AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Complete Sequences of *mcr-1*-Harboring Plasmids from Extended-Spectrum-β-Lactamase- and Carbapenemase-Producing *Enterobacteriaceae*

Aiqing Li,[a] Yong Yang,[a] Minhui Miao,[a] Kalyan D. Chavda,[b] José R. Mediavilla,[b] Xiaofang Xie,[a] Ping Feng,[a] Yi-Wei Tang,[c] Barry N. Kreiswirth,[b] Liang Chen,[b] Hong Du[a]

Department of Clinical Laboratory, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China[a]; Public Health Research Institute Tuberculosis Center, New Jersey Medical School, Rutgers University, Newark, New Jersey, USA[b]; Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, New York, USA[c]

Here we completely sequenced four *mcr-1*-haboring plasmids, isolated from two extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* and two carbapenemase-producing *Klebsiella pneumoniae* clinical isolates. The *mcr-1*-harboring plasmids from an *E. coli* sequence type 2448 (ST2448) isolate and two *K. pneumoniae* ST25 isolates were identical (all pMCR1-IncX4), belonging to the IncX4 incompatibility group, while the plasmid from an *E. coli* ST2085 isolate (pMCR1-IncI2) belongs to the IncI2 group. A nearly identical 2.6-kb *mcr-1-pap2* element was found to be shared by all *mcr-1*-carrying plasmids.

The plasmid-mediated colistin resistance gene, *mcr-1*, has recently been reported from animals and hospitalized patients in China (1). Since then, *mcr-1* has been found in ~20 countries on four different continents (2). Alarmingly, *mcr-1* has also been identified in several multidrug-resistant bacteria, including extended-spectrum-β-lactamase (ESBL)-producing and carbapenemase-producing *Enterobacteriaceae* (CPE) (3–9). However, knowledge regarding the structure of *mcr-1*-harboring plasmids is limited. Here we completely sequenced four *mcr-1*-harboring plasmids (three of which are identical), isolated from two ESBL-producing *Escherichia coli* and two carbapenemase-producing *Klebsiella pneumoniae* clinical isolates (4).

In a recent study, we identified *mcr-1* in two ESBL-producing *E. coli* (SZ01 and SZ02) and two carbapenemase-producing *K. pneumoniae* (SZ03 and SZ04) clinical isolates from a tertiary hospital in eastern China (4). SZ01, SZ02, and SZ04 carry ESBL gene $bla_{\text{CTX-M-55}}$, while SZ03 and SZ04 harbor carbapenemase gene $bla_{\text{NDM-5}}$. Multilocus sequence typing (MLST) (10, 11) showed that the two *E. coli* isolates, SZ01 and SZ02, belong to two unrelated sequence types (STs) (ST2448 and ST2085), while the two *K. pneumoniae* strains (isolated from the same patient) both belong to ST25. The *mcr-1*-harboring plasmids from all four isolates were subsequently transferred to recipient strain *E. coli* J53 AZ^r via conjugation, along with the $bla_{\text{NDM-5}}$-harboring plasmids from SZ03 and SZ04. Susceptibility testing revealed that the four *mcr-1*-harboring *E. coli* transconjugants were resistant to colistin but not to any of the other antimicrobial agents tested. The two $bla_{\text{NDM-5}}$ transconjugants were resistant to all β-lactams, except for aztreonam, but remained susceptible to other classes of antimicrobial agents (data not shown). The *mcr-1*- and $bla_{\text{NDM-5}}$-harboring plasmids from these transconjugants were extracted and subjected to sequencing using the Illumina MiSeq platform (12). The sequencing reads were assembled *de novo* using SPAdes (13), and gaps were closed by standard PCR and Sanger sequencing as described previously (12).

The *mcr-1*-harboring plasmids from SZ01, SZ03, and SZ04 (subsequently named pMCR1-IncX4) were all identical, belonging to the IncX4 incompatibility group, and were 33,287 bp in length with a G+C content of 41.8%. The backbone of pMCR1-IncX4 is similar to that of other IncX4 plasmids, including pJIE143 (GenBank accession no. JN194214) (14), pBS512_33 (CP001059), pCROD2 (FN543504) (15), and pSH146_32 (JX258655) (16). BLASTn analysis showed that pMCR1-IncX4 has a query coverage of 87% and maximal 97% identity to pSH146_32, isolated from a *Salmonella enterica* Heidelberg strain from a porcine diagnostic specimen from Minnesota in 2002 (16), and a query coverage of 77% and maximal 99% identity to pJIE143, isolated from an *E. coli* ST131 strain from Australia in 2006 (14) (Fig. 1). Plasmid pMCR1-IncX4 possesses a replication region highly similar to the one on pJIE143 (Fig. 1), including the identical replication initiation protein gene *pir*, vegetative origins *oriV*-α and *oriV*-β, and highly similar *oriV*-γ (one less iteron). The region from *traM* to *oriV*-γ, encompassing the majority of the transfer region and including the *taxABC* and *pilX* operons, shares >99.9% nucleotide identity with pMCR1-IncX4 and pJIE143 (Fig. 1). However, the region in pMCR1-IncX4 between *taxD* and the histone-like nucleotide-structuring protein gene *hns*, where colistin resistance gene *mcr-1* is located, is absent in pJIE143. In contrast, this region is highly similar to that of pSH146_32, except for the insertion of an *mcr-1-pap2* element and an IS*26* element in pMCR1-IncX4 (Fig. 1). A 2,610-bp *mcr-1-pap2* fragment (nucleotides [nt] 2339 to 4948 in pMCR1-IncX4) was inserted into a hypothetical gene (locus tag pSH146_32_13). Interestingly, insertion element IS*Apl1*, initially found to be associated with *mcr-1* in pHNSHP45 (1), was not present in pMCR1-IncX4.

Received 10 March 2016 **Accepted** 3 April 2016

Accepted manuscript posted online 18 April 2016

Citation Li A, Yang Y, Miao M, Chavda KD, Mediavilla JR, Xie X, Feng P, Tang Y-W, 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. doi:10.1128/AAC.00550-16.

Address correspondence to Liang Chen, chen11@njms.rutgers.edu, or Hong Du, hong_du@126.com.

A.L., Y.Y., and M.M. contributed equally to this work.

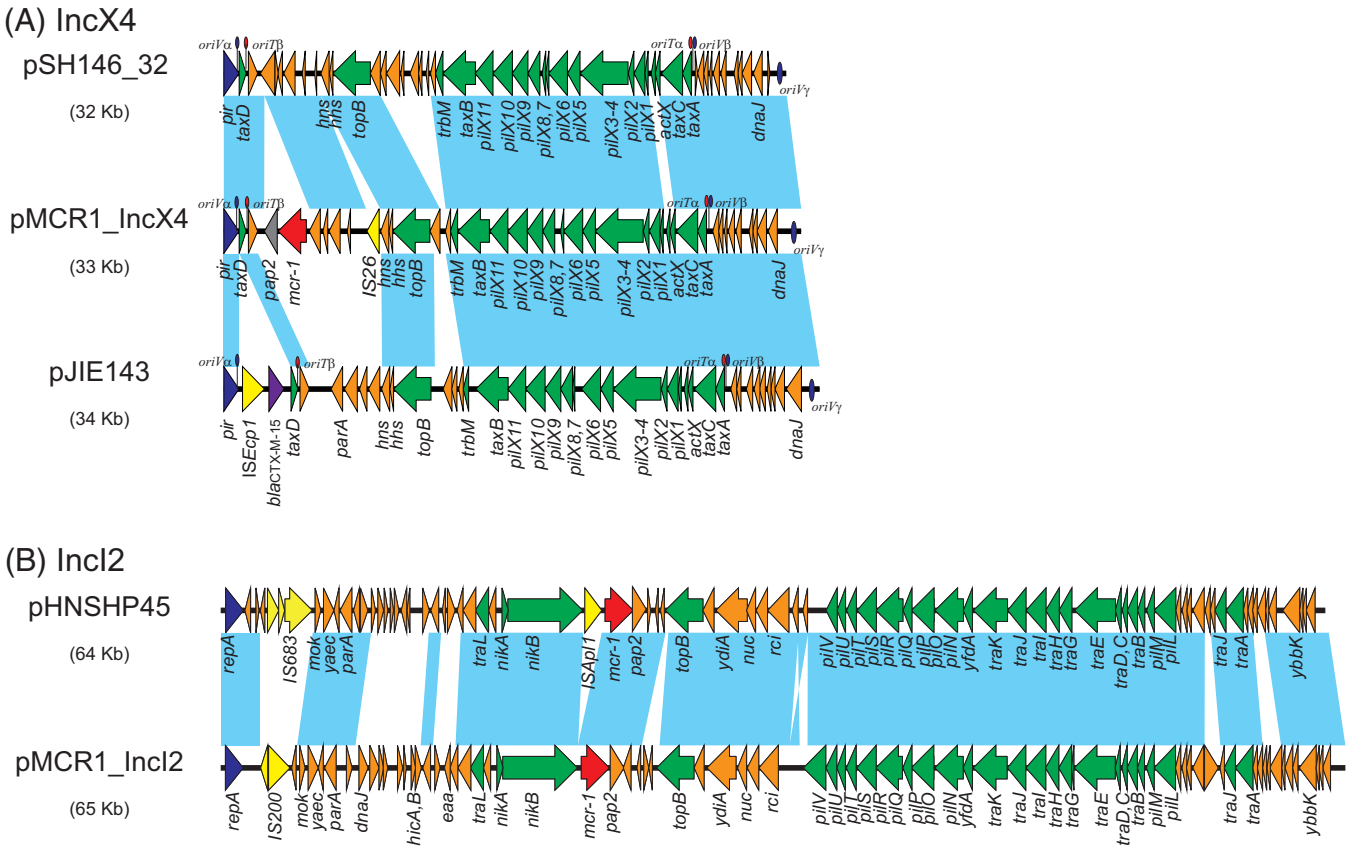

FIG 1 Structures of plasmids pMCR1-IncX4 and pMCR1-IncI2. (A) Comparison of IncX4 plasmids pSH146_32 (GenBank accession no. JX258655), pMCR1_IncX4 (KU761327, this study), and pJIE143 (JN194214); (B) comparison of IncI2 plasmids pHNSHP45 (KP347127) and pMCR1_IncI2 (KU761326, this study). Colored arrows represent open reading frames, with dark blue, yellow, green, red, purple, and orange arrows representing replication genes, mobile elements, plasmid transfer genes, the *mcr-1* gene, other antimicrobial resistance genes, and plasmid backbone genes, respectively. Blue shading denotes regions of shared homology among different plasmids.

The *mcr-1*-harboring plasmid from *E. coli* strain SZ02, pMCR1-IncI2, belongs to the IncI2 group, the same plasmid incompatibility group as pHNSHP45, the first plasmid reported to harbor *mcr-1* (1). Plasmid pMCR1-IncI2 is 64,964 bp in length and harbors 83 predicted open reading frames (ORFs), with a G+C content of 42.7%. The plasmid backbone of pMCR1-IncI2 is similar to that of other IncI2 plasmids, such as pSH146_65 (GenBank accession no. JN983044)(16), pBK15692 (KC845573) (17), and pHNSHP45 (KP347127) (1). BLASTn search results showed that pMCR1-IncI2 exhibits only 86% query coverage and 97% overall identity to pHNSHP45. In addition, sequence alignment of both plasmids identified >1,400 nucleotide differences (single nucleotide polymorphisms [SNPs]), suggesting that pMCR1-IncI2 and pHNSHP45 are distinct, a finding that suggests that the *mcr-1* gene may be repeatedly acquired. Further analysis showed that the *mcr-1* gene in pMCR1-IncI2 integrated downstream of the *nikB* gene, in the same location as in pHNSHP45. Similar to the analysis of pMCR1-IncX4, the *mcr-1*-associated IS*Apl1* element was not found in pMCR1-IncI2 (Fig. 1).

Thus far, *mcr-1* has been found in different plasmid incompatibility groups, including IncI2 (1, 9), X4 (6, 18), HI2 (19), and P (18). However, little is known regarding the mechanism whereby this gene can be mobilized between different plasmids. We therefore compared the *mcr-1* neighboring regions of pHNSHP45, pMCR1-IncI2, and pMCR1-IncX4, as well as additional *mcr-1*-harboring contig sequences from the NCBI WGS database (20, 21) (Fig. 2). The comparison identified a nearly identical 2,600-bp region (nt 2349 to 4948 on pMCR1-IncX4) shared by all sequences examined, encompassing the *mcr-1* and *pap2* (encoding a putative PAP family transmembrane protein) genes. Our analysis suggests that the 2.6-kb *mcr-1-pap2* element has been horizontally transferred into different plasmid backbones (Fig. 2). Further inspection of the upstream and downstream junctions of this *mcr-1-pap2* element failed to identify any direct or inverted repeat sequences. In pHNSHP45, IS*Apl1* is inserted directly upstream of the *mcr-1-pap2* element, and a 25-bp inverted reverse repeat is located adjacent to the 2.6-kb element. The 25-bp invert repeat was also identified at the same position in strain 2013LSAL02374, which contains a contig with a sequence from an IncP plasmid (Fig. 2). Consistent with the analysis of pMCR1-IncI2 and pMCR1-IncX4, IS*Apl1* was not always associated with *mcr-1*, and it was absent in several contigs belonging to the IncI2, IncX4, and IncP plasmids (Fig. 2). One possible explanation regarding the transfer mechanism of *mcr-1* is that the *mcr-1-pap2* element was initially translocated by the integration of IS*Apl1* (20), and the latter was subsequently lost following integration. Nevertheless, the exact mechanism underpinning *mcr-1* transfer requires additional study.

In addition to the two aforementioned *mcr-1* plasmids, we also

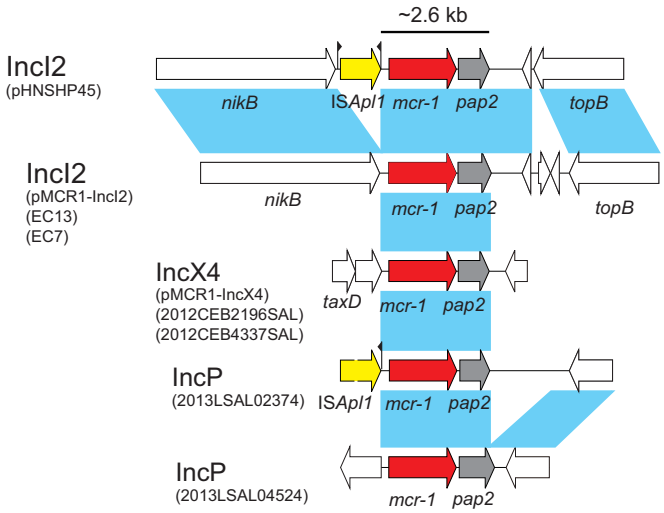

FIG 2 Genetic structure of *mcr-1*-harboring elements in selected plasmids. Plasmid incompatibility groups are noted on the left, along with the plasmids and/or strains harboring the genetic structure depicted. Arrows denote open reading frames (ORF), with red, gray, yellow, and white arrows denoting *mcr-1*, *pap2*, IS*Apl1*, and neighboring genes, respectively. Following BLAST analysis of NCBI draft genomes, only contigs long enough to unambiguously identify plasmid replicon groups were included in this figure. The dashed yellow outline of the IncP IS*Apl1* ORF therefore indicates that only a partial sequence is available. The small black arrows adjacent to IS*Apl1* denote the inverted reverse repeats (IRR). The GenBank accession numbers of the *mcr-1* contig sequences for EC5, EC7, EC13, 2012CEB2196SAL, 2012CEB4337SAL, 2013LSAL02374, and 2013LSAL04524 are JWKF01000084, JWKG01000081, JUJZ01000081, LKJJ01000031, LKJK01000087, LNCZ01000024, and LKJD01000010, respectively.

sequenced the two bla_{NDM-5}-harboring plasmids from strains SZ03 and SZ04, which were found to be identical and subsequently named pNDM5-IncX3. Plasmid pNDM5-IncX3 is 46,161 bp in length, with a G+C content of 46.7%, and harbors 58 putative ORFs. The sequence of pNDM5-IncX3 showed 100% query coverage and overall >99.9% nucleotide identity to bla_{NDM-5}-harboring plasmids pEc1929 (GenBank accession no. KT824791) (22) and pNDM_MGR194 (KF220657) (23), as well as bla_{NDM-4}-harboring pJEG027 plasmid (KM400601) (24) and bla_{NDM-7}-harboring plasmid pKpN01-NDM7 (CP012990) (25). Notably, IncX3 plasmids harboring different bla_{NDM} variants were frequently found in different hospitals among isolates of different multilocus sequence types and species in China (22, 26–28), suggesting that IncX3 plasmids are the primary type of vector responsible for the wide dissemination of NDM metallo-β-lactamases in China. Alarmingly, pNDM5-IncX3 was found to coexist with pMCR1-IncX4 within the same clinical isolate (strains SZ03 and SZ04), resulting in resistance to both colistin and carbapenems (4).

In summary, this study characterizes two *mcr-1*-harboring plasmids from ESBL-producing *E. coli* and carbapenemase-producing *K. pneumoniae*. The identification of the same plasmid (pMCR1-IncX4) in isolates of different species (SZ01, SZ03, and SZ04) suggests that plasmid transfer is contributing to the dissemination of *mcr-1* in hospital settings in China. The potential for further spread of *mcr-1*-harboring plasmids within multidrug-resistant bacterial strains poses significant challenges for successful clinical treatment and infection control strategies.

Nucleotide sequence accession numbers. The complete nucleotide sequences of plasmids pMCR1-IncI2, pMCR1-IncX4, and pNDM5-IncX3 have been deposited in GenBank under accession no. KU761326, KU761327, and KU761328, respectively.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (grants R01AI090155 and R21AI117338) and the National Natural Science Foundation of China (grant 81572032).

B.N.K. discloses that he holds two patents that focus on using DNA sequencing to identify bacterial pathogens.

FUNDING INFORMATION

This work, including the efforts of Liang Chen, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (R21AI117338). This work, including the efforts of Barry N. Kreiswirth, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (R01AI090155). This work, including the efforts of Hong Du, was funded by National Natural Science Foundation of China (NSFC) (81572032).

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. http://dx.doi.org/10.1016/S1473-3099(15)00424-7.
2. **Skov R, Monnet D.** 2016. Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds. Euro Surveill **21**(9): pii=30155. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=21403.
3. **Falgenhauer L, Waezsada SE, Yao Y, Imirzalioglu C, Kasbohrer A, Roesler U, Michael GB, Schwarz S, Werner G, Kreienbrock L, Chakraborty T, RESET consortium.** 2016. Colistin resistance gene *mcr-1* in extended-spectrum beta-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. Lancet Infect Dis **16:** 282−283 http://dx.doi.org/10.1016/S1473-3099(16)00009-8.
4. **Du H, Chen L, Tang YW, Kreiswirth BN.** 2016. Emergence of the *mcr-1* colistin resistance gene in carbapenem-resistant *Enterobacteriaceae*. Lancet Infect Dis **16:**287–288. http://dx.doi.org/10.1016/S1473-3099(16)00056-6.
5. **Zurfuh K, Poirel L, Nordmann P, Nüesch-Inderbinen M, Hächler H, Stephan R.** 2016. Occurrence of the plasmid-borne *mcr-1* colistin resistance gene in ESBL-producing *Enterobacteriaceae* in river water and imported vegetable samples in Switzerland. Antimicrob Agents Chemother **60:**2594−2595. http://dx.doi.org/10.1128/AAC.00066-16.
6. **Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agerso Y, Zankari E, Leekitcharoenphon P, Stegger M, Kaas RS, Cavaco LM, Hansen DS, Aarestrup FM, Skov RL.** 2015. Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. Euro Surveill **20**(49)pii=30085. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=21331.
7. **Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P.** 2016. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. Lancet Infect Dis **16:**281. http://dx.doi.org/10.1016/S1473-3099(16)00006-2.
8. **Haenni M, Poirel L, Kieffer N, Chatre P, Saras E, Metayer V, Dumoulin R, Nordmann P, Madec JY.** 2016. Co-occurrence of extended spectrum beta lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis **16:**281−282. http://dx.doi.org/10.1016/S1473-3099(16)00007-4.
9. **Yao X, Doi Y, Zeng L, Lv L, Liu JH.** 2016. Carbapenem-resistant and colistin-resistant *Escherichia coli* co-producing NDM-9 and MCR-1. Lancet Infect Dis **16:**288−289. http://dx.doi.org/10.1016/S1473-3099(16)00057-8.
10. **Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S.** 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol **43:**4178–4182. http://dx.doi.org/10.1128/JCM.43.8.4178-4182.2005.

11. **Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M.** 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol **60:** 1136–1151. http://dx.doi.org/10.1111/j.1365-2958.2006.05172.x.
12. **Chen L, Hu H, Chavda KD, Zhao S, Liu R, Liang H, Zhang W, Wang X, Jacobs MR, Bonomo RA, Kreiswirth BN.** 2014. Complete sequence of a KPC-producing IncN multidrug-resistant plasmid from an epidemic *Escherichia coli* sequence type 131 strain in China. Antimicrob Agents Chemother **58:**2422–2425. http://dx.doi.org/10.1128/AAC.02587-13.
13. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA.** 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol **19:**455–477. http://dx.doi.org/10.1089/cmb.2012.0021.
14. **Partridge SR, Ellem JA, Tetu SG, Zong Z, Paulsen IT, Iredell JR.** 2011. Complete sequence of pJIE143, a *pir*-type plasmid carrying IS*Ecp1*-$bla_{\text{CTX-M-15}}$ from an *Escherichia coli* ST131 isolate. Antimicrob Agents Chemother **55:**5933–5935. http://dx.doi.org/10.1128/AAC.00639-11.
15. **Petty NK, Bulgin R, Crepin VF, Cerdeno-Tarraga AM, Schroeder GN, Quail MA, Lennard N, Corton C, Barron A, Clark L, Toribio AL, Parkhill J, Dougan G, Frankel G, Thomson NR.** 2010. The *Citrobacter rodentium* genome sequence reveals convergent evolution with human pathogenic *Escherichia coli*. J Bacteriol **192:**525–538. http://dx.doi.org/10.1128/JB.01144-09.
16. **Han J, Lynne AM, David DE, Tang H, Xu J, Nayak R, Kaldhone P, Logue CM, Foley SL.** 2012. DNA sequence analysis of plasmids from multidrug resistant *Salmonella enterica* serotype Heidelberg isolates. PLoS One **7:**e51160. http://dx.doi.org/10.1371/journal.pone.0051160.
17. **Chen L, Chavda KD, Al Laham N, Melano RG, Jacobs MR, Bonomo RA, Kreiswirth BN.** 2013. Complete nucleotide sequence of a bla_{KPC}-harboring IncI2 plasmid and its dissemination in New Jersey and New York hospitals. Antimicrob Agents Chemother **57:**5019–5025. http://dx.doi.org/10.1128/AAC.01397-13.
18. **Webb HE, Granier SA, Marault M, Millemann Y, den Bakker HC, Nightingale KK, Bugarel M, Ison SA, Scott HM, Loneragan GH.** 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis **16:** 144–145. http://dx.doi.org/10.1016/S1473-3099(15)00538-1.
19. **Tse H, Yuen KY.** 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis **16:**145–146. http://dx.doi.org/10.1016/S1473-3099(15)00532-0.
20. **Petrillo M, Angers-Loustau A, Kreysa J.** 2016. Possible genetic events producing colistin resistance gene *mcr-1*. Lancet Infect Dis **16:**280. http://dx.doi.org/10.1016/S1473-3099(16)00005-0.
21. **Hu Y, Liu F, Lin IY, Gao GF, Zhu B.** 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis **16:**146–147. http://dx.doi.org/10.1016/S1473-3099(15)00533-2.
22. **Chen D, Gong L, Walsh TR, Lan R, Wang T, Zhang J, Mai W, Ni N, Lu J, Xu J, Li J.** 2016. Infection by and dissemination of NDM-5-producing *Escherichia coli* in China. J Antimicrob Chemother **71:**563–565. http://dx.doi.org/10.1093/jac/dkv352.
23. **Krishnaraju M, Kamatchi C, Jha AK, Devasena N, Vennila R, Sumathi G, Vaidyanathan R.** 2015. Complete sequencing of an IncX3 plasmid carrying $bla_{\text{NDM-5}}$ allele reveals an early stage in the dissemination of the bla_{NDM} gene. Indian J Med Microbiol **33:**30–38. http://dx.doi.org/10.4103/0255-0857.148373.
24. **Espedido BA, Dimitrijovski B, van Hal SJ, Jensen SO.** 2015. The use of whole-genome sequencing for molecular epidemiology and antimicrobial surveillance: identifying the role of IncX3 plasmids and the spread of $bla_{\text{NDM-4}}$-like genes in the *Enterobacteriaceae*. J Clin Pathol **68:**835–838. http://dx.doi.org/10.1136/jclinpath-2015-203044.
25. **Chen L, Peirano G, Lynch T, Chavda KD, Gregson DB, Church DL, Conly J, Kreiswirth BN, Pitout JD.** 2016. Molecular characterization using next generation sequencing of plasmids containing $bla_{\text{NDM-7}}$ in *Enterobacteriaceae* from Calgary, Canada. Antimicrob Agents Chemother **60:**1258−1263. http://dx.doi.org/10.1128/AAC.02661-15.
26. **Yang Q, Fang L, Fu Y, Du X, Shen Y, Yu Y.** 2015. Dissemination of NDM-1-producing *Enterobacteriaceae* mediated by the IncX3-type plasmid. PLoS One **10:**e0129454. http://dx.doi.org/10.1371/journal.pone.0129454.
27. **Qu H, Wang X, Ni Y, Liu J, Tan R, Huang J, Li L, Sun J.** 2015. NDM-1-producing *Enterobacteriaceae* in a teaching hospital in Shanghai, China: IncX3-type plasmids may contribute to the dissemination of $bla_{\text{NDM-1}}$. Int J Infect Dis **34:**8–13. http://dx.doi.org/10.1016/j.ijid.2015.02.020.
28. **Ho PL, Li Z, Lo WU, Cheung YY, Lin CH, Sham PC, Cheng VC, Ng TK, Que TL, Chow KH.** 2012. Identification and characterization of a novel incompatibility group X3 plasmid carrying $bla_{\text{NDM-1}}$ in *Enterobacteriaceae* isolates with epidemiological links to multiple geographical areas in China. Emerg Microbes Infect **1:**e39. http://dx.doi.org/10.1038/emi.2012.37.