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ORIGINAL RESEARCH

A Novel Transposon, Tn*6518*, Mediated Transfer of *mcr-3* Variant in ESBL-Producing *Aeromonas veronii*

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Purpose: The aim of this study was to determine the prevalence and transmission mechanism of *mcr-3* in *Aeromonas* spp. isolated from chicken cloaca.

Materials and Methods: *A. veronii* w55 was isolated from chicken in 2008. PCR assay was used to detect *mcr* genes and putative circular intermediate. Susceptibility testing was identified by the microdilution method. WGS was performed to obtain the whole sequence. S1-PFGE and DNA southern hybridization were used to study the location of *mcr-3.6*.

Results: PCR-based analysis indicated that 1 out of 55 *Aeromonas* spp. isolates was *mcr-3*-positive. Whole-genome sequencing revealed that the strain *A. veronii* w55 belonged to novel sequence type ST514 and had two adjacent chromosomally located *mcr* variants, *mcr-3.6* and *mcr-3*-like. The *mcr-3.6* and *mcr-3*-like genes showed 93.67% and 82.84% nucleotide sequence identity, respectively, to original *mcr-3* from *E. coli*. *A. veronii* w55 also exhibited resistance to extended-spectrum β-lactams and was positive for bla_{PER-3}, and this is the first time to report bla_{PER-3} in *A. veronii*. Genetic environment analysis revealed that the segment of *mcr-3.6-mcr-3*-like-*dgkA* was flanked by five insertion sequence elements originated from *Aeromonas* species, and the structure of IS*As2*-IS*Ahy2*-IS*As20-mcr-3.6-mcr-3*-like-*dgkA*-IS*As2* was designated as a novel transposon Tn*6518*, in which an 8405-bp circular intermediate carrying two *mcr-3* variants can be looped out.

Conclusion: This result suggested the *mcr-3* variant genes could be disseminated between various *Aeromonas* species via transposon-mediated transmission.

Keywords: *mcr-3.6*, Tn*6158*, *Aeromonas veronii*

Introduction

Aeromonas species are Gram-negative, rod-shaped bacteria with a broad host range. To date, they have been shown to infect a vast number of hosts, including fish, domestic chickens, lower and higher vertebrates, and humans.[1–3] In animals, *Aeromonas* species cause illnesses ranging from furunculosis and diarrhea to septicemia.[4] However, because of the wile use of broad-spectrum antibiotics in clinical settings, agriculture, and aquaculture, there has been an increase in antibiotic resistance among disease-causing *Aeromonas* species.

Colistin, a polycationic peptide, is considered as one of the last-resort drugs for the treatment of infections caused by multi-drug resistant Gram-negative bacteria.[5] In 2016, the first plasmid-mediated colistin resistance gene *mcr-1* was characterized in Enterobacteriaceae.[6] To date, nine mobile colistin resistance determinants (*mcr-1~mcr-9*) have been reported in *Enterobacteriaceae, Moraxella* and *Aeromonas*.[7–10] Subsequently, multiple *mcr-3* variants and one *mcr-5* gene were identified in *Aeromonas* species, implying *Aeromonas* species plays an important role in the

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dissemination of mobile colistin resistance genes.[11–13] Among them *mcr-3* was first found in *Escherichia coli* isolated from pig,[14] then some papers illustrated that this gene emerged in *Aeromonas* spp.[12] Subsequently, Ling et al study showed that *mcr-3.3* and *mcr-3*-like have emerged in *Aeromonas veronii* isolated from retail chicken meat,[8] and Eichhorn et al found four *Aeromonas* isolates were positive for *mcr-3*, each of the four *mcr-3* genes showed amino acid identities of 95.0–98.0% to the original *mcr-3* protein. These variants were designated *mcr-3.6* (*Aeromonas allosaccharophila* from golden orfe), *mcr-3.7* (*Aeromonas media* from), *mcr-3.8* (*Aeromonas jandaei* from koi carp) and *mcr-3.9* (*Aeromonas hydrophila* from koi carp).[14] Herein, we investigated the presence of *mcr*-type genes in *Aeromonas* spp. from chicken, and identified a novel transposon Tn*6518* carrying *mcr-3* variant in ESBL-producing *Aeromonas veronii*.

Materials and Methods

Bacterial Isolates, Screening for *mcr*-Type, and Species Confirmation

Fifty-five *Aeromonas* spp. isolates were collected from commercial chickens in Sichuan Province, China, in 2008, and were screened for the presence of *mcr-1* to *mcr-9* genes using a polymerase chain reaction (PCR)-based assay with primers listed in Table S1. The PCR-positive isolate was then subjected to further PCR and sequencing analysis of the 16S *rRNA*, *gyrB*, and *rpoB* loci for species identification as per Kupfer et al.[15]

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of 13 antimicrobial agents (listed in Table S2) against the *mcr-3*-positive isolate were then determined by broth microdilution according to the recommendations of the Clinical and Laboratory Standards Institute (document VET01-A4). The breakpoints for each antimicrobial agent were interpreted according to CLSI (document M45-A2) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org) guidelines.[16] *E. coli* ATCC 25922 was used as a quality control strain.

Genomic DNA Sequencing and Analysis of Antibiotic Resistance Genes

Genomic DNA from the *mcr-3*-positive isolate was subjected to sequence using both the Illumina Hiseq platform and the PacBio RSII System (Sinobiocore, Beijing, China). CLC Genomics Workbench 9.0 (CLC Bio, Aarhus, Denmark) was used for assembling data from Illumina Hiseq platform, and all contigs were screened for the presence of *mcr*-3 using standalone BLAST analysis. The data from PacBio RSII System were assembled using the Hierarchical Genome Assembly Process (HGAP) and Quiver as part of the SMRT analysis program (version 2.3) using the HGAP3 protocol, and then corrected using Pilon. Reference sequences for antibiotic resistance genes were obtained from the ARG-ANNOT database (https://omictools.com/antibiotic-resistance-gene-annotation-tool). Multilocus sequence typing (MLST) was carried out using the MLST tool from the Center for Genomic Epidemiology (http://genomicepidemiology.org/), and annotation of the genomic sequences was performed using ORF Finder (www.ncbi.nlm.nih.gov/gorf/orfig.cgi), Vector NTI (Invitrogen, Carlsbad, CA, USA), and ISFinder (http://www-is.biotoul.fr).

S1 Nuclease Pulsed-Field Gel Electrophoresis (S1-PFGE) and Southern Blotting

S1-PFGE and Southern blotting were performed to locate the *mcr-3* gene within the genome. Briefly, whole cells were embedded in agarose gel plugs and digested with S1 nuclease (TaKaRa, Dalian, China). The DNA was then separated by PFGE as described previously.[17] For Southern blotting, a 450-bp digoxigenin-labelled *mcr-3* PCR amplicon was used as a probe. Genomic DNA from *Salmonella enterica* serovar Braenderup strain H9812 restricted with *Xba*I was used as the DNA marker.

Detection of Circular Intermediates

Based on the knowledge that IS*30*-family elements can form circular DNA intermediates, and that IS*As2* belongs to the IS*30* family,[18,19] a set of reverse primers (P-F: GGCTGGTTGTGATTGTAGAG and P-R: TCGCTCGGT CTGTTTGCTTT) were designed to investigate the potential of the IS*As2*-IS*Ahy2*-IS*As20-mcr-3.12-mcr-3*-like-*dgkA*-IS*As2* segment to circularize. The designed primers allowed us to obtain a complete map of the circular intermediate.

Functional Cloning of *mcr-3.6* and *mcr-3*-Like Genes in *E. coli* and *Aeromonas* Species

To determine the function of *mcr-3* variant genes, 1983-bp and 2176-bp DNA fragments, corresponding to the *mcr-3.6* and *mcr-3*-like regions along with their upstream sequences, respectively, were amplified and cloned into

pUC19, as previously reported.[8] The resulting recombinant plasmids, pUC19-*mcr-3.6* and pUC19-*mcr-3*-like, were then separately electroporated into *E. coli* DH5α (TaKaRa, Dalian, China) and *Aeromonas salmonicida* cells originally isolated from chicken cloaca swabs. The recombinant strains were then subjected to antibiotic susceptibility testing.

Results

Prevalence of *mcr-1* to *mcr-9* in *Aeromonas* Species Isolates

The use of colistin as animal growth enhancers has been banned in China since April, 2017. However, prior to that time, the extensive use of colistin in food animal production, especially in commercial poultry farming, increased the risk of transfer of colistin resistance determinants in bacteria. Our retrospective survey of *mcr-1* to *mcr-9* in 55 *Aeromonas* spp. isolates showed that only one ESBL-producing *A. veronii* isolate w55 was *mcr-3*-positive, indicating that the presence of *mcr-3* in *Aeromonas* in China for at least 10 years.

WGS Analysis and Functional Identification of *mcr-3.6* and *mcr-3*-Like Genes

The *mcr-3.6* variant has been deposited into NCBI with GenBank number: MF598076.1, and was first detected from *A. allosaccharophila* strain IMT43045 isolated from German *Leuciscus idus*.[19] Illumina HiSeq 2500-based WGS revealed the presence of two adjacent *mcr-3* variants, *mcr-3.6* and *mcr-3*-like separated by only 66 bp, on a 36.9-kb contig of *A. veronii* w55. The two variants showed 93.7% and 82.8% nucleotide sequence identity, respectively, to the original *mcr-3* from porcine *E. coli*.[20] Both variants carried several missense mutations, resulting in 94.8% and 84.2% amino acid sequence similarity, respectively, to MCR-3.

In order to determine the two *mcr-3* variants function, we expressed *mcr-3.6* and *mcr-3*-like in *E. coli* DH5α. Transformants containing pUC19-*mcr-3.6* or pUC19-*mcr-3*-like had colistin MIC of 2 μg/mL and 0.25 μg/mL, respectively, which was 8- and 1-fold higher MIC than that of *E. coli* DH5α containing pUC19 alone (0.25 μg/mL), respectively. Furthermore, to investigate the function of the two variants in *Aeromonas* species, recombinant plasmids pUC19-*mcr-3.6* and pUC19-*mcr-3*-like were separately electroporated into competent *A. salmonicida* cells, and the MICs of colistin against these two transformants were 32 μg/mL (carrying pUC19-*mcr-3.6*) and 1 μg/mL (carrying pUC19-*mcr-3*-like), which were 32- and 1-fold higher MIC than that of the wild-type *A. salmonicida* strain (1 μg/mL), respectively. These results suggested that only *mcr-3.6* could confer colistin resistance in both *E. coli* and other *Aeromonas* species, which agrees with only pUC19-*mcr-3.3* strain confers colistin resistance.[8] Although most of the identified *mcr-3* variants in *Aeromonas* species so far derived from animals, the surveillance of these potential high-colistin resistance genes in clinical *Aeromonas* isolates are also needed.

Antimicrobial Susceptibility Profiles

Antimicrobial susceptibility testing showed that *A. veronii* w55 was resistance to amoxicillin-clavulanate (MIC, 32/16 μg/mL), cefotaxime (8 μg/mL), ceftriaxone (16 μg/mL), ceftazidime (16 μg/mL), gentamycin (16 μg/mL), and chloramphenicol (32 μg/mL), and was susceptible to aztreonam (2 μg/mL), imipenem (0.03 μg/mL), meropenem (0.03 μg/mL), ciprofloxacin (0.5 μg/mL) and tetracycline (4 μg/mL) (Table S2). Interestingly, despite the presence of the two *mcr-3* variants, the MIC value for both colistin and polymyxin B against *A. veronii* w55 was 2 μg/mL, which was determined as "susceptible" based on the EUCAST clinical breakpoints (http://www.eucast.org).

MLST and Other Resistance Genes

MLST analysis revealed that *A. veronii* w55 belonged to novel sequence type ST514 (https://cge.cbs.dtu.dk/services/MLST/). WGS analysis showed that *A. veronii* w55 not only carried colistin resistance genes *mcr-3.6* and *mcr-3*-like but also contained ESBL-producing gene $bla_{\text{PER-3}}$. PER-3 was initially characterised from an *Aeromonas punctata* in France (GenBank accession no. AY740681.1) and recently in a medical centre in Taiwan,[21] sharing 99% homology to $bla_{\text{PER-1}}$. Here we report the first identification of a $bla_{\text{PER-3}}$-producing *A. veronii* isolate. In addition, aminoglycoside resistance genes *strA, strB, aac(6')Ib-cr*, and *aacA4*, macrolide resistance gene *mph*(A), phenicol resistance gene *catB3*, sulfonamide resistance gene *sul1*, and tetracycline resistance gene *tet*(E) were also detected in the genome of *A. veronii* w55. In these resistance genes, *mcr-3*.6, *mcr-3*-like, *strA, mph*(A), *aacA4, catB3, sul1, tet*(E) located on chromosome, and others located on plasmid.

Location and Genetic Environment of *mcr-3.6*

S1-nuclease PFGE and Southern blotting using an *mcr-3*-specific probe showed that *mcr-3.6* was located on the chromosome of *A. veronii* w55 (Figure 1). The segment of

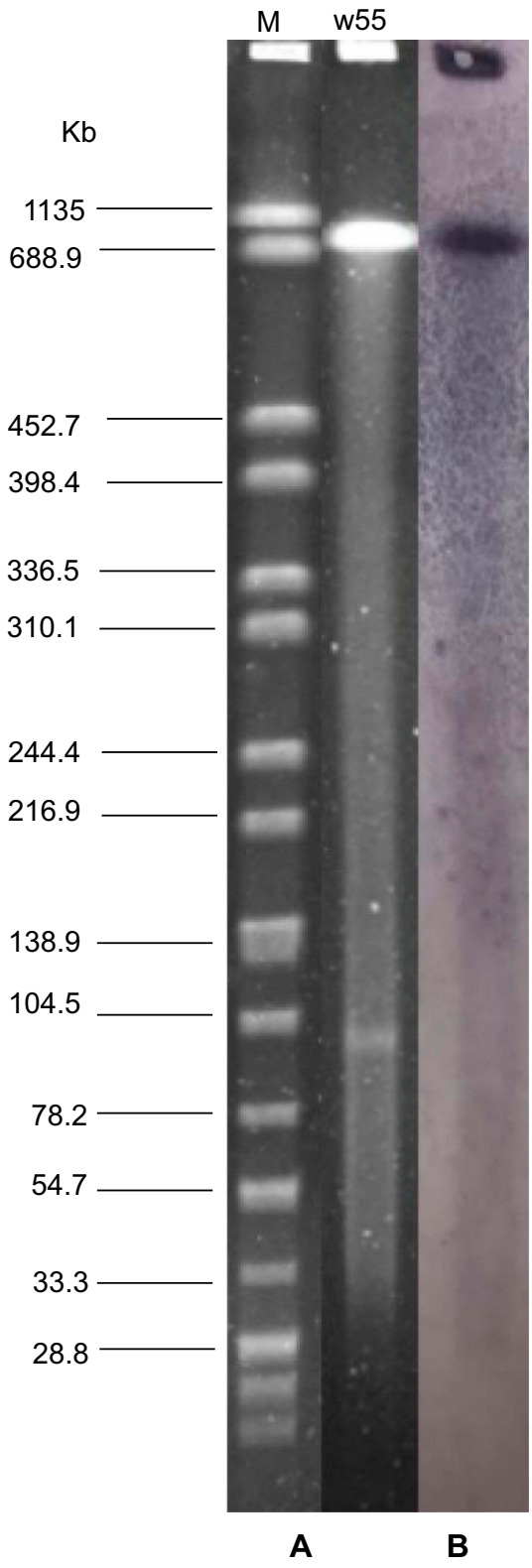

Figure 1 Location of *mcr-3.6* on the chromosome of *Aeromonas veronii* w55. (**A**) S1-PFGE and (**B**) the corresponding Southern hybridization using the *mcr-3.6* probe. Lane M, *Xbal*-digested *Salmonella enterica* serovar Braenderup strain H9812 genomic DNA marker; lane w55, *A. veronii* strain w55 genomic DNA.

mcr-3.6-mcr-3-like-*dgkA* in a 36.9-kb-size contig was surrounded by five IS elements (two IS*As2*, one IS*As20* and two truncated IS*Ahy2*, all of these elements were originally identified from various *Aeromonas* species, https://www-is.biotoul.fr/scripts/search-db.php), which was different from that in the original *mcr-3.6*-carrying *A. allosaccharophila* IMT43045, where this segment was flanked by a truncated IS*3* element (Figure 2). In detail, the upstream of *mcr-3.6-mcr-3*-like-*dgkA* segment contained one copy of IS*As2*, IS*As20*, and truncated Δ1IS*Ahy2* elements. The IS*As2* and IS*As20* elements harbored 50-bp imperfect inverted repeats (IR) at both ends (as shown in Figure 2). In addition, direct target site duplications (5′-CTT-3′ and 5′-AAGG-3′) were also observed immediately up- and downstream of the IS*As2* and IS*As20*, respectively (Figure 2). While immediate downstream of *mcr-3.6-mcr-3*-like-*dgkA* segment, another copy of IS*As2* and a truncated Δ2IS*Ahy2* were observed. The IR elements of the downstream IS*As2* were identical to that in upstream; however, the direct target site duplications of the downstream IS*As2* (5′-AAG-3′) were differed from that in the upstream (5′-CCT-3′) (Figure 2).

Formation of a Circular Intermediate Containing the *mcr-3.6* Gene

Some members of the IS family, including IS*3*, IS*30*, IS*110*, IS*26*, and IS*CR1* elements, utilize circular DNA intermediates containing accessory genes to undergo gene translocation via copy-and-paste mechanisms.[22] IS*As2*, which is first identified in *A. salmonicida*, disrupts a range of genes in *Aeromonas* species,[23] also belongs to the IS*3* family and is flanked by left and right inverted repeats. Therefore, we hypothesize that *mcr-3.6* translocation could be mediated via a circular intermediate with the assistance of IS*As2* element. The reverse primers P-F and P-R targeting the Δ1IS*Ahy2* and *dgkA* loci, respectively (Figure 2), were used to amplify a putative circular intermediate harboring *mcr-3.6* in *A. veronii* w55. PCR products with a size of ~2.0 kb were obtained and composed of the partial sequence of Δ1IS*Ahy2* and *dgkA* and one IS*As2* element. When combined with the sequence between primers P-F and P-R, we concluded that the circular intermediate was 8405-bp in size, and contained one copy of IS*As2*, Δ1IS*Ahy2*, IS*As20*, and a segment of *mcr-3.6-mcr-3*-like-*dgkA* (Figure 2). This new composite transposon was thereafter designated as Tn*6518* (IS*As2*-Δ1IS*Ahy2*-IS*As20-mcr-3.6-mcr-3*-like-*dgkA*-IS*As2*). Nevertheless, the molecular mechanisms

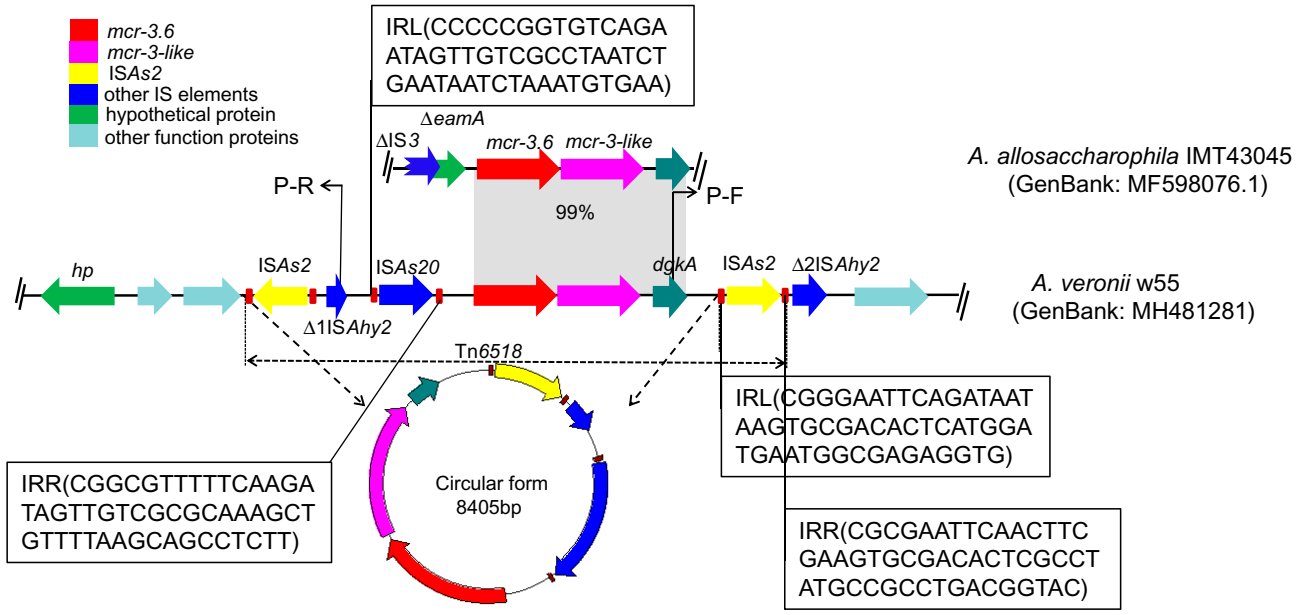

Figure 2 Genetic organization of scaffolds containing *mcr-3.6* obtained from *Aeromonas veronii* strain w55 in this study, and structural comparison with *A. allosaccharophila* IMT43045 (GenBank accession no. MF598076.1). The positions and orientations of the genes are indicated by arrows, with the direction of transcription shown by the arrowhead. Grey shading indicates >99% nucleotide sequence identity. The genetic structure of transposon Tn*6518* is shown in circular form.

underlying the formation of the circular intermediate form of Tn*6518*, and how it evolves to become a genetically stable fragment in the chromosome, require further investigation.

Discussion

Aeromonas spp. species are recognized enteric pathogens, and it is possible that the presence of *Aeromonas* isolates that carry these *mcr-3* variants in the intestine will facilitate the spread of these genes to other bacterial species living in the same habitat.[15,24] Until now, there are several *mcr-3* variants, including *mcr-3.3* and *mcr-3.13* to *mcr-3.18*, identified among 10 *Aeromonas* isolates from different sources.[12] Shen et al reported samples from the aquatic environment had the highest prevalence (10.0%) of *mcr*-3-positive *Aeromonas* isolates,[12] which indicated that *mcr-3* have been disseminated throughout various environments, the aquatic environment might be the major reservoir. For this study, it is the first case about a novel transposon, Tn*6518*, mediated transfer of *mcr-3.6* in ESBL-producing *Aeromonas veronii* isolated from chicken faeces.

The first reported *mcr-3*-carrying *E. coli* WJ1 exhibited MICs of 8 ug/mL for both colistin and polymyxin B.[14] However, five previously identified *Aeromonas* isolates carrying *mcr-3.3* to *mcr-3.7* exhibited colistin MICs ranging from 2 to128 ug/mL. In the original report on *mcr-3.3* in *A. veronii*, the *mcr-3.3*-positive isolate demonstrated borderline susceptibility to colistin (MIC of 2 mg/liter).[11] However, *E. coli* and *A. salmonicida* transformants carrying pUC19-*mcr-3.3* exhibited 8- and 64-fold higher MIC values (MICs of 2 mg/liter and 64 mg/liter, respectively) than the transformant carrying pUC19 alone, which was similar with our findings. These observations not only confirmed that the *mcr-3* variants can confer colistin resistance in both *Enterobacteriaceae* strains and *Aeromonads* but also suggested that the colistin resistance levels conferred by these genes may vary among different *Aeromonas* species. In the previous report, the *mcr-3* variants were surrounded by many different types of IS, like IS*26*, IS*6100*, IS*Kpn*3 and IS*AS17*.[12,15] However, in our study, the genetic environment of *mcr-3.6* was IS*As2*-IS*Ahy2*-IS*As20*-*mcr-3.6*-*mcr-3*-like-*dgkA*-IS*As2*, which can form a circular intermediate, and may increase the transmission of colistin resistance.

In conclusion, two *mcr-3* variants, *mcr-3.6* and *mcr-3*-like, were identified in ESBL-producing *A. veronii* isolate w55. This isolate has the novel sequence type ST514 and was isolated from a chicken cloacal sample in 2008. In addition, WGS analysis revealed that the segment of *mcr-3.6*-*mcr-3*-like-*dgkA* was surrounded by multi-copy IS elements and termed as a novel transposon Tn*6518*, which may be responsible for the transmission of *mcr-3.6* among various *Aeromonas* species. More worrying, *Aeromonas* species are prevalent in aquatic environments, where they can interact with different bacterial

species and genus. Overall, the presence of transposon-carrying *mcr-3* variants in *Aeromonas* species is highly concerning with regards to public health and highlights the need to investigate the prevalence of *mcr-3* in high-level colistin-resistant clinical *Aeromonas* isolates.

Nucleotide Sequence Accession No

The nucleotide sequence reported in this study has been submitted to GenBank with NCBI no. MH481281.

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Disclosure

The authors report no conflicts of interest in this work.

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