



Promoter Variation and Gene Expression of *mcr-1*-Harboring Plasmids in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* from a Chinese Hospital

Bin Huang,^a Yuting He,^a Xingyan Ma,^a Renxin Cai,^{b,c} Jianming Zeng,^{b,c} Yang Lu,^{b,c} Weizheng Zhang,^{b,c} Kai Lan,^{b,c} Sunmei E,^{b,c} Yi-Wei Tang,^d Barry N. Kreiswirth,^e Cha Chen,^{b,c} Liang Chen^e

^aDepartment of Laboratory Medicine, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

^bDepartment of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China

^cDepartment of Laboratory Medicine, Guangdong Provincial Hospital of Chinese Medicine, Guangzhou, China

^dDepartment of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, New York, USA

^ePublic Health Research Institute Tuberculosis Center, New Jersey Medical School, Rutgers University, Newark, New Jersey, USA

ABSTRACT Next-generation sequencing of 6 *mcr-1*-harboring *Escherichia coli* and *Klebsiella pneumoniae* isolates collected from a tertiary care hospital in China revealed significant sequence variations in the regions flanking the *mcr-1* gene. While sequence variations significantly affected the expression and promoter activity of *mcr-1*, the *mcr-1* gene expression levels did not correlate with the *in vitro* colistin resistance levels, which warrants further in-depth investigations.

KEYWORDS colistin, genomics, plasmids, promoter, gene expression

Following the initial report (1), the plasmid-mediated colistin resistance gene *mcr-1* has been found in over 35 countries on 5 continents, among various bacterial species (2, 3). Meanwhile, *mcr-1* has been identified in plasmids from different incompatibility groups, including IncX4, IncI2, IncHI1, IncHI2, IncFI, IncFII, IncP, IncI2-FIB, IncX1-X2, and IncX3-X4 groups (4). Comparative sequence analysis showed that *mcr-1* is harbored on an ~2,600-bp *mcr-1-pap2* cassette in different plasmids and the immediate downstream sequences of *mcr-1* are commonly conserved, whereas the upstream *mcr-1* sequences are more diverse, even among plasmids from the same incompatibility group (4, 5). Previous studies on *mcr-1* plasmids focused largely on plasmid sequence analysis, but the effects of sequence variations on *mcr-1* gene expression and susceptibility were largely unexplored. Here we conducted a comprehensive molecular characterization of *mcr-1*-harboring *Enterobacteriaceae* isolates collected from a tertiary care hospital in southern China in 2015 to early 2016, using next-generation sequencing, gene expression, and promoter activity analyses.

A total of 905 nonduplicated clinical isolates (569 *Escherichia coli* isolates, 278 *Klebsiella pneumoniae* isolates, 36 *Enterobacter cloacae* isolates, and 22 *Citrobacter freundii* isolates) collected from a tertiary hospital in Guangdong Province in China between 2015 and March 2016 were randomly selected for molecular screening for *mcr-1*, using the primers described elsewhere (1). Among the 905 clinical isolates, 1 *K. pneumoniae* isolate (GZ49271) and 5 *E. coli* isolates (GZ49260, GZ49263, GZ49266, GZ49269, and GZ49273) were found to harbor *mcr-1*. The 6 isolates were isolated from different types of clinical specimens (urine, blood, and cervical swab specimens) in 2015 ($n = 4$) or 2016 ($n = 2$) (see Table S1 in the supplemental material). The overall prevalence of *mcr-1* among *E. coli* isolates was 0.88% (5/569 isolates) and that among *K. pneumoniae* isolates was 0.36% (1/278 isolates). All 6 patients had a history of

Received 3 January 2018 Returned for modification 22 January 2018 Accepted 11 February 2018

Accepted manuscript posted online 20 February 2018

Citation Huang B, He Y, Ma X, Cai R, Zeng J, Lu Y, Zhang W, Lan K, E S, Tang Y-W, Kreiswirth BN, Chen C, Chen L. 2018. Promoter variation and gene expression of *mcr-1*-harboring plasmids in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from a Chinese hospital. *Antimicrob Agents Chemother* 62:e00018-18. <https://doi.org/10.1128/AAC.00018-18>.

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Cha Chen, chencha906@163.com, or Liang Chen, chen11@jms.rutgers.edu.

B.H., Y.H., and X.M. contributed equally to this article.

previous hospitalization but without exposure to colistin, and none had a history of recent overseas travel. All 6 isolates were resistant to colistin but susceptible to imipenem and meropenem, with various profiles of resistance to other antibiotics (Table S1). Among them, GZ49260, GZ49266, and GZ49269 were resistant to multiple antimicrobials, including ceftriaxone and aztreonam.

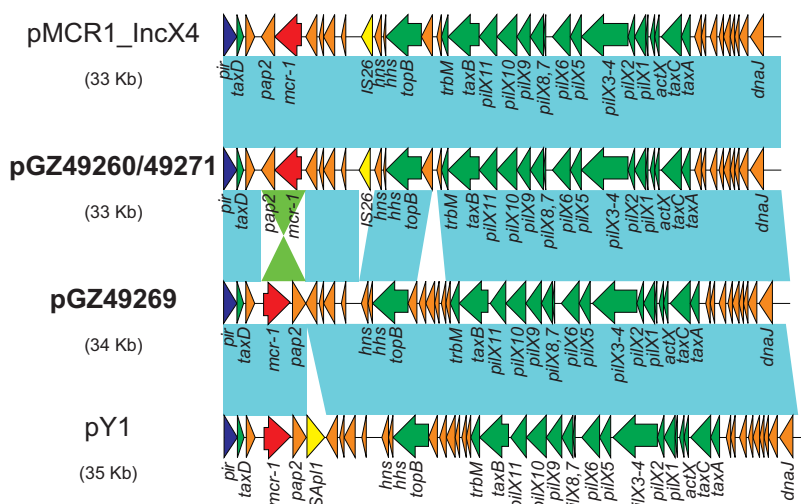
The *mcr-1* genes from all isolates except GZ49263 were successfully transferred to recipient *E. coli* J53^{AZ-R} strains by conjugation. Electroporation of plasmids extracted from GZ49263 into *E. coli* DH5 α was also unsuccessful; therefore, the subsequent complete plasmid sequence characterization focused on the other 5 isolates. The genomic DNA from the 6 clinical isolates and 5 *mcr-1* harboring plasmids from the *E. coli* J53^{AZ-R} transconjugants were sequenced using next-generation sequencing (Illumina NextSeq 500), as described previously (6). *In silico* multilocus sequence typing (MLST) analysis (7) showed that the five *E. coli* strains belonged to five different sequence types (STs), i.e., ST744, ST7542, ST156, ST4204, and ST1196, while *K. pneumoniae* GZ49271 was an ST976 strain (Table S1). *In silico* mining of acquired resistance genes (8) identified the presence of *mcr-1* in all 6 isolates and several other antimicrobial resistance genes in GZ49260, GZ49263, GZ49266, GZ49269, and GZ49271 (Table S1). Notably, GZ49260, GZ49266, and GZ49269 carried the extended-spectrum β -lactamase gene *bla*_{CTX-M} and, among them, GZ49269 harbored two different *bla*_{CTX-M} variants (*bla*_{CTX-M-14} and *bla*_{CTX-M-55}). In contrast, *mcr-1* was the only resistance gene found in *E. coli* GZ49273.

Sequence analysis showed that the five plasmids belonged to two plasmid replicon types, namely, IncX4 (pGZ49260, pGZ49269, and pGZ49271) and IncI2 (pGZ49273 and pGZ49266) types. Two IncX4 plasmids, from *E. coli* GZ49260 and *K. pneumoniae* GZ49271 (pGZ49260 and pGZ49271, respectively), were highly similar (5 single-nucleotide variations), with the same size of 33,309 bp and GC contents of 42%, suggesting horizontal transfer of the same *mcr-1* plasmids into two different species. BLAST-n results showed that they had high levels of sequence homology (>99.9% identity, with 100% query coverage) to a number of completely sequenced *mcr-1*-harboring plasmids, including pCSZ4 (GenBank accession no. [KX711706](#)) (5) and pMCR1_IncX4 (GenBank accession no. [KU761327](#)) (9) from China. In contrast, the *mcr-1* plasmid from *E. coli* GZ49269 (pGZ49269) was 33,858 bp in length, with a GC content of 42%, showing the best match (>99.9% identity, with 100% query coverage) to plasmid pPY1 (GenBank accession no. [KX711708](#)), which was isolated from an *E. coli* strain cultured from a pork specimen in China (5). Of note, the *mcr-1-pap2* cassette in pGZ49269 had an inverted orientation, in comparison to pGZ49260 and pGZ49271, which are similar to plasmid pY1 except that pY1 carries an IS*Apl1* insertion downstream of the *mcr-1-pap2* cassette (Fig. 1A).

The two IncI2 plasmids, pGZ49273 and pGZ49266, were 66,183 and 62,701 bp in length, respectively, with the same GC content of 43%. The overall backbones of the two plasmids were similar to those of several other *mcr-1* IncI2 plasmids, including the prototype pHNSHP45 (GenBank accession no. [KP347127](#)) (1) and pMCR1_IncI2 (GenBank accession no. [KU761326](#)) (9) (Fig. 1B). In comparison to pHNSHP45 and pMCR1_IncI2, the main variable regions in pGZ49273 and pGZ49266 were the *mcr-1-pap2* cassette upstream sequences. An IS*Apl1* element was inserted upstream of *mcr-1* in pHNSHP45; in contrast, IS*Apl1* was absent in the corresponding region in pMCR1_IncI2. Interestingly, novel variants of insertion sequences were identified upstream of *mcr-1* in pGZ49273 and pGZ49266. An IS5 element was inserted 62 bp upstream of *mcr-1* in pGZ49273 and was located between the -10 and -35 promoter sequences, as reported previously (10). Interestingly, three truncated IS*Apl1* elements (446, 443, and 234 bp) were found to be integrated 60 bp upstream of *mcr-1* in pGZ49266, disrupting the -35 promoter region. It is not clear how the mosaic structures of the three truncated IS*Apl1* elements were generated, and we speculate that they could be the result of multiple recombination events.

A 4,737-bp *mcr-1*-harboring element in GZ49263 was resolved using the combination of *de novo* assembly and regular PCR with Sanger sequencing. The *mcr-1* in

(A) IncX4



(B) IncI2

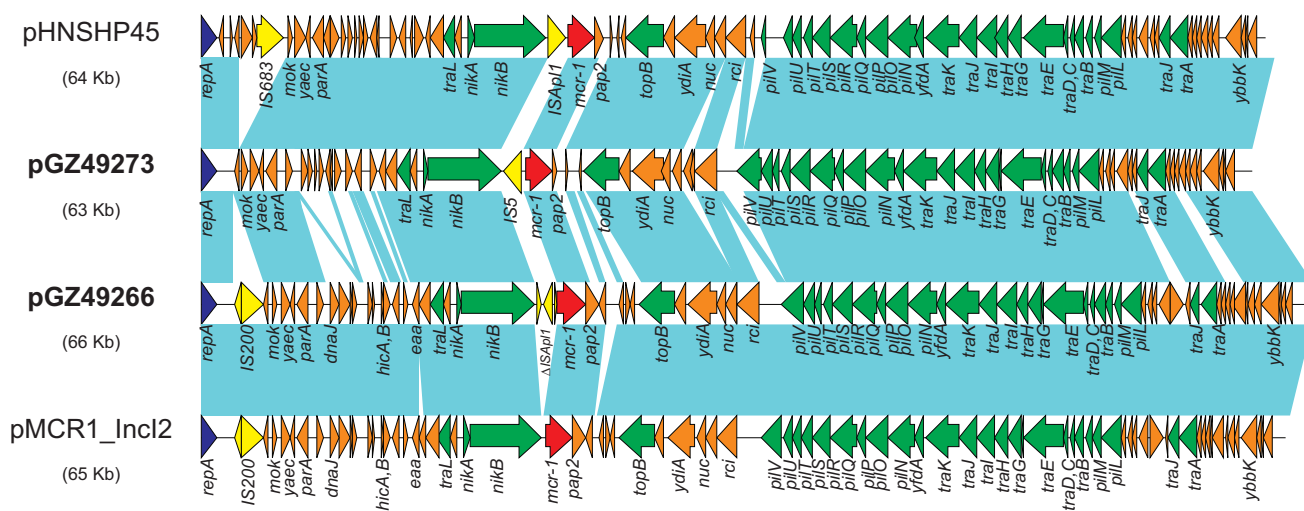


FIG 1 Plasmid structures. (A) Comparison of IncX4 plasmids pMCR1_IncX4 (GenBank accession no. [KU761326](#)), pGZ49260 (GenBank accession no. [MG210937](#)), pGZ49269 (GenBank accession no. [MG210939](#)), and pY1 (GenBank accession no. [KX711708](#)). (B) Comparison of IncI2 plasmids pHNSHP45 (GenBank accession no. [KP347127](#)), pMCR1_IncI2 (GenBank accession no. [KU761327](#)), pGZ49273 (GenBank accession no. [MG210940](#)), and pGZ49266 (GenBank accession no. [MG210938](#)). Colored arrows indicate open reading frames, with dark blue, yellow, green, red, and orange arrows representing replication genes, mobile elements, plasmid transfer genes, the *mcr-1* gene, and plasmid backbone genes, respectively. Blue shading denotes regions of shared homology among different plasmids, while green shading indicates inverted shared homology.

GZ49263 is harbored by a transposon-like element, with *mcr-1* being flanked by two directly repeated *ISAp1* elements. This region is nearly identical (1 SNP difference) to that of the corresponding region (nucleotides 21,539 to 26,275) in plasmid pSCC4 (GenBank accession no. [CP021078](#)), which was isolated from a *Citrobacter braakii* strain from a chicken in China.

Sequence comparisons of the 5' untranslated region of the *mcr-1* gene among all six genomes identified a 62-bp conserved sequence, encompassing the previously reported -10 promoter (Fig. 2A). However, the -35 promoter sequence regions were variable. pGZ49263, pGZ49260, pGZ49271, and pGZ49269 carried -35 and -10 promoter sequences similar to those of the *mcr-1* prototype plasmid pHNSHP45. In contrast, the original -35 promoters in pGZ49266 and pGZ49273 were disrupted due to insertion of a partial *ISAp1* element and IS5. BPROM promoter prediction software (Softberry, Inc.) identified two putative promoter regions in pGZ49266 and pGZ49273.

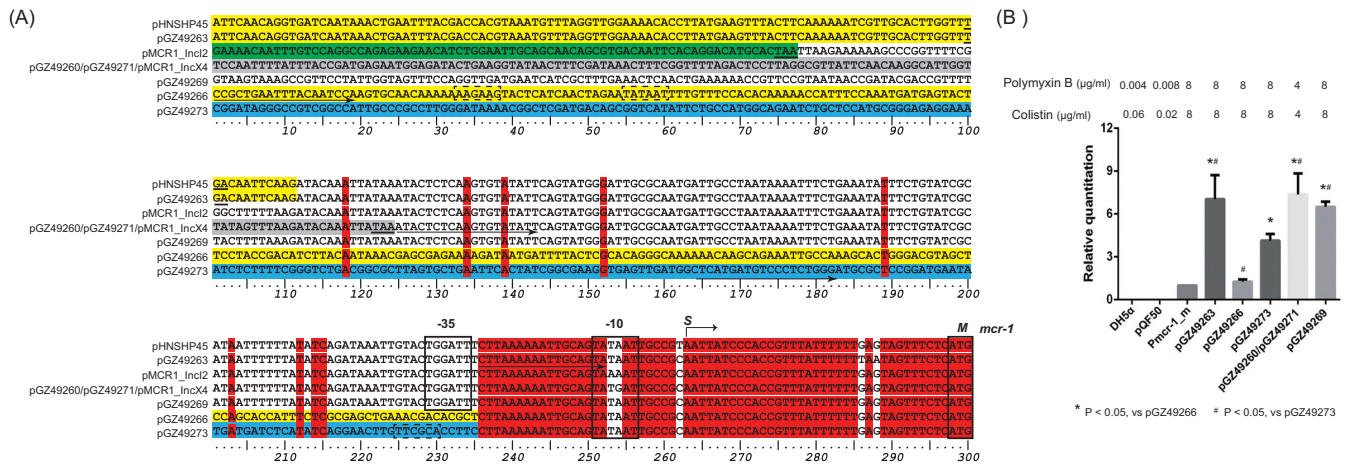


FIG 2 (A) Sequence analyses of the *mcr-1* promoter region. Red shading denotes identical residues, yellow shading shows ISAp1 or partial ISAp1 sequences, and blue shading indicates IS5 sequences. The putative -35 and -10 promoter sequences in pGZ49266 and pGZ49273 are shown in dashed boxes. The cloning primer sequences of Pmcr-1_m, pGZ49260/pGZ49271, pGZ49266, and pGZ49273 are shown as underlining arrows. The primer sequences of pGZ49263 and pGZ49269 are located upstream of this region. S, transcription start site; M, methionine and translation initiation site. (B). Levels of *mcr-1* gene expression in *E. coli* DH5 α strains with different plasmid promoter sequence constructs (pQF50). The MIC values for colistin and polymyxin B are shown above the graph.

In pGZ49273, a putative -35 promoter sequence (TCGCA) was found 67 bp upstream of the start codon, located at the 3' inverted reverse repeat of IS5 (Fig. 2A). In pGZ49266, a new set of -35 (AAGAAG) and -10 (TATAAT) sequences were identified 237 bp upstream of the start codon of *mcr-1*, located within the partial ISAp1 sequence (Fig. 2A).

We cloned each promoter sequence and the full-length *mcr-1* gene into a pQF50 expression plasmid in an *E. coli* DH5 α strain and evaluated the *mcr-1* expression using real-time reverse transcription (RT)-PCR (Table S2). The 62-bp conserved sequence and the full-length *mcr-1* were cloned from pGZ49263 (Pmcr-1_m), and the strain was used as the baseline control strain for quantitative RT-PCR (qRT-PCR) (the expression level was taken as 1) (Fig. 2B). The results showed that the *E. coli* DH5 α constructs with the *mcr-1* promoter regions from pGZ49263, pGZ49260, pGZ49271, and pGZ49269 had the highest *mcr-1* expression levels ($P < 0.05$, compared with pGZ49266 and pGZ49273 promoter clones) (Fig. 2B), in contrast with the promoter sequence analysis, in which the four strains harbored promoter regions similar to that of the prototype plasmid pHNSHP45. In contrast, the pGZ49273 promoter clone had lower *mcr-1* expression, while the pGZ49266 clone had the lowest *mcr-1* expression levels, suggesting that the promoter sequence changes associated with partial ISAp1 rearrangement and IS5 insertion, respectively, had significant effects on *mcr-1* expression. Furthermore, we evaluated the promoter activity using a promoterless *lacZ* reporter system, which demonstrated results similar to those of the *mcr-1* qRT-PCR analysis, in that pGZ49273 and pGZ49266 promoter clones had significantly lower β -galactosidase activities than did the other four plasmid promoters (pGZ49263, pGZ49260/pGZ49271, and pGZ49269) (data not shown). Interestingly, despite the differences in *mcr-1* expression and promoter activity described above, the MIC values of pQF50 *E. coli* DH5 α constructs with different promoter sequences, including the baseline control clone, were similar, with values of 4 to 8 $\mu\text{g/ml}$ for colistin and polymyxin (Fig. 2B).

In this study, we demonstrated that *mcr-1*-harboring plasmids possessed significant sequence variation, especially in the regions flanking the *mcr-1* gene. The sequence variation could significantly affect the promoter activity and *mcr-1* expression, but the *mcr-1* gene expression did not correlate with *in vitro* colistin resistance levels. MCR-1 acts as a phosphoethanolamine transferase and modifies the chemical structure of the lipid A moiety on bacterial lipopolysaccharide (LPS) through the addition of phosphoethanolamine, which reduces the negative charge and the binding affinity for colistin and ultimately produces colistin resistance. One explanation for the lack of correlation

of *mcr-1* expression levels and MICs could be that colistin is sensitive to structure modifications of lipid A and a minimal phosphoethanolamine modification (as a result of low-level *mcr-1* expression) may be sufficient to increase colistin MIC levels. Further studies, such as matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis of these clones with different promoter sequences, are needed to resolve the structural changes caused by phosphoethanolamine additions and to identify the correlations with MICs.

Accession number(s). The complete nucleotide plasmid sequences were deposited in GenBank under accession numbers [MG210937](#) to [MG210940](#). The raw whole-genome sequencing data from this study were submitted to NCBI under BioProject record number [PRJNA354234](#).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00018-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

The study was supported in part by the National Natural Science Foundation of China (grants 81572058, 81672081, and 81772249), the Guangdong Natural Science Foundation (grant 2014A030313143), and the State Education Ministry Scientific Research Foundation for the Returned Overseas Chinese Scholars. This work was in part supported by grants from the National Institute of Allergy and Infectious Diseases (grant R01AI090155 to B.N.K. and grant R21AI117338 to L.C.).

REFERENCES

1. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. 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* 16:161–168. [https://doi.org/10.1016/S1473-3099\(15\)00424-7](https://doi.org/10.1016/S1473-3099(15)00424-7).
2. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 30:557–596. <https://doi.org/10.1128/CMR.00064-16>.
3. Schwarz S, Johnson AP. 2016. Transferable resistance to colistin: a new but old threat. *J Antimicrob Chemother* 71:2066–2070. <https://doi.org/10.1093/jac/dkw274>.
4. Wang Q, Li Z, Lin J, Wang X, Deng X, Feng Y. 2016. Complex dissemination of the diversified *mcr-1*-harbouring plasmids in *Escherichia coli* of different sequence types. *Oncotarget* 7:82112–82122. <https://doi.org/10.18632/oncotarget.12621>.
5. Sun J, Fang LX, Wu Z, Deng H, Yang RS, Li XP, Li SM, Liao XP, Feng Y, Liu YH. 2017. Genetic analysis of the IncX4 plasmids: implications for a unique pattern in the *mcr-1* acquisition. *Sci Rep* 7:424. <https://doi.org/10.1038/s41598-017-00095-x>.
6. Eilertson B, Chen L, Chavda KD, Kreiswirth BN. 2017. Genomic characterization of two KPC-producing *Klebsiella* isolates collected in 1997 in New York City. *Antimicrob Agents Chemother* 61:e02458-16. <https://doi.org/10.1128/AAC.02458-16>.
7. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, Jelsbak L, Sicheritz-Ponten T, Ussery DW, Aarestrup FM, Lund O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. *J Clin Microbiol* 50:1355–1361. <https://doi.org/10.1128/JCM.06094-11>.
8. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 67:2640–2644. <https://doi.org/10.1093/jac/dks261>.
9. Li A, Yang Y, Miao M, Chavda KD, Mediavilla JR, Xie X, Feng P, Tang YW, Kreiswirth BN, Chen L, Du H. 2016. Complete sequences of *mcr-1*-harboring plasmids from extended-spectrum- β -lactamase- and carbapenemase-producing *Enterobacteriaceae*. *Antimicrob Agents Chemother* 60:4351–4354. <https://doi.org/10.1128/AAC.00550-16>.
10. Poirel L, Kieffer N, Brink A, Coetze J, Jayol A, Nordmann P. 2016. Genetic features of MCR-1-producing colistin-resistant *Escherichia coli* isolates in South Africa. *Antimicrob Agents Chemother* 60:4394–4397. <https://doi.org/10.1128/AAC.00444-16>.