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LETTER TO THE EDITOR

mcr-3 and *mcr-4* Variants in Carbapenemase-Producing Clinical *Enterobacteriaceae* Do Not Confer Phenotypic Polymyxin Resistance

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The worldwide distribution of plasmid-mediated colistin resistance determinants (*mcr-1*, *mcr-2*, *mcr-3*, and *mcr-4*) coupled to the emerging observation that colistin resistance is more prevalent in carbapenem-resistant *Enterobacteriaceae* (CRE) (1, 2) presents a daunting challenge in combatting antimicrobial resistance. Undoubtedly, next-generation sequencing approaches have expedited the discovery of mobile colistin resistance determinants (3). In this study, we undertook the *in silico* screening of 500 phenotypically carbapenem-resistant carbapenemase-producing *Enterobacteriaceae* whole genomes for the presence of the *mcr* gene, using CLC Genomics Workbench (CLC Bio-Qiagen, Aarhus, Denmark). The isolates comprised clinical and screening pure cultures submitted to the national reference laboratory for mandatory CRE surveillance. Locally, the presence of *mcr-1* as well as its cocarriage with KPC-2 had been previously well described (4, 5); hence, we did not look further into the distribution of *mcr-1*. *mcr-2* was not detected among the genomes analyzed. *mcr-3* was identified in one *Escherichia coli* genome (ENT1955) by the use of both read mapping and *de novo* assembly. To date, several *mcr-3* variants have been observed (6, 7). The *mcr-3* gene identified in this study shared 99.94% nucleotide identity with the first *mcr-3* gene discovered by Yin et al. (8) (GenBank accession no. KY924928.1) due to a "C" deletion at nucleotide position 218; the deletion was confirmed by Sanger sequencing. This resulted in a truncated protein of only 88 amino acids (Table 1) that was deemed to be nonfunctional (10). This *mcr-3*-like gene has been deposited in GenBank (see below). The genome of ENT1955 was *de novo* assembled using all the reads, and the draft genome was annotated. No plasmid-related genes were found in the approximately 9-kb contig containing the *mcr-3*-like gene. We also performed read mapping using *E. coli* Y5 (GenBank accession no. CP013483.1) as the reference because it was the most closely related genome available in GenBank. Reads that did not match the *E. coli* Y5 chromosome were separately assembled. Using this approach, the derived *mcr-3*-like contig was almost identical to the one obtained from *de novo* assembly using all reads and, again, no plasmid-related genes were observed. This led us to believe that, in contrast to those reported in several previous studies (8, 11, 12), our *mcr-3*-like gene was not plasmid associated. Interestingly, downstream of the *mcr-3*-like gene, a gene encoding IS*2*

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TABLE 1 Characteristics of KPC-2 carbapenemase-producing *Enterobacteriaceae* cocarrying *mcr* variants

Isolate name	Species	Source[a]	MIC (mg/liter)[b]		Date of isolation	Multilocus sequence type	MCR mutation(s)	Other acquired resistance determinants[d]	Replicon of *mcr* plasmid	Reference *mcr* plasmid (GenBank accession no.)
			Colistin	Polymyxin B						
mcr-3-like isolate										
ENT1955	*E. coli*	Rectal swab	0.25	0.25	4 April 2015	167	MCR-3-like truncated at amino acid position 88	bla_{KPC-2}, bla_{TEM-1B}, *aadA1*, *erm*(B), *mph*(A), *floR*, *sul3*, *tet*(A)		
mcr-4.2 isolates										
ENT1164	*E. cloacae*	Rectal swab	32[c]	16[c]	11 May 2014	54	V179G, V236F in comparison to MCR-4	bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, *aac(6′)Ib-cr*, *aacA4*, *fosA*, *mph*(A), *catB8*, *catA1*, *sul1*	ColE10	*Salmonella* sp. plasmid pMCR_R3445 (MF543359)
ENT1344	*E. cloacae*	Rectal swab	16[c]	16[c]	28 July 2014	54	V179G, V236F in comparison to MCR-4	bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{SHV-12}, *aac(6′)Ib-cr*, *aacA4*, *fosA*, *mph*(A), *catA1*, *catB8*, *sul1*, *QnrS1*	ColE10	*Salmonella* sp. plasmid pMCR_R3445 (MF543359)
ENT1606	*E. cloacae*	Rectal swab	16[c]	16[c]	24 October 2014	54	V179G, V236F in comparison to MCR-4	bla_{KPC-2}, bla_{TEM-1A}, *aac(6′)Ib-cr*, *aacA4*, *fosA*, *mph(A)*, *catA1*, *catB8*, *sul1*	ColE10	*Salmonella* sp. plasmid pMCR_R3445 (MF543359)
ENT1017	*E. cloacae*	Rectal swab	0.25	0.25	2 February 2014	54	V179G, V236F in comparison to MCR-4	bla_{KPC-2}, bla_{TEM-1B}, bla_{SHV-12}, *fosA*, *mph*(A), *catA1*, *qnrS1*	ColE10	*Salmonella* sp. plasmid pMCR_R3445 (MF543359)
ENT1018	*E. cloacae*	Rectal swab	0.125	0.125	2 February 2014	54	V179G, V236F in comparison to MCR-4	bla_{KPC-2}, bla_{TEM-1B}, bla_{SHV-12}, *fosA*, *mph*(A), *catA1*, *qnrS1*	ColE10	*Salmonella* sp. plasmid pMCR_R3445 (MF543359)
ENT1019	*E. cloacae*	Rectal swab	0.25	0.25	2 February 2014	54	V179G, V236F in comparison to MCR-4	bla_{KPC-2}, bla_{TEM-1B}, bla_{SHV-12}, *fosA*, *mph*(A), *catA1*, *qnrS1*	ColE10	*Salmonella* sp. plasmid pMCR_R3445 (MF543359)

[a]Isolates were from a routine surveillance study suggesting colonization.
[b]European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for interpretation of colistin MIC results (susceptible, ≤2 mg/liter; resistant, >2 mg/liter).
[c]A single "skip well" was observed during testing, which suggests heteroresistance to polymyxins in these isolates (9).
[d]Determined by ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/).

TABLE 2 Broth microdilution polymyxin susceptibility testing of cloned *mcr-3*-like and *mcr-4.2* expressed in *E. coli* BL21(DE3) host

	MIC (mg/liter)	
E. coli BL21(DE3) strain[a]	Colistin	Polymyxin B
pET48b—*mcr-3*-like	1	1
pET48b—*mcr-4.2*	1	1
pET48b	1	0.5
No vector	0.5	1

[a]Expression of *mcr* was induced by the addition of 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) to cation-adjusted Mueller-Hinton broth media.

transposase TnpB, matching the gene encoding the transposase carried by *mcr-3*-bearing plasmid pWJ1 (8), was detected. Phenotypically, the isolate was susceptible to both colistin and polymyxin B (Table 1) as determined by the reference broth microdilution method (13).

There were no exact matches to *mcr-4* in the screened genomes. Instead, the *mcr-4*-like gene was found in six *Enterobacter cloacae* isolates (Table 1), which had 100% nucleotide identity to a putative sulfatase from *Shewanella frigidimarina*. Compared to *mcr-4* (GenBank accession no. MF543359), this *mcr-4*-like gene carried 2 missense mutations resulting in codon changes at positions V179G and V236F. The *mcr-4*-like gene was located on a 7.7-kb contig, and a BLAST search showed 95% coverage and 99% similarity to the *mcr-4* gene in plasmid pMCR_R3445 (GenBank accession no. MF543359). Thus, we named this *mcr-4*-like gene "*mcr-4.2*" (see below).

Interestingly, the genomes of all of the *mcr-4.2*-positive isolates also possessed another putative phosphoethanolamine transferase (EPT) (GenBank accession no. WP_012477388). This putative EPT was not encoded on the plasmid. An alignment of the MCR-4.2 amino acid sequence and the putative EPT against MCR-1 and MCR-2 showed conservation in active site residues (data not shown) (10). However, it appears that the presence of *mcr-4.2* and the predicted novel EPT genes did not confer overt phenotypic resistance to polymyxins (Table 1). The finding of heterogeneously elevated drug MICs for *E. cloacae* isolates is likely attributable to heteroresistance causing a "skip well" phenomenon observed during the broth microdilution susceptibility testing (Table 1) (9).

Expression vector pET-48b(+) (Novagen, WI, USA) was used for functional cloning of the full coding sequences of the *mcr-3*-like and *mcr-4.2* genes but without the fusion tags. There was essentially no difference in the polymyxin MICs for the cloned *mcr-3*-like and *mcr-4.2* genes, which suggests that they did not confer phenotypic resistance (Table 2). Therefore, we concluded that the *mcr-3*-like gene carrying a nonsense mutation was nonfunctional and that *mcr-4.2* alone was unlikely to be the major mechanism of resistance to polymyxins.

In summary, we describe the discovery of new *mcr*-like elements, although phenotypic susceptibility testing indicates that the presence of these genes alone was unlikely to contribute to colistin resistance.

Accession number(s). The *mcr-3*-like and *mcr-4.2* genes identified in this study have been deposited in GenBank under accession no. MG026622 and MG026621, respectively.

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REFERENCES

1. Giani T, Antonelli A, Caltagirone M, Mauri C, Nicchi J, Arena F, Nucleo E, Bracco S, Pantosti A; AMCLI-CoSA survey participants, Luzzaro F, Pagani L, Rossolini GM. 2017. Evolving beta-lactamase epidemiology in Enterobacteriaceae from Italian nationwide surveillance, October 2013: KPC-carbapenemase spreading among outpatients. Euro Surveill 22:30583. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30583.
2. Huang TD, Bogaerts P, Berhin C, Hoebeke M, Bauraing C, Glupczynski Y. 2017. Increasing proportion of carbapenemase-producing Enterobacteriaceae and emergence of a MCR-1 producer through a multicentric study among hospital-based and private laboratories in Belgium from September to November. Euro Surveill 22:30530. https://doi.org/10.2807/1560-7917.ES.2017.22.19.30530.
3. Kluytmans J. 2017. Plasmid-encoded colistin resistance: mcr-one, two, three and counting. Euro Surveill 22:30588. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30588.
4. Teo JQM, Ong RTH, Xia E, Koh TH, Khor CC, Lee SJY, Lim TP, Kwa AL. 2016. mcr-1 in multidrug-resistant blaKPC-2-producing clinical Enterobacteriaceae isolates in Singapore. Antimicrob Agents Chemother 60:6435–6437. https://doi.org/10.1128/AAC.00804-16.
5. Teo JW, Chew KL, Lin RT. 2016. Transmissible colistin resistance encoded by mcr-1 detected in clinical Enterobacteriaceae isolates in Singapore. Emerg Microbes Infect 5:e87. https://doi.org/10.1038/emi.2016.85.
6. Roer L, Hansen F, Stegger M, Sönksen UW, Hasman H, Hammerum AM. 2017. Novel *mcr-3* variant, encoding mobile colistin resistance, in an ST131 *Escherichia coli* isolate from bloodstream infection, Denmark, 2014. Euro Surveill 22:22846. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30584.
7. Ling Z, Yin W, Li H, Zhang Q, Wang X, Wang Z, Ke Y, Wang Y, Shen J. 24 October 2017. Chromosome-mediated mcr-3 variants in Aeromonas veronii from chicken meat. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.01272-17.
8. 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.
9. Landman D, Salamera J, Quale J. 2013. Irreproducible and uninterpretable polymyxin B MICs for Enterobacter cloacae and Enterobacter aerogenes. J Clin Microbiol 51:4106–4111. https://doi.org/10.1128/JCM.02129-13.
10. Hu M, Guo J, Cheng Q, Yang Z, Chan EWC, Chen S, Hao Q. 2016. Crystal structure of Escherichia coli originated MCR-1, a phosphoethanolamine transferase for colistin resistance. Sci Rep 6:38793. https://doi.org/10.1038/srep38793.
11. Hernández M, Iglesias MR, Rodríguez-Lázaro D, Gallardo A, Quijada N, Miguela-Villoldo P, Campos MJ, Píriz S, López-Orozco G, de Frutos C, Sáez JL, Ugarte-Ruiz M, Domínguez L, Quesada A. 2017. Co-occurrence of colistin-resistance genes mcr-1 and mcr-3 among multidrug-resistant Escherichia coli isolated from cattle, Spain, September 2015. Euro Surveill 22:30586. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30586.
12. Litrup E, Kiil K, Hammerum AM, Roer L, Nielsen EM, Torpdahl M. 2017. Plasmid-borne colistin resistance gene mcr-3 in Salmonella isolates from human infections, Denmark, 2009–17. Euro Surveill 22:30587. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30587.
13. Clinical and Laboratory Standards Institute. 2015. M07-A10. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: ninth edition. Clinical and Laboratory Standards Institute, Wayne, PA.