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Detection of a novel *mcr-5.4* gene variant in hospital tap water by shotgun metagenomic sequencing

Giuseppe Fleres[1], Natacha Couto[1], Leonard Schuele[1], Monika A. Chlebowicz[1], Catarina I. Mendes [1], Luc W. M. van der Sluis[2], John W. A. Rossen[1], Alex W. Friedrich[1] and Silvia García-Cobos[1]*

[1]University of Groningen, University Medical Center Groningen, Department of Medical Microbiology, Groningen, The Netherlands; [2]Center of Dentistry and Oral Hygiene, University Medical Center Groningen, 9712 CP Groningen, The Netherlands

*Corresponding author. E-mail: s.garcia.cobos@umcg.nl

Sir,

Colistin is considered a last-resort antibiotic for treating serious infections caused by MDR Gram-negative bacteria. The efficacy of this antibiotic is challenged by the emergence and global spread of mobile colistin resistance (*mcr*) determinants, which threaten human, animal and environmental health. The first mobile colistin resistance gene (*mcr-1*) was reported in 2015 and since then up to eight different variants have been described.[1] In 2017, Borowiak *et al.*[2] described a new transposon-associated phosphoethanolamine transferase mediating colistin resistance, named *mcr-5*, in d-tartrate-fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B isolated from poultry. The *mcr-5.3* variant has been recently reported in *Stenotrophomonas* spp. from sewage water.[3] Here we report for the first time (to the best of our knowledge) the detection of an *mcr-5* gene in a hospital water environment using short-read metagenomic sequencing (SRMseq) and subsequent characterization using long-read metagenomic sequencing (LRMseq) to reveal its genetic environment.

In June 2017, eight tap-water samples (900 mL) were collected at the University Medical Center Groningen. Water samples were filtered (0.2 μm) and after DNA extraction (PowerWater DNA Extraction Kit, QIAGEN), SRMseq was performed on a MiSeq instrument (500 cycles) (Illumina). Antibiotic resistance genes were identified in the metagenome assemblies (CLC Genomics Workbench v10.1.1, QIAGEN) using ABRicate-0.7 (https://github.com/tseemann/abricate) and applying the following thresholds: >70% identity and >80% coverage. One sample contained an *mcr*-type gene (5× sequencing depth), with the nucleotide change 313C>T (amino acid change F105L) with respect to the original *mcr-5.1* gene, which was designated *mcr-5.4* by NCBI (accession no. MK965519). This sample was selected for LRMseq; the DNA libraries were prepared using the Rapid PCR Barcoding Kit (SQK-RPB004) from Oxford Nanopore Technologies (ONT) and loaded into a FLO-MIN106 R9.4 flow cell. The run was performed on a MinION device (ONT) and it proceeded for 24 h. The data were basecalled using Albacore (https://github.com/rrwick/Basecalling-comparison) and further processed with Poretools[4] and Porechop (https://github.com/rrwick/Porechop). Trimmed reads from SRMseq and LRMseq were used for hybrid-assembly analysis by metaSPAdes-3.13.0.[5] After a BLAST search using the hybrid contig containing the *mcr-5.4* gene, the plasmid pSE13-SA01718 (accession no. KY807921.1) was listed as one of the hits with the highest identity and we used it as a reference for genome comparison with the Artemis Comparison Tool (ACT) v1.0.[6] The *mcr-5.4*-carrying contig from the hybrid assembly was annotated using PATRIC v3.5.27.[7] Trimmed reads from SRMseq were used to investigate the bacterial composition by OneCodex.[8] Finally, in order to predict the bacterial host of the *mcr-5.4* gene, a contig-binning analysis of the hybrid-assembled metagenome was performed using MaxBin2 v2.2.4 (https://sourceforge.net/projects/maxbin2/), probability threshold 0.9 and minimum contig length 1000 bp. The resulting bin containing the *mcr-5.4* gene was selected for taxonomy classification using Kraken2 (https://github.com/DerrickWood/kraken2) (minikraken2 DB v1).

SRMseq showed the *mcr-5.4* gene detected in a contig of 2113 bp flanked by two truncated protein-coding sequences (CDSs), encoding the ChrB domain protein (involved in chromate resistance) and the Major Facilitator Superfamily (MFS) transporter. The hybrid-assembly analysis resulted in a contig of 8456 bp consisting of nine CDSs and four truncated CDSs (Figure 1). Comparative analysis of the genetic environment of the *mcr-5* gene, between the annotated hybrid metagenome contig and the reference plasmid pSE13-SA01718, showed a region of 4670 bp with 98% identity, corresponding to the backbone of the Tn*6452* transposon (Figure 1). We observed three truncated CDSs for the MFS-type transporter in our contig instead of two as previously described in the reference sequence pSE13-SA01718. These differences did not appear to be due to sequencing errors when we checked the sequence MK965519, (i) using pilon (https://github.com/broadinstitute/pilon) to correct for errors in short-read sequencing data and (ii) using CLC Genomic Workbench to update the hybrid contig by mapping both long and short reads against the hybrid contig. We also observed a region of 3786 bp, with no identity either with the reference plasmid pSE13-SA01718 (Figure 1) or with any other sequence in the GenBank database.

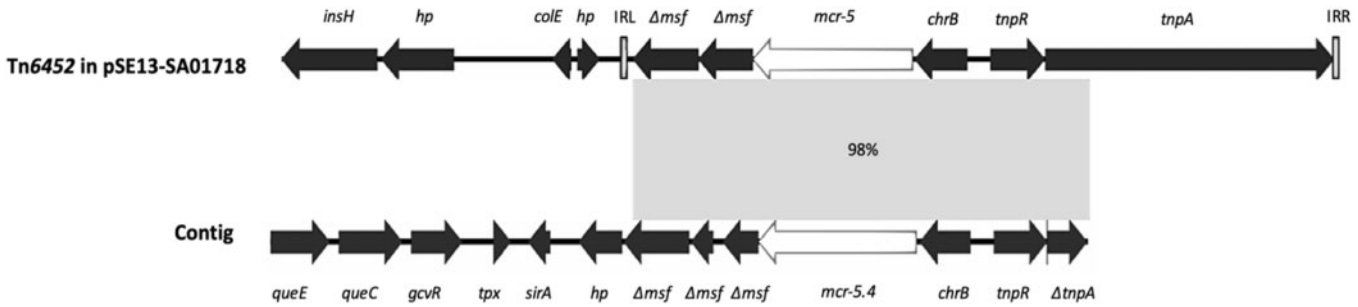

Figure 1. Comparative analysis of the genetic environment of *mcr-5* between the reference plasmid pSE13-SA01718 (accession no. KY807921.1) and the annotated hybrid metagenome contig (accession no. MK965519). The contig carrying the *mcr-5.4* gene consists of the following putative gene products: 7-carboxy-7-deazaguanine synthase (*queE*), 7-cyano-7-deazaguanine synthase (*queC*), glycine cleavage system transcriptional anti-activator GcvR (*gcvR*), thiol peroxidase (*tpx*), sulphurtransferase TusA family protein (*sirA*), hypothetical protein (*hp*), truncated MFS-type transporter (Δ*msf*), lipid A phosphoethanolamine transferase (*mcr-5.4*), ChrB domain protein (*chrB*), transposon resolvase (*tnpR*) and truncated transposon transposase (Δ*tnpA*). Areas with 98% identity between sequences are represented in light grey. Arrows indicate the position and direction of the genes. The transposon Tn*6452* sequence in the reference plasmid pSE13-SA01718 is bounded by inverted repeats: IRL and IRR.

Species previously described to harbour an *mcr-5* gene are *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Aeromonas hydrophila* and *Cupriavidus gilardii*. The bacterial composition analysis of the water sample using SRMseq showed the presence of *Pseudomonas* spp. (relative abundance: 0.004%), *Cupriavidus* spp. (relative abundance: 0.001%) and *Aeromonas* spp. (relative abundance: 0.0003%). The binning analysis produced a bin positive for the *mcr-5.4* gene consisting of 1336 contigs (genome size: 5 175 285 bp; genome completeness: 68.2%). This bin was taxonomically classified as bacteria (70.73%) and proteobacteria (64.90%), and from this the most abundant class was Gammaproteobacteria (37.20%) (order Pseudomonadales, 15.57%), followed by Betaproteobacteria (14.90%) (order Burkholderiales, 10.63%).

Colistin resistance determinants (*mcr*) have been rarely reported in water environments; *mcr-1* has been detected in both hospital sewage and in environmental water streams and *mcr-3* in environmental water.[9,10] To the best of our knowledge, this is the first-time description of an *mcr-5* gene in an indoor and healthcare water environment. Despite the fact that the comparative analysis showed the hybrid contig covering a large region of Tn*6452*, neither the left inverted repeat (IRL) nor the right inverted repeat (IRR) have been found. In addition, the lack of the right transposon region does not allow us to search for other possible inverted repeats. Thus, it is not possible to conclude whether the described *mcr-5.4* gene is transferable or not. Taxonomic analysis suggested the order of Pseudomonadales as the most probable host of the *mcr-5.4* gene in the water sample. Further studies are needed to determine the frequency of this gene in hospital water and other water environments and to evaluate the potential risks for patients and healthcare workers.

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Transparency declarations

None to declare.

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Detection of chromosome-mediated *tet*(X4)-carrying *Aeromonas caviae* in a sewage sample from a chicken farm

Chong Chen[1], Liang Chen[2], Yan Zhang[1], Chao-Yue Cui[1], Xiao-Ting Wu[1], Qian He[1], Xiao-Ping Liao[1], Ya-Hong Liu[1] and Jian Sun[1]*

[1]*National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China;* [2]*Hackensack-Meridian Health Center for Discovery and Innovation, Nutley, NJ, USA*

*Corresponding author. E-mail: jiansun@scau.edu.cn

Sir,

Recently, novel plasmid-mediated tigecycline resistance mechanisms, Tet(X3) and Tet(X4), have been described in Enterobacteriaceae and *Acinetobacter* isolates from animals and humans.[1] It raises a global antimicrobial resistance concern because these tetracycline-inactivating enzymes are able to inactivate the entire family of tetracycline antibiotics, including the newly FDA-approved eravacycline and omadacycline.[1] The plasmid-borne *tet*(X3) and *tet*(X4) genes have been identified in >15 different Gram-negative species, with *Escherichia coli* being the most common.[1,2] Here we report, to the best of our knowledge, the first identification of the *tet*(X4) gene on the chromosome of an *Aeromonas caviae* strain from sewage in China. *Aeromonas* species, including *A. caviae*, are important zoonotic pathogens of poikilotherms, but are now emerging as important human pathogens,[3] and have been frequently found to carry antimicrobial resistance genes (e.g. bla_{NDM-1} and *mcr-3* variants) on the chromosome.[4–6]

During a routine antimicrobial resistance surveillance study, a *tet*(X4)-positive strain, WCW1-2, was isolated on an LB agar plate containing tigecycline (2 mg/L) from a sewage sample from a chicken farm in 2018 in Guangdong, China. The 16S rRNA sequencing analysis further suggested that it belonged to *A. caviae*, which shared >99.9% nucleotide identity with the isolates from patients in USA and Brazil (accession numbers CP026055 and CP024198). Antimicrobial susceptibility testing was conducted by broth microdilution, with *E. coli* ATCC 25922 as the quality control strain, and interpreted according to the CLSI guideline.[7] The *tet*(X4)-positive *A. caviae* WCW1-2 was resistant to tetracycline, amoxicillin/clavulanic acid, ciprofloxacin and trimethoprim/sulfamethoxazole, but remained susceptible to amikacin, cefotaxime, colistin, gentamicin and meropenem (Table S1, available as Supplementary data at *JAC* Online). In addition, WCW1-2 exhibited high MICs of tigecycline (16 mg/L), eravacycline (4 mg/L) and omadacycline (8 mg/L).

The tigecycline resistance in *A. caviae* WCW1-2 failed to transfer to sodium azide-resistant *E. coli* J53 by filter mating, but further gene cloning of *tet*(X4) and its putative promoter into a pUC18 vector (primers in Table S2) confirmed the *tet*(X4)-mediated tigecycline resistance. Susceptibility testing results showed that the *tet*(X4) construct had 64- to 512-fold increases in MICs of tetracycline (128 mg/L), chlortetracycline (256 mg/L), oxytetracycline (128 mg/L), doxycycline (32 mg/L), minocycline (16 mg/L), tigecycline (8 mg/L), eravacycline (2 mg/L) and omadacycline (8 mg/L), which were consistent with the results for the parental strain WCW1-2 (Table S1).

Genomic DNA of *A. caviae* WCW1-2 was then completely sequenced using a combination of the Nanopore GridION and Illumina HiSeq platforms (Nextomics, Wuhan, China), followed by assembling with Unicycler.[8] The results of WGS revealed that WCW1-2 belonged to a novel ST, ST645, and harboured one chromosome of 4684096 bp (CP039832), but without plasmids. The *tet*(X4) gene was found to be on the chromosome of WCW1-2, which explained the failure of transfer of tigecycline resistance, and shared a homology region (namely upstream of the Δ*merR* gene and downstream of the *ucpA* gene) with the chromosome of another *A. caviae* strain (Figure 1a). Moreover, WCW1-2 harboured an additional 15 antimicrobial resistance genes encoding resistance to β-lactams (bla_{MOX-5} and bla_{OXA-10}), aminoglycosides [*aadA1*, *aph(3')-Ia*, *aph(3")-Ib* and *aph(6)-Id*], fluoroquinolones [*qnrVC4* and *aac(6')-Ib-cr*], phenicols (*cmlA1*, *catB3* and *floR*), trimethoprim/sulfamethoxazole (*sul1*, *dfrA14* and *dfrB4*) and tetracyclines [*tet*(A)].

A further BLASTn search for the *tet*(X4) gene against the NCBI database identified a series of Enterobacteriaceae carrying the same subtype from humans (e.g. NZ_NQAI01000053) and pigs (e.g. NZ_NQBP01000050), including the first described *tet*(X4)-harbouring plasmid, p47EC (MK134376) (Figure 1b). Analysis of their genetic environments revealed that the *tet*(X4) gene was usually