Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016

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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' TGGTACAGCCCCTTATT 3'; MCR2-R 5'GCTTGAGATTGGGTATGA 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, bioMérieux, 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

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

FIGURE 2*MCR-2* and *MCR-1* predicted tertiary structures

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-harboring *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*-harboring pKP37-BE (1.71×10^{-3}) compared with the *mcr-1* harboring IncFII plasmid, pKP81-BE (1.39×10^{-6}) [4]. Both *mcr-1* and *mcr-2* transconjugants exhibited colistin MICs of 4–8 mg/L (macrobroth dilution).

Structure predictions and phylogenetic analyses of the MCR-2 protein

MCR-2 protein was predicted to have two domains, with domain 1 (1 to 229 residues) as a transporter and domain 2 (230 to 538 residues) as a transferase domain (Figure 2).

The best template for domain 1 was 4HE8, a secondary membrane transport protein with a role in transferring solutes across membranes [12]. The best-fit template for domain 2 was 4kav ($p=4.13 \times 10^{-13}$), a lipooligosaccharide phosphoethanolamine transferase A from *Neisseria meningitidis*, also previously shown to be the best-fit template for *MCR-1* [1]. 4kav belongs to the YhjW/YjdB/YijP superfamily and its role in conferring polymyxin resistance has been experimentally validated [13]. Overall, the un-normalised global distance test (uGDT) was 318 (GDT: 58) and all 538 residues were modelled (Figure 2).

MCR-1 and *MCR-2* proteins showed 80.65% identity (supplementary material [9]). In addition, *MCR-2* showed 64% identity to the phosphoethanolamine transferase of *Moraxella osloensis* (WP_062333180) 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' TGTTGCTTGTCGATTGGA 3' and MCR2-IR 5' AGATGGTATTGTTGGTTGCTG 3', and the following cycling conditions: 33 cycles of $95^{\circ}\text{C} \times 3 \text{ min}$, $65^{\circ}\text{C} \times 30 \text{ s}$, $72^{\circ}\text{C} \times 1 \text{ min}$, followed by 1 cycle of $72^{\circ}\text{C} \times 10 \text{ min}$. 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).

FIGURE 3

Phylogenetic analysis of the entire MCR-2 protein sequence

Maximum likelihood tree generated by bootstrap analysis using 1,000 replicates. The analysis was carried out using CLC Genomics workbench v9.0.1 (clcbio, Qiagen) in-built tool for this evolutionary relationship with other related sequences. Branch length is proportional to the number of substitutions per site. Bootstrap values are indicated in the nodes.

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 house-keeping 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 – 10^5 -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]

FIGURE 4

Countries (n = 30) reporting presence of *mcr-1* in samples of animal, environmental or human origin (data collected till 27 June 2016)

Adapted from [15]; updated using data from [14,16,17,25-27].

makes this plasmid replicon a highly effective vehicle for dissemination of *mcr-2*. IncX4 plasmids have also been previously shown to harbour *mcr-1* [21] as well as extended spectrum beta-lactamase genes, *bla*_{CTX-M} [20]. Interestingly, the pKP37-BE backbone, which likely originated from *Salmonella* spp., harboured a battery of virulence genes including the *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 *E. coli* remains to be explored.

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

* Authors' correction

The number of countries in which *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.

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The complete plasmid sequence of pKP37-BE was deposited at the European Nucleotide Archive accession numbers PRJEB14596 (study) and LT598652 (plasmid sequence).

Conflict of interest

None declared.

Authors' contributions

This study was designed by SMK. Isolates were collected by PB. Experimental work was done by BBX and CL. Data was analysed and interpreted by BBX, RR, SKS, HG and SMK. The manuscript was drafted by BBX, SKS and SMK, and was reviewed by all authors.

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