J Antimicrob Chemother 2018; **73**: 1777–1780
doi:10.1093/jac/dky110 Advance Access publication 11 April 2018

Journal of Antimicrobial Chemotherapy

Mobile colistin resistance gene *mcr-5* in porcine *Aeromonas hydrophila*

Shizhen Ma[1], Chengtao Sun[1], Anette Hulth[2,3], Jiyun Li[1], Lennart E. Nilsson[4], Yuqing Zhou[1], Stefan Börjesson[5], Zhenwang Bi[6], Zhenqiang Bi[6], Qiang Sun[7,8] and Yang Wang[1]*

[1]Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agricultural University, Beijing, China; [2]Public Health Agency of Sweden, Stockholm, Sweden; [3]Global Health - Health Systems and Policy, Department of Public Health Sciences, Karolinska Institutet, Solna, Sweden; [4]Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden; [5]Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), Uppsala, Sweden; [6]Shandong Provincial Key Laboratory of Infectious Disease Control and Prevention, Shandong Center for Disease Control and Prevention, Jinan, Shandong, China; [7]Center for Health Management and Policy, Shandong University, Jinan, Shandong, China; [8]Key Lab of Health Economics and Policy Research of Ministry of Health, Shandong University, Jinan, Shandong, China

*Corresponding author. Tel: +8610-62734255; E-mail: wangyang@cau.edu.cn

Received 16 January 2018; returned 1 February 2018; revised 25 February 2018; accepted 7 March 2018

Objectives: To characterize the mobile colistin resistance gene *mcr-5* in *Aeromonas hydrophila* from backyard pigs in rural areas of China.

Methods: Pig faecal samples from 194 households were directly tested for the presence of *mcr-5* by PCR assay and the phenotypic antimicrobial susceptibility profiles of the *mcr-5*-positive isolates were determined using the broth dilution method. The genomic location and transferability of *mcr-5* were analysed by S1-PFGE with Southern blotting and DNA hybridization, and natural transformation, respectively. One strain isolated from an *mcr-5*-positive sample was subjected to WGS and the stability of the *mcr-5*-harbouring plasmid over successive generations was examined by subculturing.

Results: One *mcr-5*-positive *A. hydrophila* isolate showing resistance, with a colistin MIC of 4 mg/L, was isolated from a backyard pig faecal sample. *mcr-5* was located on a 7915 bp plasmid designated pI064-2, which could naturally transform into a colistin-susceptible *A. hydrophila* strain of porcine origin and mediated colistin resistance in both the original isolate and its transformants. The plasmid backbone (3790 bp) of pI064-2 showed 81% nucleotide sequence identity to the corresponding region of the ColE2-type plasmid pAsa1 from *Aeromonas salmonicida*, while similar replication primases are widely distributed among aeromonads, Enterobacteriaceae and *Pseudomonas* species.

Conclusions: To the best of our knowledge, this is the first identification of the novel colistin resistance gene *mcr-5* in an *A. hydrophila* isolate from the faeces of a backyard pig. *mcr-5* is expected to be able to disseminate among different bacterial species and genera.

Introduction

Colistin is one of the 'last-resort' drugs for treatment of serious clinical infections caused by MDR Gram-negative bacteria, especially carbapenem-resistant Enterobacteriaceae.[1] However, increasing use of this antibiotic in clinical and veterinary practice has led to the emergence of mobile colistin resistance genes, including *mcr-1*, which was first reported in *Escherichia coli* in 2015, and *mcr-2*, identified in *E. coli* in 2016.[2,3] Recently, three further plasmid-mediated colistin resistance genes, *mcr-3*, *mcr-4* and *mcr-5*, were identified, with Enterobacteriaceae, particularly *E. coli* and *Salmonella* spp., being the predominant hosts.[4–6] However, MCR-5 is distinct from MCR-1, MCR-2, MCR-3 and MCR-4, showing only 34%–36% amino acid sequence identity to the other four proteins. Despite the sequence discrepancy, all five proteins are phosphoethanolamine transferases, adding phosphoethanolamine to the lipid A moiety of LPS, leading to a more cationic LPS structure and consequently resistance to colistin.[7] To date, only *mcr-1* and *mcr-3* have been widely reported among Enterobacteriaceae, while the *mcr-5* gene has only been found in *Salmonella* spp. from poultry and animal-derived food products in Germany.[6]

Bacteria belonging to the genus *Aeromonas*, especially *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii*, frequently cause diarrhoeal diseases and wound infections in both humans and animals, and it appears to be ubiquitous in the aquatic environment.[8] The *mcr-3* variants have been identified in 10 *Aeromonas* species of various origins across four continents,[4,9] with *mcr-3.3* and *mcr-3.7* identified in *A. veronii* and *A. caviae* from chicken meat and a domestic duck, respectively, in China.[10,11] Thus, *Aeromonas* species may play an important role in the acquisition and dissemination of the *mcr* genes. Here, we report the identification of an *mcr-5*-positive *A. hydrophila* isolate from the faeces of backyard pigs from rural areas of Shandong Province, China.

Materials and methods

Sample collection, bacterial identification and susceptibility testing

A total of 336 faecal samples were collected from backyard pigs from 194 households across 12 villages in rural areas of Shandong Province, China, in August 2017, using the ESwab Collection and Transport System (Copan, Brescia, Italy). The village and household selection methods have been described previously.[12] All samples were enriched in 1 mL of LB broth containing 10 mg/L vancomycin at 37°C overnight. Total DNA was extracted from 500 μL of each of the enriched cultures using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and the DNA used for screening of *mcr-5* by PCR, as previously reported.[6] The enriched samples positive for *mcr-5* were inoculated onto *Salmonella Shigella* agar (Binhe Microorganism Reagent Co., Hangzhou, China) containing 2 mg/L colistin. One random colony of each morphotype was selected for *mcr-5* gene detection. Species identification of *mcr-5*-positive isolates was conducted by 16S rRNA gene sequencing and MALDI-TOF MS analysis (Bruker Daltonik GmbH, Bremen, Germany).[11] *mcr-5*-positive isolates were also subjected to antimicrobial susceptibility testing for colistin and eight other commonly used antibiotics, using the broth dilution method and interpreted according to CLSI M100-S25,[13] VET01-S2[14] and EUCAST (version 6.0, http://www.eucast.org/clinical_breakpoints/). *E. coli* strain ATCC 25922 served as the quality control strain.

Location and transferability of mcr-5

S1-PFGE followed by Southern blotting and DNA hybridization with a digoxigenin-labelled *mcr-5* probe were performed to determine the genomic location of *mcr-5*, as previously described.[6] The transferability of *mcr-5* was then examined using natural transformation, with plasmids extracted from an *mcr-5*-positive strain used as donor DNA and a colistin-susceptible porcine *A. hydrophila* strain (2ZF0081a) used as the recipient, as previously reported.[15] Competent cells of the control strain 2ZF0081a were induced in 20% nutrient broth (NB) in late stationary phase after 24 h of incubation at 30°C. A 40 μL aliquot of competent cells was added to 100 μL of transformation buffer consisting of 53.5 mM Tris, pH 7.9, 20 mM $MgSO_4$ and 33 mM NaCl. Plasmids extracted from an *mcr-5*-positive strain using a Plasmid Midi Kit (Omega, Norcross, GA, USA) were added in a volume of 10 μL (final concentration of 3.3 ng/μL). The mixture was incubated at 30°C for 120 min, then diluted in 0.85% NaCl and inoculated onto LB agar with 2 mg/L colistin at 30°C for 48 h. Transformants were confirmed by *mcr-5* PCR assays.

WGS analysis of the mcr-5-carrying isolate

DNA was extracted from the *mcr-5*-positive isolate using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). DNA libraries were prepared using a KAPA Hyper Prep Kit (Kapa Biosystems, Wilmington, MA, USA) and 150 bp paired-end sequencing was conducted using an Illumina HiSeq 2500 platform (Annoroad Genomics Co., Beijing, China). The draft genomes were assembled using SPAdes with ×50 minimum assembled coverage.[16] Identification of antibiotic resistance genes and plasmid replicon typing were carried out via the Center for Genomic Epidemiology website (http://www.genomicepidemiology.org/). The *mcr-5*-carrying contigs were annotated using the online automated PATRIC server (version 3.5.1, https://www.patricbrc.org).

Stability of the mcr-5-carrying plasmid

The stability of the *mcr-5*-carrying plasmid was confirmed by subculturing successive generations in triplicate without colistin, as previously reported.[17] Briefly, three colonies were enriched in 5 mL of LB broth containing 2 mg/L colistin for 24 h at 30°C. Colistin was removed by centrifuging 1 mL of culture (4000 **g**, 5 min) and resuspending the pellet in 1 mL of saline. A total of 4.88 μL of the suspension was then inoculated into 5 mL of fresh LB broth minus antibiotic (timepoint zero) and incubated at 30°C with shaking at 200 rpm for 24 h to obtain approximately 10 generations. Thereafter, 4.88 μL of each culture was transferred to 5 mL of fresh LB broth every 24 h. Cultures were diluted and plated onto LB plates every second day. A total of 50 resulting colonies were randomly selected for *mcr-5*-specific PCR assays to determine the proportion of *mcr-5*-positive bacteria in each population.

Results and discussion

PCR assays showed that eight pig faecal samples from seven households in four villages were positive for *mcr-5*, while all the samples were negative for the other three novel *mcr-2*, *mcr-3* and *mcr-4* genes. However, we were only able to detect one *mcr-5*-positive isolate (I064-2), identified as *A. hydrophila*, from one sample, while *E. coli*, *A. veronii* and *A. caviae* strains isolated from the other seven samples were negative for *mcr* genes. The considerably higher rate of positive results obtained from direct sample testing compared with bacterial isolation is indicative of a substantial segment of unseen resistome, which is in line with the previous observation of the 'phantom resistome' of bla_{NDM} and *mcr-1* resistance genes in samples from commercial chicken farms.[18] The identified *A. hydrophila* isolate, I064-2, was resistant to colistin (MIC = 4 mg/L) and intermediate to tetracycline (MIC = 8 mg/L), but was susceptible to amikacin (MIC = 4 mg/L), gentamicin (MIC = 2 mg/L), florfenicol (MIC = 1 mg/L), ceftazidime (MIC = 0.5 mg/L), ciprofloxacin (MIC = 0.5 mg/L), tigecycline (MIC = 0.5 mg/L) and meropenem (MIC = 0.03 mg/L). S1-PFGE and DNA hybridization analyses revealed that the *mcr-5* gene was located on an ~8 kb plasmid (data not shown).

The draft genome of *A. hydrophila* I064-2, assembled from WGS reads, contained 135 contigs (N50=112971 bp). Among these, contig 56 (8064 bp, ×476.625 assembled coverage) harboured a 3670 bp segment consisting of *chrB-mcr-5-*Δ*msf-*Δ*msf* that showed 100% nucleotide sequence identity to the *mcr-5*-carrying operon of plasmid pSE13-SA01718 (GenBank accession number KY807921.1) from a *Salmonella* Paratyphi B isolate from Germany (Figure 1).[6] We also observed 149 bp repeats at each end of contig 56. Reverse PCR using primers pI0642-F (5′-CGGCT CGTATTATGGCTGTCG-3′) and pI0642-R (5′-CGCTCGGGTGCGA AATCA-3′) (Figure 1) yielded a 1940 bp amplicon that successfully closed the contig, fully covering the 149-repeated base, confirming that *mcr-5* was located on a 7915 bp plasmid,

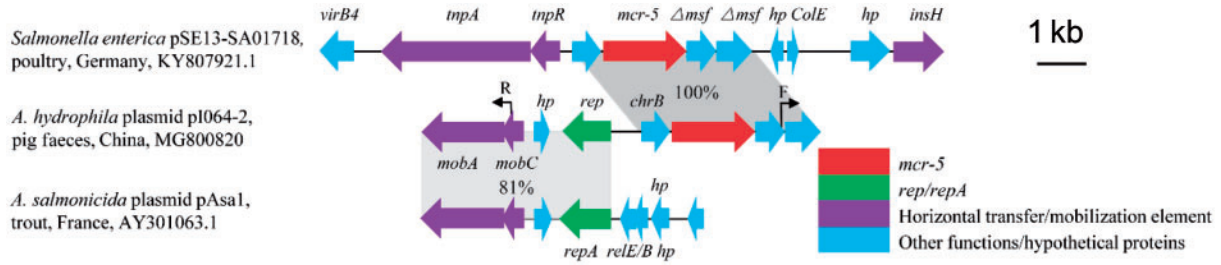

Figure 1. Comparison of the genetic environment of *mcr-5* and the plasmid backbone of pI064-2 with pSE13-SA01718 (GenBank accession no. KY807921.1) and pAsa1 (GenBank accession no. AY301063.1). Arrows indicate the positions and directions of the genes. Δ indicates a truncated gene. Regions with 100% homology are indicated by dark grey shading and those with 81% homology are indicated by light grey shading. F and R represent the primers pI0642-F and pI0642-R used in the reverse PCR assay. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

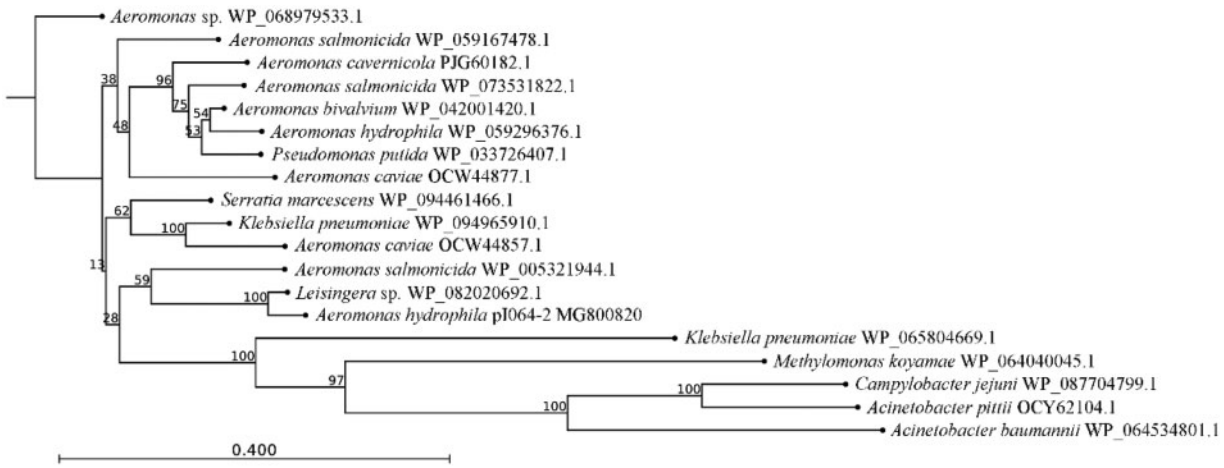

Figure 2. Phylogenetic tree generated from the deduced amino acid sequences of 18 replication primases from different bacterial species and from pI064-2. The tree was generated using CLC Genomics Workbench 9 (CLC Bio-Qiagen, Aarhus, Denmark).

designated pI064-2 (GenBank accession number MG800820). The 100% nucleotide sequence identity between the *mcr-5*-carrying fragments of pSE13-SA01718 from *Salmonella* Paratyphi B and pI064-2 implied high stability of the *mcr-5*-carrying operon, as well as the dissemination of *mcr-5* between different bacterial species and genera.

In addition to the *mcr-5*-carrying operon, pI064-2 contains a 3790 bp segment consisting of genes involved in plasmid replication (*rep*) and mobilization (*mobC* and *mobA*) and coding for proteins of unknown function. This region showed 81% nucleotide sequence identity to the corresponding region of the ColE2-type plasmid pAsa1 (GenBank accession number AY301063.1) from a piscine *Aeromonas salmonicida* isolate (Figure 1). The 1967 bp segment containing *mobC-mobA* showed 91% nucleotide sequence identity and 82% query coverage to the corresponding region of pAsa1, while the replicase-encoding gene *rep* showed 76% nucleotide sequence identity and 95% coverage to the *repA* region of pAsa1.[19] BLASTp analysis of the pI064-2-encoded replicase (311 amino acids) revealed that a large section of this protein shows high amino acid identity to the replicase of a *Leisingera* sp. isolate obtained from an *Euprymna scolopes* sample from the USA[20] (GenBank accession number WP_082020692.1; 91% identity and 75% query coverage) and to the deduced amino acid sequence of *repA* from pAsa1 (75% identity and 99% query coverage) (Figure 2). In addition, the *A. hydrophila* pI064-2 replicase showed 65%–76% amino acid sequence identity to the replication primases from *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas putida*, *A. caviae*, *A. salmonicida* and *A. hydrophila* isolates derived from humans, chicken, fish, sewage and sand samples collected from eight countries in Asia, Europe and North America [Figure 2 and Table S1 (available as Supplementary data at *JAC* Online)]. Similarities between ColE-like replication primases in Enterobacteriaceae, *Pseudomonas* species and aeromonads suggests the likelihood of potential wide dissemination of *mcr-5* among these bacteria, especially *Aeromonas* species containing closely related plasmids. BLAST analysis revealed the presence of other resistance genes in *A. hydrophila* I064-2, including the AmpC β-lactamase gene bla_{MOX-6} and the tetracycline resistance gene *tet*(E).

Conjugation experiments did not result in the transfer of pI064-2 into a recipient *E. coli* J53Az^R strain using filter mating, but purified plasmid DNA obtained from I064-2 was successfully transformed into a colistin-susceptible *A. hydrophila* strain in transformation buffer. The *mcr-5*-positive transformant had an MIC of

2 mg/L for colistin, 8-fold higher than the control recipient (MIC = 0.25 mg/L). Stability testing showed that all I064-2 colonies were positive for *mcr-5* for ∼200 generations (20 days) in the absence of colistin selection, confirming that pI064-2 is stable in the WT parent. However, pI064-2 was only maintained in the *mcr-5*-positive transformant for 20 generations without the pressure of colistin. As previously described, natural transformation is a general property of *Aeromonas* environmental isolates.[15] Therefore, *mcr-5* might have the potential to be transferred among the aquatic *Aeromonas* species in the environment.

In conclusion, to the best of our knowledge, this is the first report of *mcr-5* in *A. hydrophila* isolated from backyard pigs in rural areas of China. The ColE-like replication primase in *mcr-5*-harbouring plasmid, which is widely distributed among Enterobacteriaceae, *Pseudomonas* species and aeromonads, indicates that pI064-2 has the potential to disseminate among different bacterial genera. In addition, the observed 100% nucleotide sequence identity between the *mcr-5*-carrying operons in *Salmonella* and *Aeromonas* spp. also suggests the possibility of horizontal gene transfer of the *mcr-5*-carrying segment among these bacterial species and genera. Further studies should focus on the surveillance of *mcr-5* in colistin-resistant Gram-negative pathogens derived from animals, humans and the environment.

Funding

This study is part of the Sino-Swedish IMPACT project, which is funded by the National Natural Science Foundation of China (grant number 81361138021) and the Swedish Research Council (grant number D0879801).

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

References

1 Falagas ME, Karageorgopoulos DE, Nordmann P. Therapeutic options for infections with Enterobacteriaceae producing carbapenem-hydrolyzing enzymes. *Future Microbiol* 2011; **6**: 653–66.

2 Liu Y, 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.

3 Xavier BB, Lammens C, Ruhal R *et al*. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 2016; **21**: pii=30280.

4 Yin W, Li H, Shen Y *et al*. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *MBio* 2017; **8**: e00543-17.

5 Carattoli A, Villa L, Feudi C *et al*. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill* 2017; **22**: pii=30589.

6 Borowiak M, Fischer J, Hammerl JA *et al*. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. *J Antimicrob Chemother* 2017; **72**: 3317–24.

7 Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 2017; **30**: 557–96.

8 Janda JM, Abbott SL. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clin Microbiol Rev* 2010; **23**: 35–73.

9 Eichhorn I, Feudi C, Wang Y *et al*. Identification of novel variants of the colistin resistance gene *mcr-3* in *Aeromonas* spp. from the national resistance monitoring programme GERM-Vet and from diagnostic submissions. *J Antimicrob Chemother* 2018; **73**: 1217–21.

10 Ling Z, Yin W, Li H *et al*. Chromosome-mediated *mcr-3* variants in *Aeromonas veronii* from chicken meat. *Antimicrob Agents Chemother* 2017; **61**: e01272-17.

11 Wang X, Zhai W, Li J *et al*. Presence of *mcr-3* variant in *Aeromonas caviae*, *Proteus mirabilis*, and *Escherichia coli* from one domestic duck. *Antimicrob Agents Chemother* 2017; **62**: e02106-17.

12 Sun Q, Wang Y, Hulth A *et al*. Study protocol for One Health data collections, analyses and intervention of the Sino-Swedish integrated multisectoral partnership for antibiotic resistance containment (IMPACT). *BMJ Open* 2018; **8**: e017832.

13 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Fifth Informational Supplement M100-S25*. CLSI, Wayne, PA, USA, 2015.

14 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk and Diffusion Susceptibility Tests for Bacteria Isolated From Animals: Second Informational Supplement VET01-S2*. CLSI, Wayne, PA, USA, 2013.

15 Huddleston JR, Brokaw JM, Zak JC *et al*. Natural transformation as a mechanism of horizontal gene transfer among environmental *Aeromonas* species. *Syst Appl Microbiol* 2013; **36**: 224–34.

16 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

17 De Gelder L, Ponciano JM, Joyce P *et al*. Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. *Microbiology* 2007; **153**: 452–63.

18 Wang Y, Zhang R, Li J *et al*. Comprehensive resistome analysis reveals the prevalence of NDM and MCR-1 in Chinese poultry production. *Nat Microbiol* 2017; **2**: 16260.

19 Boyd J, Williams J, Curtis B *et al*. Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. *Plasmid* 2003; **50**: 131–44.

20 Collins AJ, Fullmer MS, Gogarten JP *et al*. Comparative genomics of *Roseobacter* clade bacteria isolated from the accessory nidamental gland of *Euprymna scolopes*. *Front Microbiol* 2015; **6**: 123.