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We identified a novel plasmid-mediated colistin-resistance gene in porcine and bovine colistin-resistant Escherichia coli that did not contain mcr-1. The gene, termed mcr-2, a 1,617 bp phosphoethanolamine transferase harboured on an IncX4 plasmid, has 76.7% nucleotide identity to mcr-1. Prevalence of mcr-2 in porcine colistin-resistant E. coli (11/53) in Belgium was higher than that of mcr-1 (7/53). These data call for an immediate introduction of mcr-2 screening in ongoing molecular epidemiological surveillance of colistin-resistant Gram-negative pathogens.

Following the report of mcr-1 detection in China in November 2015 [1], we screened 105 colistin-resistant Escherichia coli (colistin minimum inhibitory concentration (MIC) 4–8 mg/L [2]) isolated during 2011–12 from passive surveillance of diarrhoea in 52 calves and 53 piglets in Belgium [3]. mcr-1 was detected in 12.4% (n = 13) of the E. coli isolates, of which six and seven were from calves and piglets, respectively [3,4]. In the present study, we analysed porcine and bovine colistin-resistant Escherichia coli isolates that did not show presence of mcr-1 and identified a novel plasmid-mediated colistin-resistance-conferring gene, mcr-2.

Identification of mcr-2 in colistin-resistant E. coli isolates not harbouring mcr-1

Of 92 porcine and bovine colistin-resistant Escherichia coli isolates not harbouring mcr-1, 10 were randomly selected for further analysis. Plasmid DNA was isolated (PureLink HiPure Plasmid Miniprep Kit, Invitrogen, Carlsbad, CA, United States), sequenced by Illumina (2 x 250 bp) (Nextera XT sample preparation kit, MiSeq), de novo assembled and annotated using SPAdes [v3.8.1] and RAST [5,6]. Plasmids from three of the 10 E. coli isolates showed the presence of a gene for a putative membrane protein, which was identified as a phosphoethanolamine transferase (sulfatase) using pfam and Interproscan protein databases [7,8].

The mcr-2 gene, as we termed it, is 1,617 bp long, nine bases shorter than mcr-1 (1,626 bp), and shows 76.75% nt identity to mcr-1 (supplementary material [9]).

The entire mcr-2 gene was amplified (PCR primers: MCR2-F 5’ TGGTACAGCCCCTTTATT 3’; MCR2-R 5’GCTTGAGATTGGGTTATGA 3’), cloned (vector pCR 2.1, TOPO TA Cloning kit, Invitrogen) and electroporated into DH-5 α E. coli. Transformants exhibited colistin MICs of 4–8 mg/L (E-test, bioMerieux, Marcy l’Etoile, France), which were reconfirmed by macrobroth dilution (European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [2]).

mcr-2 is harboured on IS1595 with likely origins in Moraxella spp.

mcr-2-harbouring plasmids from all three E. coli isolates were analysed. The mobile element harbouring mcr-2 was identified as an IS element of the IS1595 superfamily, which are distinguished by the presence of an ISXO2-like transposase domain [10].

We also identified a 297 bp open reading frame downstream of mcr-2 on this element, which encodes a PAP2 membrane-associated lipid phosphatase with 41% identity to Moraxella osloensis phosphatidic acid phosphatase (71% query coverage). Interestingly, a blastn search of the IS1595 backbone, after removal of the mcr-2 and pap2 phosphatase gene sequences, identified a single hit to Moraxella bovoculi strain 58069 (GenBank accession number CP011374) genomic region.
Figure 1
Genetic organisation and structure of the mcr-2-harbouring plasmid pKP37-BE from a colistin-resistant *Escherichia coli* isolate not harbouring mcr-1, Belgium, June 2016

The plasmid map was generated using GenomeVx [23].
RaptorX [24] was used to generate the structures. For both MCR-2 and MCR-1, domain 1 was predicted to be a transporter and domain 2 a phosphoethanolamine transferase (sulfatase).

(1,531,602 to 1,532,255 bp) with 75% identity and 100% query coverage.

*mcr-2* is harboured on an IncX4 incompatibility-type plasmid in *E. coli* ST10

The three *mcr-2* plasmid-harbouring *E. coli* isolates belonged to ST10 (*n* = 2, porcine) and ST167 (*n* = 1, bovine). All three plasmids belonged to IncX4 incompatibility type; all three *mcr-2* genes showed 100% homology.

Plasmid pKP37-BE isolated from one of the porcine ST10 *E. coli* isolates was found to have a size of 35,104 bp, 41.3% GC content and 56 protein-encoding gene sequences (RAST) (Figure 1); European Nucleotide Archive accession numbers PRJEB14596 (study) and LT598652 (plasmid sequence).

Apart from IS1595, pKP37-BE did not carry any other resistance genes and the plasmid backbone was highly similar to *Salmonella enterica* subsp. *enterica* serovar Heidelberg plasmid pSH146-32 (GenBank accession number JX258655), with 98% identity and 90% query coverage. Several *Salmonella*-associated virulence genes were found on pKP37-BE, including *virB/D4* that encodes a type 4 secretion system [11].

Conjugation experiments using a rifampicin-resistant *E. coli* recipient (A15) showed an approximately 1,200-fold higher transfer frequency of the *mcr-2*-harbouring pKP37-BE (1.71 × 10−3) compared with that for domain 2 (87.4%). Other blastp hits for the domain 2 transferase were *Enhydrobacter aerosaccus* (WP_06233180) with 99% sequence coverage, and 65%, 65%, and 61% identity to that of *Enhydrobacter aerosaccus* (KND21726), *Paenibacillus sophorae* (WP_063619495) and *Moraxella catarrhalis* (WP_003672704), respectively, all with 97% query coverage.

We also carried out blastp searches of the two domains of MCR-2 separately. The identity level of domain 1 between MCR-1 and MCR-2 was low (72%) compared with that for domain 2 (87.4%). Other blastp hits for the domain 2 transferase were *Enhydrobacter aerosaccus* and *Moraxella osloensis* (69% identity; 100% query coverage) followed by *Paenibacillus sophorae* (68% identity; 100% query coverage) and *Moraxella catarrhalis* (68% identity; 99% query coverage). Phylogenetic analysis showed that MCR-2 might have originated from *Moraxella catarrhalis* (56% bootstrap value) (Figure 3).

**PCR-based screening identified a higher prevalence of *mcr-2* than of *mcr-1* in porcine *E. coli* in Belgium**

We screened our entire collection of porcine and bovine colistin-resistant *E. coli* isolates (*n* = 105) using an *mcr-2*-specific PCR approach using primers MCR2-IF 5′ TGGTTGCTTGCCGATTGGA 3′ and MCR2-IR 5′ AGATGGATTTGCGTTGTGCTG 3′, and the following cycling conditions: 33 cycles of 95°C × 3 min, 65°C × 30 s, 72°C × 1 min, followed by 1 cycle of 72°C × 10 min. We found *mcr-2* in 11/53 porcine and 1/52 bovine colistin-resistant *E. coli* isolates (an overall prevalence of 11.4%).

**Discussion**

Identification of plasmid-mediated colistin resistance represents a paradigm shift in colistin-resistance mechanisms, which until recently were restricted to chromosomal mutations and vertical transmission. Since *mcr-1* conferring plasmid-mediated colistin resistance was first detected in China, *mcr-1* has been identified in 30 countries across five continents [14-17] (Figure 4).
Our analysis identified a novel plasmid-mediated phosphoethanolamine transferase-encoding gene, \( mcr-2 \), which was detected at an even higher prevalence than that of \( mcr-1 \) among colistin-resistant porcine \( E. coli \) in our study. We were, however, limited by small sample numbers. It should also be noted that the calves and piglets were from different regions of the country (calves from Wallonia and piglets from Flanders).

Phylogenetic analysis of MCR-2 provided strong evidence that this protein was distinct from MCR-1, and that it might have originated from \( Moraxella \) catarrhalis. The latter set of data are further strengthened by the fact that \( mcr-2 \) is co-harboured with a lipid phosphatase gene that shows highest homology to a phosphatase from \( Moraxella \) spp., and that the genetic element IS1595 harbouring these two genes might itself have originated from \( Moraxella \) spp. While \( Moraxella \) spp. are not polymyxin producers, this bacterial genus is known to be intrinsically resistant to polymyxins [18] and potential intergeneric transfer of \( mcr-2 \) from co-habiting \( Moraxella \) spp. of animal, human or environmental origin is therefore highly likely. Phosphoethanolamine transferases are housekeeping enzymes that catalyse the addition of the phosphoethanolamine moiety to the outer 3-deoxy-D-manno-octulosonic acid (Kdo) residue of a Kdo(2)-lipid A [19]. The fact that we did not identify any chromosomal mutations in the known colistin resistance-conferring genes in our \( E. coli \) isolates (by whole genome sequencing, data not shown) additionally supports the role of the acquired phosphoethanolamine transferase in conferring colistin resistance.

Finally, the high transfer frequency of the \( mcr-2 \)-harbouring IncX4 plasmid might underlie the higher prevalence of \( mcr-2 \) in our porcine isolates. In the three \( mcr-2 \) harbouring isolates analysed, IS1595 showed presence of direct repeats and a complete \( tnpA \) gene, while inverted repeats were not found (data not shown). However, the carrier plasmid IncX4 is itself highly transmissible, showing \( 10^2 \)–\( 10^4 \)-fold higher transfer frequencies than, for instance, epidemic IncFII plasmids, as shown previously [20] as well as in our own transconjugation experiments. Importantly, a lack of fitness-burden of IncX4 carriage on bacterial hosts [20]...
makes this plasmid replicon a highly effective vehicle for dissemination of \textit{mcr-2}. IncX4 plasmids have also been previously shown to harbour \textit{mcr-1} [21] as well as extended spectrum beta-lactamase genes, \textit{bla}_{CTX-M} [20]. Interestingly, the pKP37-BE backbone, which likely originated from \textit{Salmonella} spp., harboured a battery of virulence genes including the \textit{virB4/D4} genes encoding a type-IV secretion system that has been shown to mediate downregulation of host innate immune response genes and an increased bacterial uptake and survival within macrophages and epithelial cells [11]. Outer membrane modifications leading to colistin resistance have been shown to attenuate virulence [22]: whether these co-harboured virulence genes are able to compensate the pathogenic abilities of colistin-resistant \textit{E. coli} remains to be explored.

Taken together, these data call for immediate inclusion of \textit{mcr-2} screening in ongoing molecular epidemiological surveillance to gauge the worldwide dissemination of \textit{mcr-2} in both human and animal colistin-resistant Gram-negative bacteria of medical importance.

* Authors’ correction

The number of countries in which \textit{mcr-1} has been identified was updated to 32 and supporting references were added on 11 July 2016. The references in the article were renumbered accordingly.


