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

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KEYWORDS bioinformatics, Enterobacteriaceae, next-generation sequencing, PCR, antibiotic resistance, plasmid, polymyxin

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 IS2

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<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Species</th>
<th>Source</th>
<th>MIC (mg/liter)</th>
<th>Date of isolation</th>
<th>Multilocus sequence type</th>
<th>MCR mutation(s)</th>
<th>Other acquired resistance determinants</th>
<th>Replicon of mcr plasmid (GenBank accession no.)</th>
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<td>Salmonella sp. plasmid pMCR_R3445 (MF543359)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Isolates were from a routine surveillance study suggesting colonization.

<sup>b</sup>European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for interpretation of colistin MIC results (susceptible, <2 mg/liter; resistant, >2 mg/liter).

<sup>c</sup>A single "skip well" was observed during testing, which suggests heteroresistance to polymyxins in these isolates (9).

<sup>d</sup>Determined by ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/).
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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<table>
<thead>
<tr>
<th>E. coli BL21(DE3) straina</th>
<th>Mic (mg/liter)</th>
<th>Colistin</th>
<th>Polymyxin B</th>
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<tr>
<td>No vector</td>
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</table>

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


