mcr-1-orf*	–
HN-15	60	120; 90; 78	pECHN-15–61(IncI2)	—	IS*Apl1-mcr-1-orf*	–
FJ-44	250, 33	560; 78; 60	pECFJ-44-33(IncX4)	pECFJ-44-250	*mcr-1-orf*	–
JS-56	60	33	pECJS-56-62(IncI2)	—	*mcr-1-orf*	–
JS-59	244	90	pECJS-59-244(IncHI2)	—	Tn*6330*	+
JS-61	230, 60	104;	pECJS-61-63(IncI2)	pECJS-61-230	*mcr-1-orf*; Tn*6330*	+
JS-63	230, 60	104; 33	pECJS-63-63(IncI2)	pECJS-63-230	*mcr-1-orf*	–
SD-112	33	104; 78	pECSD-112-33(IncX4)	—	*mcr-1-orf*	–
SD-137	60	138; 104; 33	pECSD-137-60(IncI2)	—	*mcr-1-orf*	–
FJ-B42	60, 33	138; 104	pECFJ-B42-33(IncX4)	—	*mcr-1-orf*	–
			pECFJ-B42-63(IncI2)		IS*Apl1-mcr-1-orf*	
FJ-B44	33	480; 270; 78; 60	pECFJ-B44-33(IncX4)	—	*mcr-1-orf*	–
JS-B60	250	560	pECJS-B60-267(IncHI2)	—	IS*Apl1-mcr-1-orf*	–
JS-B65	60, 33	90; 40	pECJS-B65-33(IncX4)	—	*mcr-1-orf*	–
			pECJS-B65-60(IncI2)		*mcr-1-orf*	
JS-64	280	560; 104; 60	—	pECJS-64-280	Tn*6330*	+
JS-B73	230	100	—	pECJS-B73-230	Tn*6330*	+

[a]The size of the plasmids was determined according to the S1-PFGE results.
[b]Plasmids for which complete sequences have been obtained. Complete sequences were not available in five IncHI2 *mcr-1*-bearing plasmids, for which only the assembly scaffolds were generated. The replicon types of samples were confirmed by PCR using specific primers listed in Table S2.
[c]The *mcr-1* locus for pECFJ-44-250 and pECJS-63-230 was unavailable.

Figure 1. Sequence alignment of IncHI2 *mcr-1* plasmids and WGS contigs in six samples. pECJS-59-244 was used as reference to compare with pHNSHP45-2 (GenBank no. KU341381), pECJS-B60-267 and other WGS data involving IncHI2 contigs detectable in *mcr-1*-positive strains. The outer circle with red arrows denotes annotation of reference plasmid. Among the five *mcr-1* IncHI2 strains (JS-44, JS-61, JS-63, JS64 and B73) without complete plasmid sequences, JS-44 exhibits sequence similarity with the reference, but alignment was not successful because of the overall low sequence homology. Note that Tn*6330* is only present in plasmids pECJS-59-244, pECJS-61-230, pECJS-64-280 and pECJS-B73-230. The number of IS*Apl1* repeats is not depicted in this figure. One MDR region was observable in the backbone of all IncHI2 plasmids; detailed information of the *mcr-1* location in the complete sequences of IncHI2 plasmids is denoted in Figure S2. Information about the IncHI2 plasmids tested in this study is provided in Table 1. This figure is available in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

which contained a large MDR region with numerous ISs. As a result, complete sequences of six IncX4-type, seven IncI2-type and two IncHI2-type plasmids were obtained using this approach (Table 1).

Plasmids belonging to each of these three types obtained from this study, and genetically similar *mcr-1*-bearing plasmids reported previously, were subjected to comparative analysis.

The ~33 kb, IncX4-type plasmids harboured by *E. coli* strains isolated from different geographical locations of China as well as other countries were found to be almost identical (Figure 2). Apart from *mcr-1*, no other resistance genes were detectable; in addition, no IS*Apl1* elements were found to flank the *mcr-1* gene.

A genetically homologous, yet *mcr-1*-negative plasmid of this type, namely pSH146_32, was previously isolated from a *Salmonella* Heidelberg strain (JX258655), suggesting that the *mcr-1-orf* gene cassette was most likely introduced into this plasmid backbone to form a *mcr-1*-bearing plasmid of this type (Figure 2).
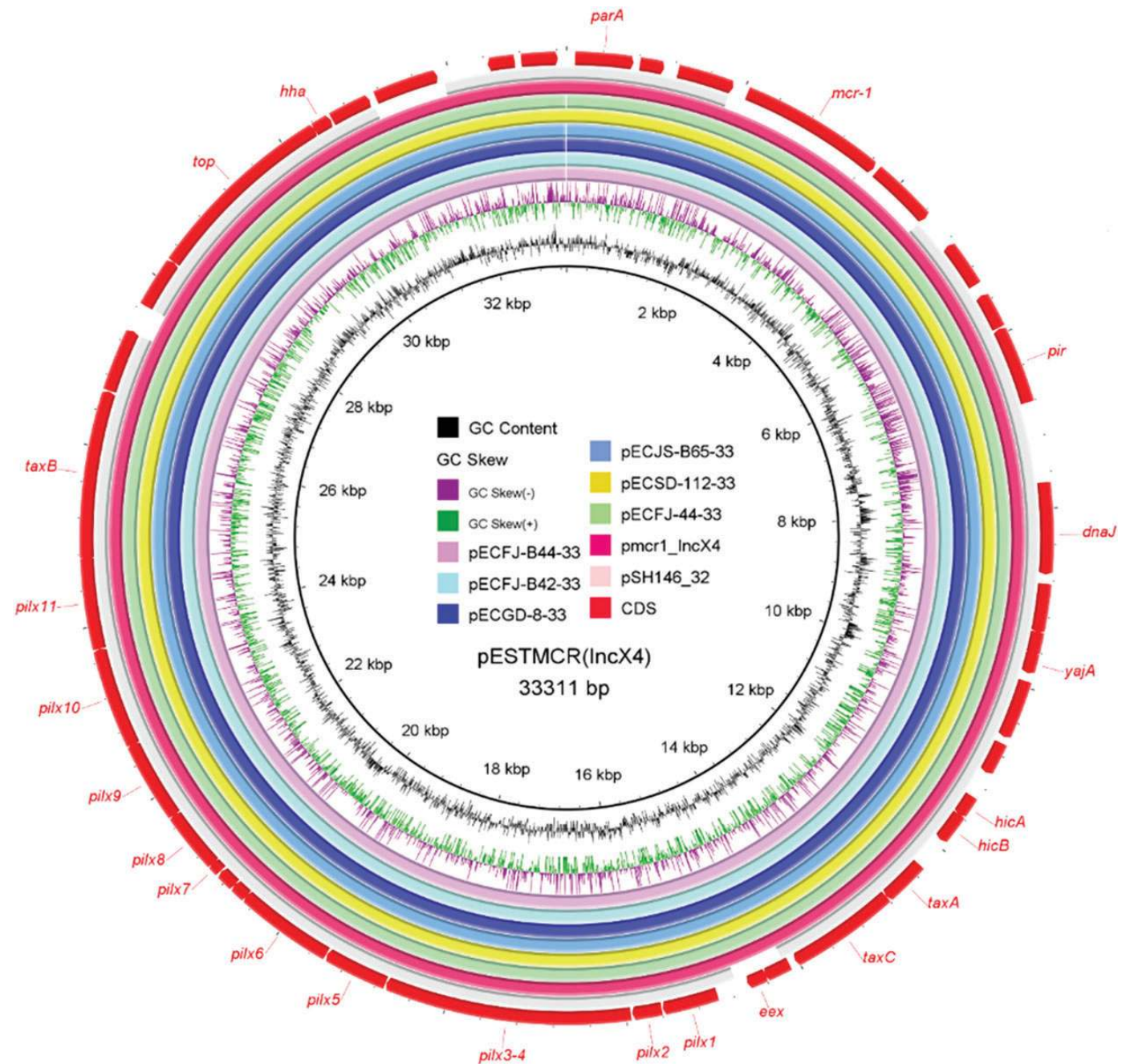
Figure 2. Sequence alignment of IncX4-type *mcr-1*-bearing plasmids. pESTMCR, which was recovered in Estonia with GenBank no. KU743383, was used as reference to match with other IncX4-type plasmids with (six plasmids in this study and pmcr1_IncX4, KU761327) and without the *mcr-1* gene (pSH146_32, JX258655). The outer circle with red arrows denotes the annotation of reference sequence. pSH146_32 exhibits a lower degree of sequence homology to the reference sequence when compared with other plasmids, and is depicted by a grey circle instead of the light pink colour chosen to represent this plasmid. The figure shows that the six IncX4 plasmids tested in this study and two previously reported IncX4 plasmids (pESTMCR and pmcr1_IncX4) share an extremely high degree of sequence homology. Information about the IncX4 plasmids tested in this study is provided in Table 1. This figure is available in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The ∼60 kb, IncI2-type *mcr-1* plasmid was present in 7 out of the 15 samples. Compared with pHNSHP45, which was first reported to encode the MCR-1 protein,[23] an IS*683* region was found to be missing in all seven IncI2-type plasmids isolated in this study (Figure 3). Two of the plasmids were found to contain an IS*Apl1-mcr-1* cassette identical to that of pHNSHP45, whereas another five plasmids lacked the IS*Apl1* gene. The 216–280 kb, IncHI2-type plasmids, which harboured a large MDR region, was the most genetically diverse plasmid type (Figure 1, Figure S2). Unlike the IncX4- and IncI2-type plasmids, for which the structure and location of the *mcr-1* cassettes were consistent among members of each plasmid type, the insertion site of the *mcr-1* cassette
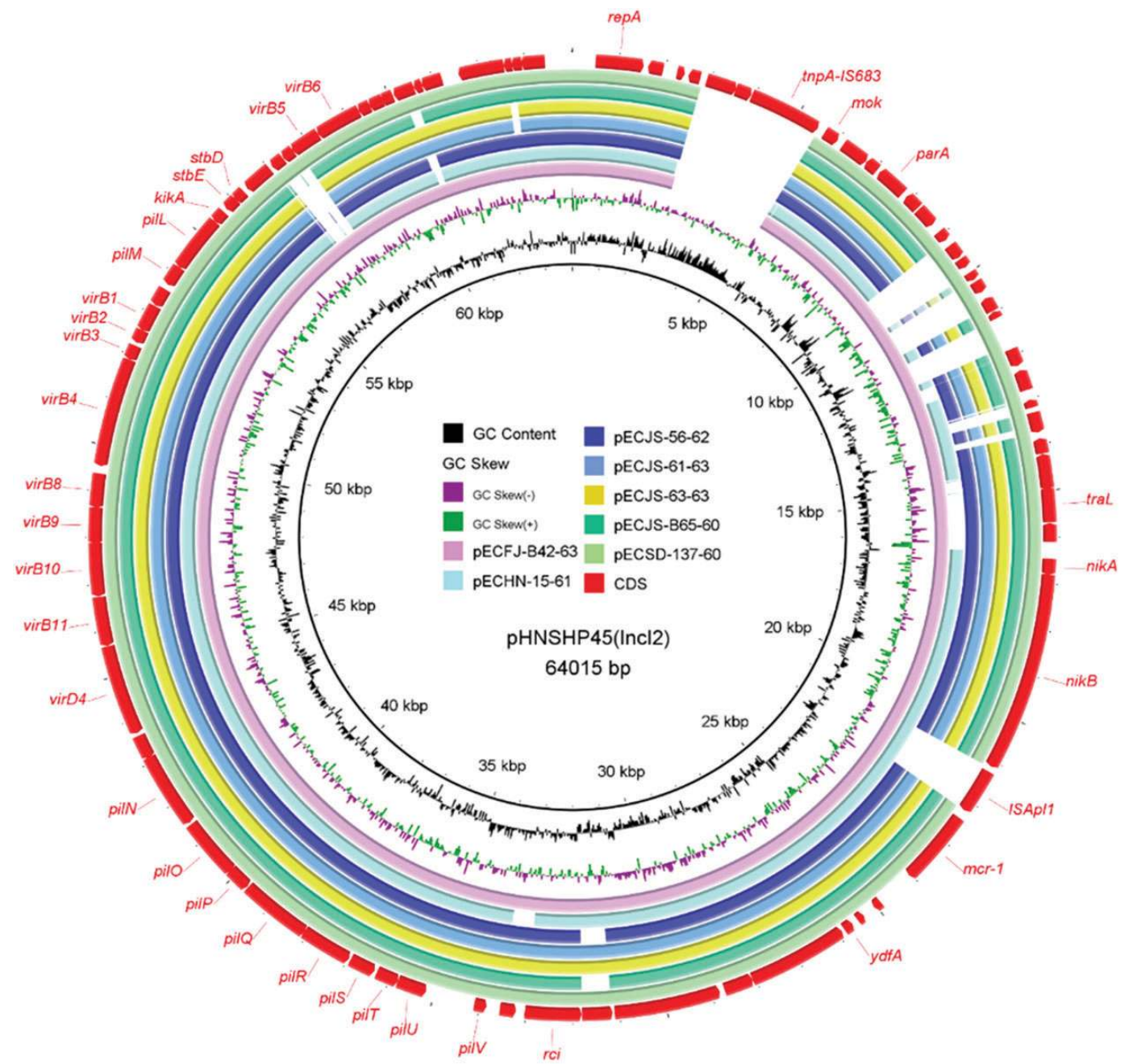
Figure 3. Sequence alignment of seven IncI2-type *mcr-1*-bearing plasmids. pHNSHP45 with GenBank no. KP347127 was used as a reference to compare with other *mcr-1*-bearing plasmids which possess the IncI2 replicon. The outer circle with red arrows signifies annotation of the reference sequence. Gaps in the circle refer to plasmid regions which are missing when compared with the reference. The IS*683* element is absent in all of the seven IncI2 plasmids tested in this study (see Table 1 for details). IncI2 plasmids can be categorized into two subtypes based on the presence or absence of IS*Apl1*. This figure is available in colour in the online version of *JAC* and in black and white in the print version of *JAC*.
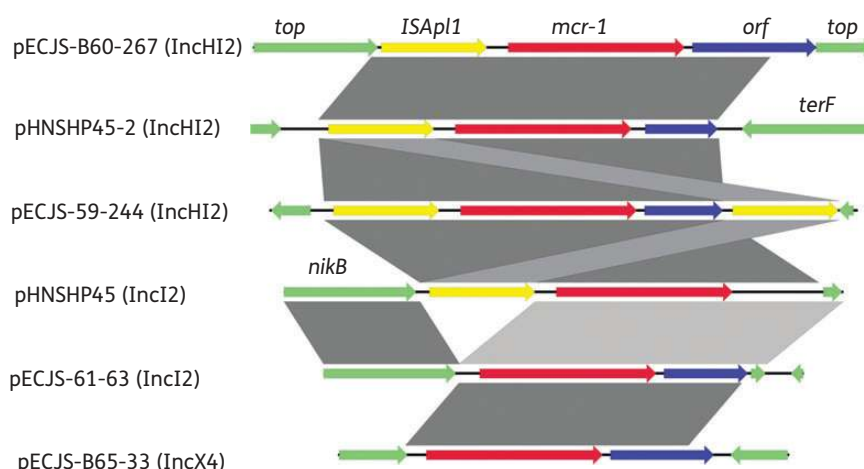
Figure 4. Sequence alignment of plasmids harbouring the *mcr-1* gene. CDSs without labels represent hypothetical proteins. The shadow parallelograms denote genetic regions that exhibit sequence homology among different segments. Light shadow denotes regions with a lower level of sequence identity (>99%). The genetic contexts of *mcr-1* in IncHI2 plasmids can be categorized into four types according to the complete sequences available to date. Another plasmid with a new format *mcr-1* gene cassette, namely pSA26-MCR-1, is described in Figure S2. IncI2-type plasmids can be divided into two subtypes based on the presence or absence of IS*Apl1*. All the IncX4-type plasmids are identical in terms of genetic arrangement in the vicinity of the *mcr-1* gene. This figure is available in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

in IncHI2 plasmids was variable and could be categorized into four types, which differed by the truncation status of *orf*, and the orientation and numbers of IS*Apl1* elements (Figure 4, Figure S2). Alignment of *mcr-1*-bearing elements revealed the core structure of all known *mcr-1* cassettes detectable in this and previous studies. Our data showed that the most conserved structure was the *mcr-1-orf* structure (the *orf* encodes the putative PAP family transmembrane protein). However, three of the six structures were found to have lost this intact *orf* due to alteration of the last four nucleotides in this element (Figure 4). Furthermore, a newly deposited *mcr-1*-bearing IncHI2 plasmid named pSA26-MCR-1 (KU743384), which was recovered from an NDM-1-producing *E. coli* ST68 strain isolated from a blood sample from a Saudi Arabian patient, was found to harbour a new *mcr-1* cassette in which the second copy of IS*Apl1* is inserted into the *orf* region in a reverse manner, indicating that the putative PAP *orf* is not required for evolution of *mcr-1* cassettes. Another 20 non-*mcr-1* IncHI2 plasmids exhibiting homology to pECJS-B60-267 (coverage >70%, identity >98% by BLASTN in the NCBI database) were extracted and the resistance gene distribution was examined (Figure S3, Table S4). The number of resistance genes in these 24 IncHI2 plasmids (including the four *mcr-1*-bearing plasmids) ranged from 2 to 17, and carbapenemase-encoding genes, such as $bla_{\text{IMP-8}}$, $bla_{\text{KPC-3}}$ and $bla_{\text{VIM-1}}$, were found in five plasmids. Since the insertion site of *mcr-1* in IncHI2 may vary, according to the four complete IncHI2 plasmids (Figure S2), we hypothesize that these four plasmids acquired the *mcr-1* gene by different genetic evolutionary routes.

Formation of a circular intermediate containing the *mcr-1* gene cassette

Reverse primers targeting the *mcr-1* gene were used to amplify the putative circular intermediate that harboured the *mcr-1* gene. PCR products with a size of ~2.5 kb in length were amplified in four samples harbouring the IncHI2 *mcr-1* plasmids (Figure 5b).

Sequence analysis showed that the ~2.5 kb PCR products were composed of partial sequences at both the 5′ and 3′ ends of the *mcr-1* gene, one *orf*, and one copy of IS*Apl1*. When combined with the central region of the *mcr-1* gene (the sequence between MCR1-R and MCR1-RC-F), we concluded that the circular intermediate was 3679 bp in size, containing one copy of IS*Apl1*, *mcr-1* and one *orf* (Figure 5a). After aligning with the pECJS-59-244 sequence, we found that this circular form was 100% homologous to position 173391–177069 (KX084394), suggesting that the IS*Apl1* element in the circular intermediate was derived from the downstream IS*Apl1* element, which differed from the upstream IS*Apl1* by one nucleotide at the potential promoter region (upstream IS*Apl1*: CTTCCAA-IS*Apl1*, downstream IS*Apl1*: TTTCCAA- IS*Apl1*). Notably, the circular intermediate can only be generated when the *mcr-1-orf* was surrounded by direct repeats of IS*Apl1*. The four plasmid samples from which the circular intermediate could be amplified were found to contain an *mcr-1* gene cassette flanked by two intact IS*Apl1* elements. This new composite transposon was designated Tn*6330* (IS*Apl1-mcr-1-orf*-IS*Apl1*), in which two directly oriented copies of IS*Apl1* flank the *mcr-1* gene and therefore exhibit the potential to form a circular intermediate (Figure 5).

Discussion

Since the discovery of the *mcr-1* element in China in late 2015,[8] the mobile colistin resistance determinant has been reported among different Enterobacteriaceae species isolated from various sources in different countries.[9,10,23] However, there is a lack of information regarding the genetic context of plasmids harbouring this gene and the underlying transmission mechanisms. In this study, we surveyed the prevalence of the *mcr-1* gene in ESBL-producing *E. coli* strains isolated from different geographical locations in China and conducted a comprehensive analysis of the plasmids that harboured the *mcr-1* gene in 15 representative strains. Three major types of plasmids, namely IncX4 (~33 kb),
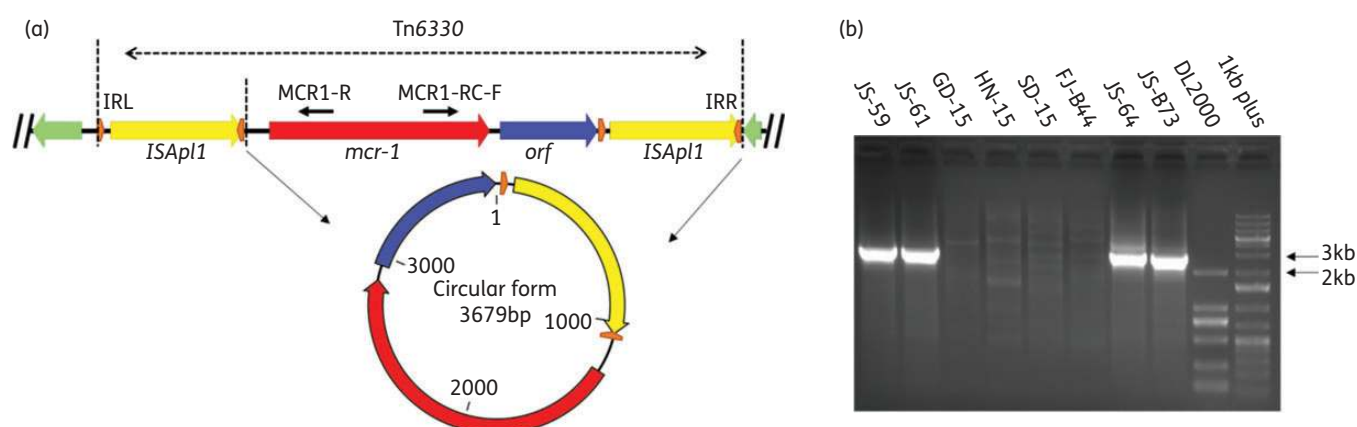
Figure 5. Formation of a circular intermediate by *mcr-1* gene cassette. (a) Genetic structure of transposon Tn*6330* (located in pECJS-59-244) and its circular intermediate, which harbors the *mcr-1* gene. (b) Gel electrophoresis of PCR amplicons corresponding to the circular intermediate of Tn*6330*, detectable in *mcr-1*-positive *E. coli* strains using the reverse primers MCR-RC-F and MCR-R. This figure is available in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

IncI2 (∼60 kb) and IncHI2 (∼216–280 kb), were detectable, alone or in various combinations, in as few as 15 *E. coli* strains, suggesting that *mcr-1* can be captured by a wide range of mobile genetic elements circulating among bacterial strains of animal origin. Among such plasmids, we noted that the IncX4 type (∼33 kb) was genetically the least variable, whereas the IncHI2 type (∼216–280 kb) was the most divergent due to the fact that this type of plasmid contains an MDR region which comprises a variable combination of antibiotic resistance genes (Figure S3). Plasmids of similar backbone but lacking the *mcr-1* gene cassette, such as pHXY0908 and pHK0683, have previously been reported in *Salmonella* spp., suggesting that the formation of this IncHI2 type of plasmid is most likely due to the acquisition of the *mcr-1* gene cassette by vectors containing such backbone structure.[24] In this work, four types of genetic cassettes harbouring the *mcr-1* gene have been identified in the IncHI2-type plasmids, suggesting that the *mcr-1* gene may be actively inserted into different genetic loci of the plasmid during dynamic gene transposition events that commonly occur in animal isolates. Although only four complete *mcr-1*-bearing IncHI2 plasmids are available, the different locations of *mcr-1* in these plasmids, and the fact that MDR IncHI2 plasmids are prevalent among human pathogens, infer that IncHI2 is the most efficient vehicle for disseminating *mcr-1* and other resistance elements including the carbapenemase-encoding genes. The clinical significance of this category of plasmid is therefore highlighted by its potential to confer, in a wide range of pathogens, phenotypic resistance to carbapenems and colistin, both last-line antibiotics used to treat Gram-negative bacterial infections. The existence of plasmid pSH146_32, which belongs to the IncX4 type (∼33 kb) in *Salmonella* Heidelberg (JX258655), supported the notion that acquisition of the *mcr-1* gene cassette by specific prototype plasmids plays an important role in the transmission of the colistin resistance element. Although no prototype plasmid of IncI2 (∼60 kb) has been reported previously, two *mcr-1* gene cassettes observable in *Salmonella enterica* and *E. coli* strains in England and Wales could be found through bioinformatics analysis,[12] suggesting that insertion of the *mcr-1* gene cassette into this type of plasmid is also a common event, and such plasmid has spread worldwide.

It is known that some members of the IS family, including IS*3*, IS*30*, IS*110*, IS*26* and IS*CR1*, utilize circular DNA intermediates that contain accessory genes to undergo gene translocation, through copy-and-paste or cut-and-paste mechanisms.[25–28] IS*Apl1* is a type of IS surrounded by a pair of left and right inverted repeats (IRL and IRR), which was first identified in *Actinobacillus pleuropneumoniae*, with the ability to disrupt a range of genes in such species.[16] The fact that the *mcr-1* gene is consistently associated with IS*Apl1* and that the *mcr-1* gene cassette may be inserted into different genetic loci in different plasmids, prompted us to hypothesize that *mcr-1* translocation could be mediated through a circular intermediate with the aid of IS*Apl1*.[13,29] In this study, we provide direct evidence that all *mcr-1* gene cassettes containing IS*Apl1*-*mcr-1*-*orf*-IS*Apl1*, designated as Tn*6330*, can form a circular intermediate which mediates the insertion of the *mcr-1* gene cassette into the IncHI2 plasmid backbone, and possibly other plasmids. In the first study on IS*Apl1*, a circular form harbouring only IS*Apl1* was detected without other accessory genes.[16] This is in agreement with the fact that no circular form containing *mcr-1* can be detected when only one copy of IS*Apl1* is located upstream of *mcr-1*, or in cases where such an element does not exist. With the help of IS*Apl1*, the generation of a circular intermediate undoubtedly confers on *mcr-1* the ability to be translocated into different genetic sites in the plasmid. We also note that the number of IS*Apl1* around *mcr-1* in the IncX4 (0), IncI2 (1 or 0) and IncHI2 (1 or 2) plasmids varies. Notably, another complete *mcr-1*-bearing IncHI2 plasmid, pSA26-MCR-1, was found to harbour three copies of IS*Apl1* (Figure S2), with the *mcr-1* gene being flanked by two IS*Apl1* elements in the form of inverted repeats, possibly constituting another transposable element format. This observation, together with the fact that the degree of genetic similarity within IncX4- and IncI2-type plasmids is high but IncHI2-type plasmids are genetically divergent, may suggest that Tn*6330* insertion into the IncHI2 type is still highly active, yet the loss of IS*Apl1* in the *mcr-1* gene cassette in the IncX4- and IncI2-type plasmids renders these plasmids more stable, since the *mcr-1* gene cassette in these vectors is no longer transposable. Nevertheless, the molecular mechanisms underlying the formation of the circular intermediate of Tn*6330*, and how it

evolves to become a genetically stable fragment in the residing plasmid, require further investigation.

In conclusion, this study characterized in detail the diverse genetic features of *mcr-1*-bearing plasmids in *E. coli* isolated from food animals, and revealed the presence of a novel transposon Tn*6330* comprising an *mcr-1* gene cassette with the structure IS*Apl1*-*mcr-1*-*orf*-IS*Apl1*, which may be regarded as the key element responsible for mediating the translocation of the *mcr-1* gene cassette into various plasmid backbones through formation of a circular intermediate. The findings in this work therefore provide important insights into the transmission mechanisms of *mcr-1*, and lay the foundation for devising effective intervention approaches aimed at preserving the value of colistin as a last-line antimicrobial agent.

Transparency declarations

None to declare.

Supplementary data

Figures S1–S3 and Tables S1–S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Norrby SR, Nord CE, Finch R *et al*. Lack of development of new antimicrobial drugs: a potential serious threat to public health. *Lancet Infect Dis* 2005; **5**: 115–9.

2 Blair JM, Webber MA, Baylay AJ *et al*. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; **13**: 42–51.

3 Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Updat* 2010; **13**: 132–8.

4 Velkov T, Thompson PE, Nation RL *et al*. Structure–activity relationships of polymyxin antibiotics. *J Med Chem* 2010; **53**: 1898–16.

5 Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clin Infect Dis* 2005; **40**: 1333–41.

6 Falagas ME, Kasiakou SK. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit Care* 2006; **10**: R27.

7 Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014; **5**: 643.

8 Liu YY, Wang Y, Walsh TR *et al*. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016; **16**: 161–8.

9 Ye H, Li Y, Li Z *et al*. Diversified mcr-1-harbouring plasmid reservoirs confer resistance to colistin in human gut microbiota. *MBio* 2016; **7**: e00177.

10 Rapoport M, Faccone D, Pasteran F *et al*. mcr-1-mediated colistin resistance in human infections caused by *Escherichia coli*: first description in Latin America. *Antimicrob Agents Chemother* 2016; **60**: 4412–3.

11 Shen Z, Wang Y, Shen Y *et al*. Early emergence of mcr-1 in *Escherichia coli* from food-producing animals. *Lancet Infect Dis* 2016; **16**: 293.

12 Doumith M, Godbole G, Ashton P *et al*. Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J Antimicrob Chemother* 2016; **71**: 2300–5.

13 Li A, Yang Y, Miao M *et al*. Complete sequences of mcr-1-harboring plasmids from extended spectrum β-lactamase (ESBL)- and carbapenemase-producing Enterobacteriaceae (CPE). *Antimicrob Agents Chemother* 2016; **60**: 4351–4.

14 Malhotra-Kumar S, Xavier BB, Das AJ *et al*. Colistin resistance gene mcr-1 harboured on a multidrug resistant plasmid. *Lancet Infect Dis* 2016; **16**: 283–4.

15 Xavier BB, Lammens C, Butaye P *et al*. Complete sequence of an IncFII plasmid harbouring the colistin resistance gene mcr-1 isolated from Belgian pig farms. *J Antimicrob Chemother* 2016; **71**: 2342–4.

16 Tegetmeyer HE, Jones SC, Langford PR *et al*. ISApl1, a novel insertion element of *Actinobacillus pleuropneumoniae*, prevents ApxIV-based serological detection of serotype 7 strain AP76. *Vet Microbiol* 2008; **128**: 342–53.

17 Luo R, Liu B, Xie Y *et al*. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience* 2012; **1**: 18.

18 Carattoli A, Zankari E, Garcia-Fernandez A *et al*. In silico detection and typing of plasmids using PlasmidFinder and Plasmid Multilocus sequence typing. *Antimicrob Agents Chemother* 2014; **58**: 3895–03.

19 Overbeek R, Olson R, Pusch GD *et al*. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014; **42**: D206–D14.

20 Alikhan NF, Petty NK, Ben Zakour NL *et al*. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 2011; **12**: 402.

21 Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. *Bioinformatics* 2011; **27**: 1009–10.

22 Kiss J, Olasz F. Formation and transposition of the covalently closed IS30 circle: the relation between tandem dimers and monomeric circles. *Mol Microbiol* 1999; **34**: 37–52.

23 Elnahriry SS, Khalifa HO, Soliman AM *et al*. Emergence of plasmid-mediated colistin resistance gene, mcr-1, in a clinical *Escherichia coli* isolate from Egypt. *Antimicrob Agents Chemother* 2016; **60**: 3249–50.

24 Zhi C, Lv L, Yu LF *et al*. Dissemination of the mcr-1 colistin resistance gene. *Lancet Infect Dis* 2016; **16**: 292–3.

25 He S, Hickman AB, Varani AM *et al*. Insertion sequence IS26 reorganizes plasmids in clinically isolated multidrug-resistant bacteria by replicative transposition. *MBio* 2015; **6**: e00762.

26 Siguier P, Gourbeyre E, Chandler M. Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiol Rev* 2014; **38**: 865–91.

27 Li R, Wong MH, Zhou Y *et al*. Complete nucleotide sequence of a conjugative plasmid carrying bla_{PER-1}. *Antimicrob Agents Chemother* 2015; **59**: 3582–4.

28 Olasz F, Farkas T, Kiss J *et al*. Terminal inverted repeats of insertion sequence IS30 serve as targets for transposition. *J Bacteriol* 1997; **179**: 7551–8.

29 Petrillo M, Angers-Loustau A, Kreysa J. Possible genetic events producing colistin resistance gene mcr-1. *Lancet Infect Dis* 2016; **16**: 280.