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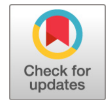

Chromosome-Mediated *mcr-3* Variants in *Aeromonas veronii* from Chicken Meat

Zhuoren Ling,[a] Wenjuan Yin,[a] Hui Li,[a] Qidi Zhang,[b] Xiaoming Wang,[a] Zheng Wang,[a] Yuebin Ke,[c] Yang Wang,[a,c] Jianzhong Shen[a,c]

Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agricultural University, Beijing, China[a]; College of Animal Science and Technology, Qingdao Agricultural University, Qingdao, Shandong, China[b]; Key Laboratory of Genetics & Molecular Medicine of Shenzhen, Shenzhen Center for Disease Control and Prevention, Shenzhen, China[c]

ABSTRACT Two adjacent colistin resistance gene variants, termed *mcr-3.3* and *mcr-3*-like, were identified in the chromosome of an *Aeromonas veronii* isolate obtained from retail chicken meat. The variants showed 95.20% and 84.19% nucleotide sequence identity, respectively, to *mcr-3* from porcine *Escherichia coli*. Functional cloning indicated that only *mcr-3.3* conferred polymyxin resistance in both *E. coli* and *Aeromonas salmonicida*. The *mcr-3.3*-*mcr-3*-like segment was also observed in other *Aeromonas* species, including *A. media*, *A. caviae*, and *A. hydrophila*.

KEYWORDS colistin resistance, *mcr-3*, *Aeromonas veronii*

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Address correspondence to Jianzhong Shen, sjz@cau.edu.cn.

Z.L. and W.Y. contributed equally to this article.

Colistin, the last line of defense against multidrug resistant Gram-negative bacteria, especially carbapenem-resistant *Enterobacteriaceae*, is nowadays increasingly used in the treatment of serious clinical infections. However, its clinical efficacy has been jeopardized since the emergence of the plasmid-mediated colistin resistance gene *mcr-1*, first identified in 2016 (1), followed by seven other *mcr-1* variants and a second resistance gene, *mcr-2* (2). Recently, a third polymyxin resistance gene, *mcr-3*, was characterized from *Escherichia coli*, further affecting the efficacy of colistin (3).

Compared with *mcr-1*, the global spread of which was diligently documented (4–11), there are few data on the prevalence of *mcr-3*-positive Gram-negative bacteria. It is particularly important to identify the presence of these strains in food products as they can have a direct impact on public health. Other than three *Enterobacteriaceae* species, including *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhimurium, which have been identified as *mcr-3* positive, all *mcr-3*-like genes, whose deduced amino acids presented 75.6–94.8% amino acid identity to MCR-3, have been identified in 10 *Aeromonas* species of various origins across four continents (3). However, whether these *mcr-3*-like genes confer colistin resistance in *Aeromonas* and *Enterobacteriaceae* remains unknown.

In the current study, 16 chicken meat samples were collected from six geographically distant supermarkets and three farmers' markets in Qingdao, Shandong Province, China, in 2015. The chicken meat samples were then macerated in 10 ml of brain heart infusion broth for 5 min in aseptic sampling bags (Hope Bio-Technology Co., Beijing, China), and the resulting infiltrated fluids were stored in ESwab tubes (Copan Diagnostics, Murrieta, CA) for further analysis. Direct screening was then performed on all 16 samples to detect the *mcr-3* gene, and only one sample was identified as *mcr-3* positive. The *mcr-3*-positive strain 172 was isolated using brain heart infusion agar (Beijing Land Bridge, Beijing, China) with 1 mg/liter colistin and then identified as *Aeromonas veronii* by 16S rRNA sequencing and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis.

Antimicrobial susceptibility testing was performed on *A. veronii* 172 using the agar dilution method described by the Clinical and Laboratory Standards Institute (docu-

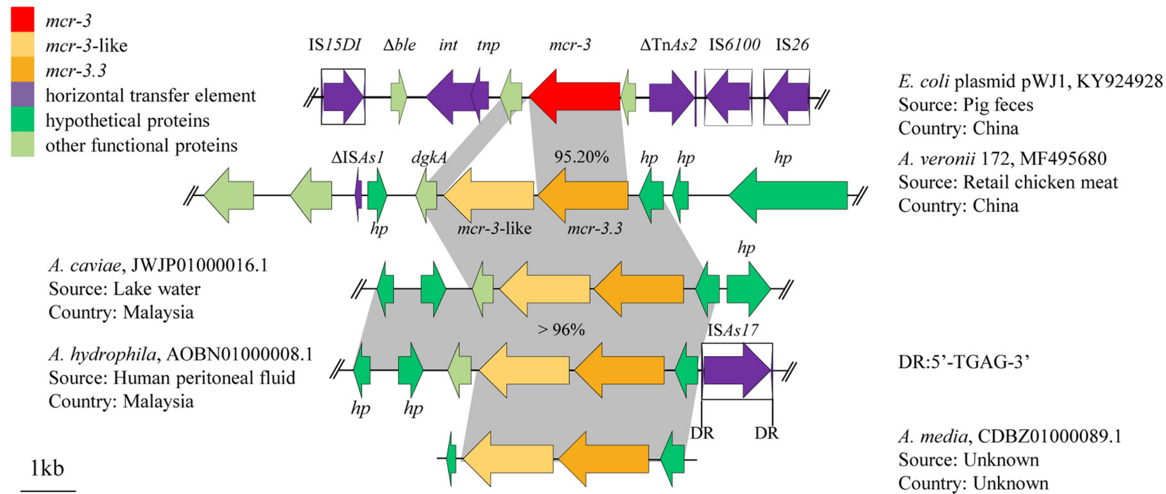

FIG 1 The genetic environment of the *mcr-3.3-mcr-3*-like segment in different *Aeromonas* isolates. Arrows indicate the directions of the genes. The regions in gray represent the two linked areas with high similarity.

ment VET01-A4) (12) and was interpreted according to Clinical and Laboratory Standards Institute document M45 (13). *E. coli* strain ATCC 25922 served as the quality control strain. *A. veronii* 172 was resistant to amoxicillin-clavulanic acid (64/32 mg/liter) and chloramphenicol (32 mg/liter) and had intermediate resistance to tetracycline (8 mg/liter) but was susceptible to ceftazidime (1 mg/liter), imipenem (1 mg/liter), meropenem (1 mg/liter), gentamicin (1 mg/liter), aztreonam (0.03 mg/liter), ciprofloxacin (0.5 mg/liter), and trimethoprim-sulfamethoxazole (0.5/9.5 mg/liter). Interestingly, this *mcr-3*-carrying isolate had an MIC for polymyxin B of 2 mg/liter and was borderline susceptible to colistin (2 mg/liter), according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (http://www.eucast.org/clinical_breakpoints/).

A. veronii 172 was then subjected to 300-bp paired-end whole-genome sequencing using the Illumina HiSeq 2500 system (Annoroad, Beijing, China). A total of 116 contigs were obtained by draft assembly using the CLC Genomics Workbench 9 (CLC Bio, Aarhus, Denmark). Among these, contig 13 (268.7 kb) contained two *mcr-3* variants that were located adjacently and separated by only 66 bp. The two variants showed 95.20% and 84.19% nucleotide sequence identity to the original *mcr-3* gene from pigs and were therefore termed *mcr-3.3* and *mcr-3*-like, respectively (Fig. 1). Both variants carried several missense mutations, resulting in 95.75% (*mcr-3.3*) and 84.84% (*mcr-3*-like) amino acid sequence similarity to *mcr-3* (Fig. 2). To identify the function of *mcr-3* variant genes, the 1853-bp and 3558-bp DNA fragments, including *mcr-3.3* and *mcr-3.3-mcr-3*-like fragments as well as their respective upstream sequences, were amplified and cloned into pUC19 before being electroporated into *E. coli* DH5α (TaKaRa). Transformants containing pUC19-*mcr-3.3* and pUC19-*mcr-3.3-mcr-3*-like had colistin MICs of 2 mg/liter and 1 mg/liter, respectively, which were 4- to 8-fold higher than the MIC of DH5α containing pUC19 alone (0.25 mg/liter), while the MICs of transformants with pUC19-*mcr-3*-like were no different than that of pUC19-DH5α. However, the gap between *mcr-3*-like and *mcr-3.3* is very small (only 66 bp), which means that the promoter region of *mcr-3*-like may be absent, leading to an insufficient expression of *mcr-3*-like. Therefore, to further confirm the invalidation of *mcr-3-like* in mediating colistin resistance, only the 1623-bp and 1626-bp coding sequences of *mcr-3.3* and *mcr-3-like* were ligated into expression vector pET-28a (Novagen, Beijing, China) and transformed into *E. coli* BL21(DE3)plys (Transgen Biotech, Beijing, China). When induced by isopropyl-β-D-thiogalactopyranoside (IPTG) with a concentration of 0.1 mM, the transformants containing pET-*mcr-3*-like did not present any change in MIC compared with BL21(DE3)plys containing pET28a alone (8 mg/liter), while the MICs of transformants carrying pET-

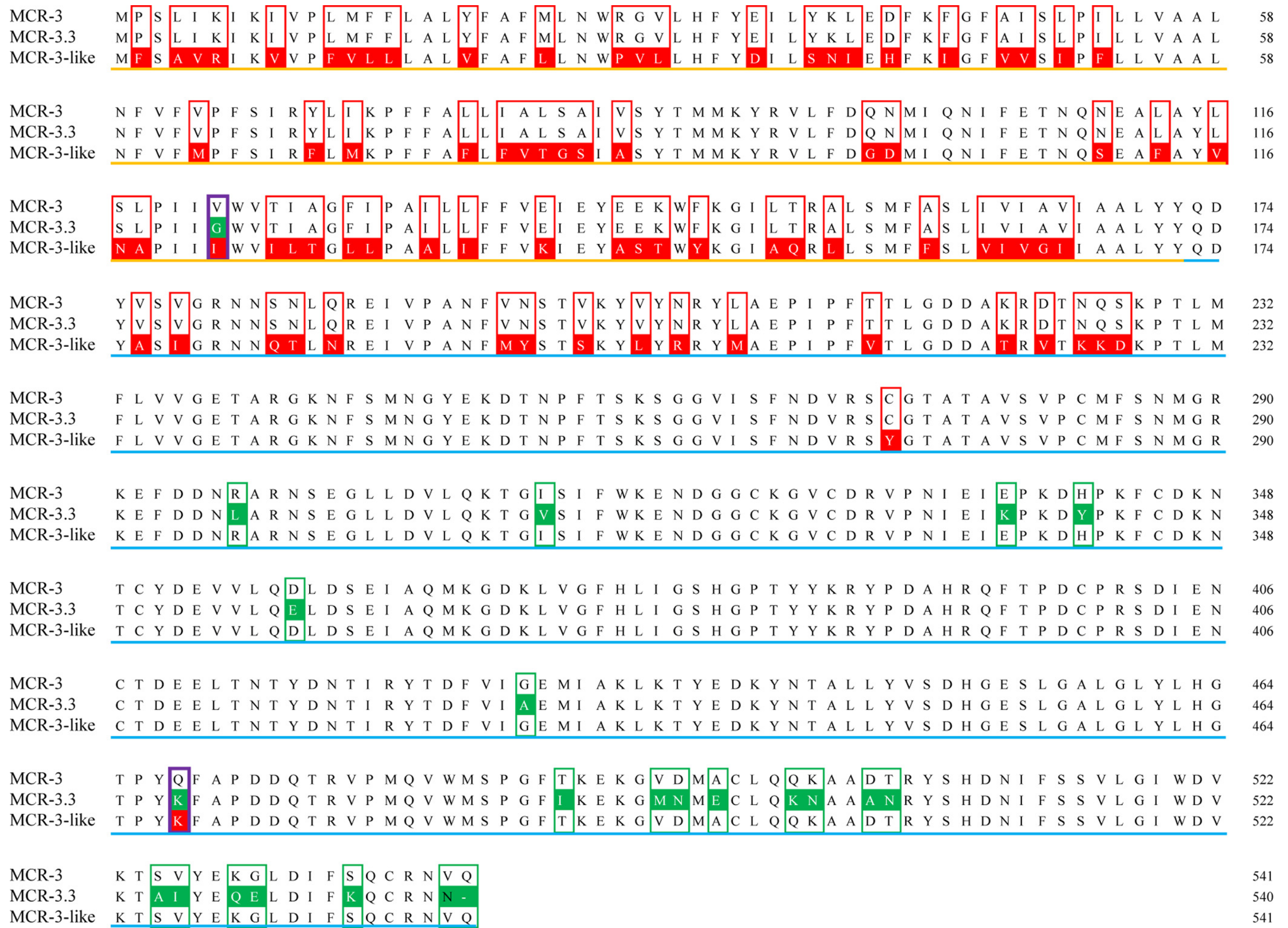

FIG 2 Alignment of the predicted amino acid sequences of MCR-3, MCR-3.3, and MCR-3-like. Orange lines denote putative transmembrane regions and blue lines indicate catalytic domains. Differences in amino acid residues between MCR-3 and MCR-3.3 are shown in green boxes, while red boxes indicate differences between MCR-3 and MCR-3-like.

mcr-3.3 increased to 16 mg/liter, suggesting that only *mcr-3.3* confers colistin resistance in *E. coli*. In addition, to investigate the function of two variants in the species of *Aeromonas*, the plasmids pUC19-*mcr-3.3* and pUC19-*mcr-3.3-mcr-3*-like were separately electroporated into a competent *Aeromonas salmonicida* strain that originated from a chicken cloaca swab. To our surprise, MICs of both transformants soared to 64 mg/liter, which was 64-fold higher than the MIC of a transformant with pUC19 alone (1 mg/liter). Stability testing revealed that pUC19-*mcr-3.3* existed stably in the host of *A. salmonicida* after 20 generations of subculture both without or with colistin (1 mg/liter). These results suggested that *mcr-3.3*, but not *mcr-3-like*, could confer colistin resistance in *E. coli* and *Aeromonas* species, the latter of which may not only serve as a reservoir for *mcr-3* variants but also have the potential to develop a highly colistin-resistant phenotype.

Comparative genomic analysis between contig 13 and the whole complete genomic sequence of *A. veronii* AVNIH1 (GenBank accession no. CP014774.1) was performed using Mauve (http://darlinglab.org/mauve/mauve.html) and showed that almost all of the block outlines in contig 13 matched their counterparts in the chromosome of AVNIH1 (see Fig. S1 in the supplemental material). S1-nuclease pulsed-field gel electrophoresis and Southern blotting also confirmed that the two *mcr-3* variants were located on the chromosome of *A. veronii* 172 (data not shown).

The *mcr-3.3-mcr-3*-like segment in *A. veronii* 172 was surrounded by both hypothetical

and functional protein-coding genes. Unlike the *mcr-3* in *E. coli* plasmid pWJ1, the fragment containing the *mcr-3.3-mcr-3*-like segment in *A. veronii* 172 lacked transfer elements (Fig. 1). However, *dgkA*, which was located downstream of *mcr-3* in pWJ1, was also identified in the downstream regions of the *mcr-3.3-mcr-3*-like segment in *A. veronii* 172, *Aeromonas caviae* (GenBank accession no. JWJP01000016.1), and *Aeromonas hydrophila* (GenBank accession no. AOBN01000008.1). The upstream region of the *mcr-3.3-mcr-3*-like segment in *A. hydrophila* contained a complete insertion sequence, IS*As17*, the open reading frame of which exhibited 99% nucleotide sequence identity to that originated from *Aeromonas salmonicida*. This IS*As17* element had imperfect inverted repeats of 50 bp right (IRR, TGGATTTGACCCCATAGATCCAGACACTTTTTCCCGCTTAGGGTTGTGAT) and 50 bp left (IRL, TAGATTGGCCCCTTGAAGATCCAGACACTTCGCCCCTCTTAACTTAAGAG) at its ends. In addition, direct target site duplications (5′-TGAG-3′) were also observed immediately upstream and downstream of IS*As17* (Fig. 1).

Apart from the *mcr-3.3-mcr-3*-like segment, BLAST analysis revealed the presence of other resistance genes in the genome of *A. veronii* 172, including β-lactam resistance genes *cphA1* and *ampS*, phenicol resistance gene *catA2*, and tetracycline resistance gene *tet*(E). This was consistent with the multidrug-resistance phenotype observed during antibiotic susceptibility testing. In addition, the multilocus sequence type of this *Aeromonas* strain was submitted and assigned as a novel sequence type ST512 (https://cge.cbs.dtu.dk/services/MLST/).

In conclusion, an *A. veronii* strain containing both *mcr-3.3* and *mcr-3*-like genes on its chromosome was isolated from chicken meat in China. This strain showed resistance to both β-lactams and phenicols and had borderline susceptibility to colistin, which was mediated by *mcr-3.3* alone. The presence of these *mcr-3* chromosomal variants is worrisome because acquisition of a plasmid carrying an *mcr-3* variant may lead to high levels of colistin resistance in these *Aeromonas* strains. Since *Aeromonas* species are prevalent in aquatic environments, where they interact with bacteria from different origins, they may act as a reservoir for *mcr-3* and contribute to its potential dissemination.

Accession number(s). The 268746-bp nucleotide sequence of contig 13 carrying *mcr-3.3-mcr-3*-like segment has been deposited as GenBank accession no. MF495680.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01272-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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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.
2. Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. Euro Surveill 21(27):pii=30280. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=22525.
3. Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, Zhang R, Walsh TR, Shen J, Wang Y. 2017. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. MBio 8:e00543-17. https://doi.org/10.1128/mBio.00543-17.
4. Bernasconi OJ, Kuenzli E, Pires J, Tinguely R, Carattoli A, Hatz C, Perreten V, Endimiani A. 2016. Travelers can import colistin-resistant *Enterobacteriaceae*, including those possessing the plasmid-mediated *mcr-1* gene. Antimicrob Agents Chemother 60:5080–5084. https://doi.org/10.1128/AAC.00731-16.
5. 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.
6. Castanheira M, Griffin MA, Deshpande LM, Mendes RE, Jones RN, Flamm RK. 2016. Detection of *mcr-1* among *Escherichia coli* clinical isolates collected worldwide as part of the SENTRY Antimicrobial Surveillance

Program in 2014 and 2015. Antimicrob Agents Chemother 60:5623–5624. https://doi.org/10.1128/AAC.01267-16.

7. Shen Z, Wang Y, Shen Y, Shen J, Wu C. 2016. Early emergence of *mcr-1* in *Escherichia coli* from food-producing animals. Lancet Infect Dis 16:293. https://doi.org/10.1016/S1473-3099(16)00061-X.
8. Gu DX, Huang YL, Ma JH, Zhou HW, Fang Y, Cai JC, Hu YY, Zhang R. 2016. Detection of colistin resistance gene *mcr-1* in hypervirulent *Klebsiella pneumoniae* and *Escherichia coli* isolates from an infant with diarrhea in China. Antimicrob Agents Chemother 60:5099–5100. https://doi.org/10.1128/AAC.00476-16.
9. Perreten V, Strauss C, Collaud A, Gerber D. 2016. Colistin resistance gene *mcr-1* in avian-pathogenic *Escherichia coli* in South Africa. Antimicrob Agents Chemother 60:4414–4415. https://doi.org/10.1128/AAC.00548-16.
10. Rapoport M, Faccone D, Pasteran F, Ceriana P, Albornoz E, Petroni A, MCR Group, Corso A. 2016. First description of *mcr-1*-mediated colistin resistance in human infections caused by *Escherichia coli* in Latin America. Antimicrob Agents Chemother 60:4412–4413. https://doi.org/10.1128/AAC.00573-16.
11. Wang Y, Tian G-B, Zhang R, Shen Y, Tyrrell JM, Huang X, Zhou H, Lei L, Li H-Y, Doi Y, Fang Y, Ren H, Zhong L-L, Shen Z, Zeng K-J, Wang S, Liu J-H, Wu C, Walsh TR, Shen J. 2017. Prevalence, risk factors, outcomes, and molecular epidemiology of *mcr-1*-positive *Enterobacteriaceae* in patients and healthy adults from China: an epidemiological and clinical study. Lancet Infect Dis 17:390–399. https://doi.org/10.1016/S1473-3099(16)30527-8.
12. Clinical and Laboratory Standards Institute. 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 4th ed. CLSI VET01-A4. Clinical and Laboratory Standards Institute, Wayne, PA.
13. Clinical and Laboratory Standards Institute. 2015. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria, 3rd ed. CLSI M45. Clinical and Laboratory Standards Institute, Wayne, PA.